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EDITORIAL

Intestinal sugar transport

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Abstract

Carbohydrates are an important component of the diet. The carbohydrates that we ingest range from simple monosaccharides (glucose, fructose and galactose) to disaccharides (lactose, sucrose) to complex polysaccharides. Most carbohydrates are digested by salivary and pancreatic amylases, and are further broken down into monosaccharides by enzymes in the brush border membrane (BBM) of enterocytes. For example, lactase-phloridzin hydrolase and sucrase-isomaltase are two disaccharidases involved in the hydrolysis of nutritionally important disaccharides. Once monosaccharides are presented to the BBM, mature enterocytes expressing nutrient transporters transport the sugars into the enterocytes. This paper reviews the early studies that contributed to the development of a working model of intestinal sugar transport, and details the recent advances made in understanding the process by which sugars are absorbed in the intestine.

Key words: Glucose; Fructose; SGLT1; GLUT2; GLUT5; Transport; Intestine; Enterocytes; Sugar

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INTRODUCTION

It has been known for decades that two different processes existed for intestinal glucose and fructose absorption. In studies using everted sacs of hamster small intestine, Crane and colleagues found that when the serosal and the mucosal side of the tissue were bathed in glucose, glucose accumulated on the serosal side[1]. This was not the case for fructose, and therefore the absorptive process was labelled as non-concentrating. The involvement of sodium (Na+) in glucose absorption was first proposed by Riklis and Quastel (1958)[2], although studies had previously demonstrated that the decrease in sugar absorption seen in adrenalectomized animals was prevented by adding NaCl to the drinking water[3]. The original Na^+/glucose cotransport hypothesis was presented by Crane in the 1960s[4]. This group showed that active glucose absorption by hamster small intestine required sodium (Na^+) in the bathing medium. Glucose transport was also blocked by ouabain that inhibits the Na^+K^+-ATPase in the basolateral membrane (BLM). This protein is responsible for maintaining the Na^+ gradient in the enterocytes, and driving Na^+ dependent transporters such as the sodium-dependent glucose transporter (SGLT1) in the brush border membrane (BBM).

Crane further developed the model of a mobile carrier in the BBM with two binding sites, one for glucose and one for Na^+[5]. He determined that the continuously maintained outward Na^+ gradient accomplished by the Na^+K^+-ATPase on the BLM was the primary asymmetry providing the driving force for active sugar transport. The phenomenon was considered to be "secondary active transport", as the hydrolysis of ATP was indirectly coupled to glucose transport via this electrochemical gradient. This pioneering work provided the framework for the further characterization of not only glucose transport, but also the transport of other co-transported solutes, and this concept is now considered to be a central tenet in cell physiology.

The pioneering work done by Crane was followed by the electrophysiological studies of Curran and colleagues[6-8] that further characterized transcellular Na^+ transport, and increased the understanding of Na^+ coupled co-transport. Further important advances were made in the 1980s. The method of expression cloning, developed by Wright and colleagues, resulted in SGLT1 being the first eukaryote cotransporter to be cloned. This technique takes advantage of the fact that Xenopus oocytes have the unique ability to translate foreign mRNA, and insert functional transporters into their plasma membrane. The researchers injected rabbit polyA RNA into the oocytes, and observed increases in glucose transport. Utilizing molecular techniques, they were able to isolate a single clone, and use it as a probe to identify human SGLT1[9].

With the continuing development of molecular techniques, the process of intestinal sugar absorption was developed further. The cloning and characterization of the sugar transporters GLUT2[10] and GLUT5[11]

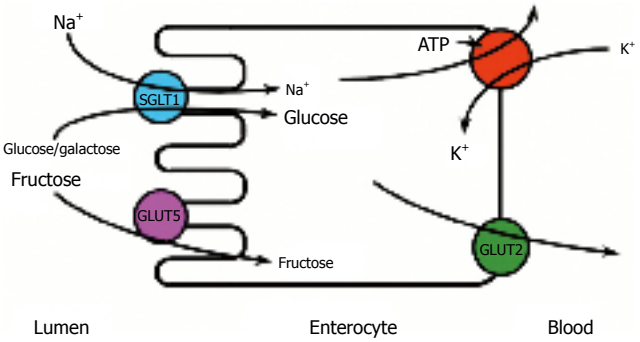

Figure 1 Classical model of intestinal sugar transport (from Wright, 1998). SGLT1 is the sodium dependent glucose/galactose transporter on the brush border membrane (BBM). The Na+K+-ATPase on the basolateral membrane (BLM) maintains the gradient necessary for the functioning of SGLT1. GLUT5 is a facilitative transporter on the BBM which transports fructose into the cell. GLUT2 on the BLM transports glucose, galactose and fructose out of the cell.

Table 1 Affinity constants of sugar transporters

Transporter	Km
SGLT1 (BBM)	Glucose:0.1-0.6 mmol/L (Wright *et al*, 2003)
GLUT2 (BLM)	Glucose:> 50 mmol/L Fructose: 66 mmol/L (Walmsley *et al*, 1998)
GLUT5 (BBM)	Fructose: 6-14 mmol/L (Walmsley *et al*, 1998)

soon followed, and the molecular aspects of the process of sugar absorption across the BBM and BLM were characterized. What is now known as the *"classical model of sugar absorption"* was developed (Figure 1), with SGLT1 actively transporting glucose and galactose across the BBM, and fructose crossing the BBM by facilitative diffusion via GLUT5. GLUT2, a low affinity transporter, was responsible for transporting these sugars across the BLM via facilitative diffusion.

SGLT1

The sodium/glucose cotransporter family (SLCA5) contains more than 200 members found in both animal and bacterial cells. There are 11 human genes expressed in tissues ranging from epithelia to the central nervous system. Hediger *et al* (1987) cloned the SGLT1 gene[9]. The cotransporter is a 73 kDa membrane protein with a Na^+-glucose stoichiometry of 2:1. The transporter has the same affinity for both glucose and galactose (Table 1), and transport is phloridzin sensitive (Ki = 0.1 mmol/L) (Table 2). The membrane topology of SGLT1 was determined using N-glycosylation scanning mutants and hydropathy plots. The transporter contains 14 transmembrane alpha-helices, with an extracellular N and C terminus[12-14]. The transporter contains a single glycosylation site between transmembrane 5 and 6; however, glycosylation is not required for functioning of the protein. Phosphorylation sites have been identified between transmembrane helices 6 and 7, and between transmembrane helices 8 and 9[15]. The importance of SGLT1 phosphorylation will be discussed below. SGLT1 is found in brush border membrane of mature enterocytes in the small intestine, with very small amounts detectable in the kidneys and the heart. Recently, SGLT1 has also been detected in the luminal membrane of intracerebral capillary endothelial cells, where it may participate in the transport of glucose across the blood-brain barrier[16].

Table 2 Inhibitors of sugar transporters

	Inhibitors
SGLT1	Phloridzin
GLUT2	Cytochalasin B Phloretin
GLUT5	Glyco-1, 3-oxazolidin-2-thiones, -ones (Girniene *et al*, 2003)
Na^+K^+-ATPase	Oubain

The process of intestinal sugar transport has been reviewed by Wright *et al* (2003)[17]. Initially, on the luminal side of the BBM, two Na^+ ions bind to SGLT1 and produce a conformational change that permits sugar binding. Another conformational change allows the substrates to enter the enterocyte. The sugar, followed by the Na^+, dissociates from SGLT1 because the affinity of the cytosolic sites is low, and also because the intracellular concentration of Na^+ is low (10 *vs* 140 mEq/L). Sodium can be replaced by H^+ or Li^+, but the affinity for glucose then decreases (apparent Michaelis affinity constant (Km) = 4-11 mmol/L).

The Na^+K^+-ATPase in the BLM is responsible for maintaining the Na^+ and K^+ electrochemical gradients across the cell membrane. The Na^+K^+-ATPase contains a 110 kDa α_1 catalytic subunit, as well as a highly glycosylated 55 kDa β_1 subunit[18,19]. The Na^+K^+-ATPase is up-regulated in experimental diabetes[20] and experimental ileitis [21], with post-translational modifications playing an important role in its regulation. This up-regulation may influence the functioning of SGLT1 and subsequently alter intestinal sugar uptake in these conditions.

Panayotova-Heiermann and Wright (2001) expressed various cDNA constructs of rabbit SGLT1 in Xenopus oocytes in order to determine the helices involved in sugar transport[22]. They found that helices 10-13 form the sugar permeation pathway for SGLT1, and they speculated that the N terminal region of SGLT1 (helices 1-9) may be required to couple Na^+ and glucose transport.

A number of factors influence the transport function of SGLT1 (Table 3). For example, the regulation of SGLT1 by dietary sugars was examined by Miyamoto *et al* (1993)[23]. Using Northern blotting, they showed that SGLT1 mRNA was increased by feeding rats 55% sugar diets containing glucose, galactose, fructose, mannose, xylose, or 3-O-methylglucose. Because 3-O-methylglucose is transported by SGLT1, but is not metabolized, and because SGLT1 does not transport fructose, mannose or xylose, the up-regulation of SGLT1 does not appear to

Table 3 Factors influencing transporter function

Factors influencing SGLT1 function	Factors influencing GLUT2 function	Factors influencing GLUT5 function
Foxl1 (Katz *et al*, 2004)	PKCbII(Helliwell *et al*, 200b)	cAMP (Mahraoui *et al*, 1994)
AMPK (Walker *et al*, 2004)	p38(Helliwell *et al*, 200b)	p38 (Helliwell *et al*, 200b)
PKA (Wright *et al*, 1997)	ERK(Helliwell *et al*, 200b)	ERK (Helliwell *et al*, 200b)
PKC (Wright *et al*, 1997)	PI3K(Helliwell *et al*, 200b)	PI3K (Helliwell *et al*, 200b)
RS1 (Veyhl *et al*, 1993)	mTOR (Helliwell *et al*, 2003)	TNF-α (Garcia-Herrera, 2004)
HNF-1 (Martin *et al*, 2000)	AMPK (Walker *et al*, 2004)	
Sp1 (Martin *et al*, 2000)		
Hsp 70 (Ikari *et al*, 2002)		
TGF-b (Ikari *et al*, 2002)		

depend on either metabolism or transport of the sugar in question (Table 3).

Wright *et al* (1997) evaluated the role of SGLT1 phosphorylation[24]. They expressed rabbit SGLT1 in Xenopus oocytes, and activated protein kinase A (PKA) or protein kinase C (PKC) using 8-Br-cAMP and *sn*-1, 2-dioctanoylglycerol (DOG), respectively. PKA activation increased glucose transport by approximately 30%, while PKC activation reduced transport by 60%. The change in maximal transport rates (Vmax) was accompanied by alterations in the number of transporters in the plasma membrane, as well as changes in the surface area of the membrane. Since endocytosis and exocytosis alter the membrane surface area, the findings of the effects of PKA and PKC on SGLT1 suggest that these proteins may be involved in the regulation of glucose transport.

Similar increases in Vmax were obtained with activation of PKA in oocytes expressing rabbit, human, and rat SGLT1 isoforms. The effects of PKC, however, may depend on the sequence of the co-transporter, as there are conflicting reports of the effect of PKC on glucose transport. For example, PKC decreases sugar transport in Xenopus oocytes expressing rabbit and rat SGLT1[25,26], and increases sugar transport when human SGLT1 is expressed[27].

Veyhl *et al* (1993) demonstrated the presence of an intracellular regulatory protein (RS1) that may modify the activity of SGLT1[28]. Co-expression of RS1 and SGLT1 in Xenopus oocytes reduced both the Vmax for glucose transport as well as SGLT1 protein levels[26]. Plasma membrane surface area was also reduced, suggesting alterations in the endo- and/or exo-cytosis of membrane vesicles.

To investigate the role of intracellular trafficking in sugar transport, oocytes were injected either with cRNA of wild type, or mutant dynamin. Dynamin is a motor protein involved in receptor-mediated endocytosis, vesicle recycling, caveolae internalization and vesicle trafficking from the Golgi[29]. The inhibition of glucose uptake by RS1 was largely reduced after co-expression of the mutant dynamin protein. The investigators concluded that RS1 modulates dynamin-dependent trafficking to the BBM of intracellular vesicles containing SGLT1.

In order to further characterize the role of the RS1 protein in the regulation of intestinal glucose transport, a knockout mouse lacking the RS1 protein was recently developed: Osswald *et al* (2005) showed that RS1-/- mice developed obesity associated with increases in food intake, glucose transport and SGLT1 expression in the small intestine[30]. The effect of RS1 deficiency was tissue-specific and occurred through post-transcriptional mechanisms, as SGLT1 mRNA abundance was unchanged. These researchers speculated that therapeutic strategies aimed at reducing glucose uptake by increasing RS1 might potentially be used to treat obesity.

Heat shock proteins (hsp) may also play a role in regulating SGLT1 function (Table 3). A study done in renal epithelial cells showed that treatment with hsp70 increased glucose transport, but not the abundance of SGLT1 protein[31]. The increase in sugar transport was inhibited by an antibody directed against transforming growth factor β (TGF- β), leading the investigators to explore the effect of TGF-β on SGLT1: there was an increase in SGLT1 activity, as well as an increase in hsp70 protein when TGF-β was added to the culture media. The researchers speculated that hsp70 might stabilize SGLT1 expression in the membrane. This concept was supported by confocal microscopy studies, which demonstrated that TGF-β appears to move both SGLT1 and Hsp70 near the apical membrane site. However, the mechanism by which TGF-β exerts these effects on Hsp70 and SGLT1 is not known.

It is not known if SGLT1 is localized to specific microdomains within the BBM. In renal proximal tubular cells, SGLT1 was found in detergent-resistant membrane microdomains, also referred to as "lipid rafts"[32]. In this model, the absence of vimentin, an intermediate filament component, decreased glucose transport and caused a reduction in the amount of SGLT1 protein in these membrane microdomains. Furthermore, fluidization of the plasma membrane, or depleting the membrane of cholesterol, dramatically decreased glucose transport. This suggests that the activity of SGLT1 is optimal in a microenvironment characterized by low fluidity. Further research is required to determine if SGLT1 is localized to lipid rafts in the intestinal BBM, if this localization is mandatory for the functioning of SGLT1, and what are the factors that may regulate the localization of SGLT1 to these specialized microdomains.

The transcription factors hepatocyte nuclear factor-1 (HNF-1) and Sp1 may also regulate SGLT1. Martin *et al*, (2000) characterized the promoter region of the human SGLT1 gene by transfecting reporter constructs into

Caco-2 cells[33]. They demonstrated that three cis-elements, HNF-1, and two sites on the SGLT1 promoter ("GC boxes" to which Sp1 binds are required for maintaining basal transcription of SGLT1. Members of the Sp1 family bind to the GC boxes, and in the presence of HNF-1, synergistically activate the promoter. Some members of the Sp1 family have been implicated in tissue- and developmental- specific regulation of genes[34,35]. HNF-1 alters the expression of many small intestinal genes, including sucrase-isomaltase (SI) and lactase. It has also been implicated in the diurnal regulation of SGLT1 in rodents[36]. If HNF-1 was required for basal SGLT1 expression, glucose-galactose malabsorption would have been expected to be observed. Of interest, HNF-1 knockout mice experience life-threatening effects on the hepatic and renal systems, but no adverse effects on the gastrointestinal tracts were reported.

Katz *et al* (2004) identified a link between a mesenchymal factor and the regulation of a specific epithelial transport process[37]. *Foxl1* is a winged-helix transcription factor expressed in the mesenchymal cells bordering the crypts in the small intestine. Using the everted sleeve method coupled with Western blotting, the researchers showed that homozygous *Foxl1* null mice had decreased intestinal glucose uptake and decreased levels of SGLT1 protein. Growth retardation and abnormal small intestinal architecture were observed, characterized by short, broad and irregular villi. The effect of the loss of Foxl1 on SGLT1 was specific, as no changes in the expression of SI, lactase, GLUT2 or Na^+K^+ATPase were observed.

The transport of water across the intestinal epithelia has always been a subject of curiosity. The discovery of aquaporins by Preston *et al* (1992) renewed interest in this topic[38]. Although aquaporins may account for a portion of water absorption in the intestine, Wright and colleagues investigated the coupling of water transport to active Na^+-glucose cotransport. Overexpression of human or rabbit SGLT1 in Xenopus oocytes revealed that activation of the transporter was associated with an increase in volume of the cell (reflecting water transport), and this effect was blocked by phlorizin. If oocytes expressing SGLT1 were incubated in a sugar-free solution, no change in oocyte volume was observed. The increase in volume could be accounted for by a stoichiometry of two Na+ ions, one glucose molecule, and 249 water molecules[39]. The transport of water was independent of the osmotic gradient across the membrane, and may be a consequence of the conformational changes in SGLT1 that occur during Na^+/glucose transport. A channel formed by five C-terminal transmembrane helices of SGLT1 is thought to transport not only water, but also urea[22,40].

It is important to note, however, that others have suggested that local osmotic gradients fully account for water fluxes. Lapointe *et al* (2002) present evidence from experiments using Xenopus oocytes expressing human SGLT1 that contradicts the water cotransport hypothesis and suggests the passive movement of water across the plasma membrane[41].

Oral rehydration therapy (ORT) was developed in the 1970's to treat diarrheal dehydration[42]. The introduction of this very simple treatment has reduced mortality due to diarrhea in children under five years of age from 5 million in 1978, to 1.3 million in 2002 (http: //www.who.int/child-adolescent-health.2002; Victora *et al*, 2000). This success led to the proclamation that ORT was the most important medical advance of the 20th century, and earned Dr Hirschhorn and colleagues the first Pollin prize for Pediatric Research.

The goals of ORT are to replace fluids and minimize malnutrition. Starting in 1978, solutions containing a mixture of glucose, sodium, chloride, potassium and citrate were being commonly distributed by the World Health Organization. In fact, 800 million packets of ORT were distributed worldwide in 1991-1992[43]. Interestingly, controversy now exists over the optimal formulation, with reduced osmolarity formulas, rice-based formulas, or formulas containing amylase-resistant starch being favored by some researchers. For example, hypoosmolar rice-based formulas produced better results in cholera patients when compared to standard formulas[44]. The advantages of this rice-based formula are that it is cheap, offers more calories than standard ORT, and rice is readily available in many cholera-stricken regions. ORT formulas containing amylase-resistant starches may be favored due to the production of short chain fatty acids, which increase colonic Na+, Cl- and fluid absorption, and reduce colonic secretions[45-47]. These effects counteract the fluid losses and hypersecretion seen with infectious diarrhea.

Several features of carbohydrate digestion contribute to the efficacy of ORT. This life-saving therapy is based on the ability of SGLT1 to co-transport water. Na^+-dependent glucose absorption is not affected by the increased cAMP levels commonly seen with infections such as Cholera, and therefore this physiological fact can be exploited as a means to achieve glucose, Na^+ and water absorption, even in the presence of chloride and water secretion. Also, the oral administration of glucose or carbohydrates up-regulates SGLT1, thereby further increasing the intestinal transport of glucose, Na^+ and water. Since ORT is commonly administered to infants, it is important to utilize a transport system that is expressed and functional early in life. SGLT1 is expressed prenatally[48], and is functional at birth, making it an ideal candidate. In contrast to glucose, the use of fructose in these ORT solutions is contraindicated, as GLUT5 in the BBM is only expressed following weaning[49].

Glucose-galactose malabsorption (GGM) is a very rare autosomal recessive disease characterized by severe life-threatening diarrhea in the neonate, that resolves when the offending sugars (glucose, galactose and lactose) are removed from the diet[15,50]. Normal intestinal mucosal histology is observed, while phlorizin binding studies show reductions in SGLT1 protein in the BBM[51,52]. Electrophysiological studies and freeze fracture electron microscopy showed that this disease is due to a failure of the SGLT1 protein to traffic normally to the BBM[53]. Approximately 300 cases of GGM have been identified worldwide, affecting all racial and ethnic groups. The majority (70%) of patients are female, with two thirds coming from a consanguineous relationship[17]. Unlike genetic diseases like cystic fibrosis, in which a single

mutation accounts for most cases, in GGM each patient appears to have a unique mutation, ranging from missense mutations, to frame-shift mutations, to split-site-conservative mutations which produce truncated protein and mistrafficking of SGLT1 to the BBM[53-56]. This variety of mutations limits the usefulness of genetic testing for GGM, although prenatal diagnosis in a family at risk may be possible.

GGM is a difficult condition to diagnose. If GGM is suspected, the first step is the elimination of glucose, galactose and lactose from the infant's diet. Oral glucose tolerance tests in GGM patients produce a flat glucose response in the blood, as glucose is malabsorbed in the intestine. A hydrogen breath test performed following oral glucose produces abnormally high concentrations of H_2 in the breath (> 20 ppm) indicating glucose malabsorption, while oral fructose tolerance tests produce normal results. GGM is treated by using glucose-, galactose- and lactose-free formulas, and by eliminating the offending sugars from the diet[17]. Normal growth and neurological development are possible if infants receive fructose-based formula, and if dietary counselling is available[15,57].

GLUT5

GLUT5 is a 43 ku protein, with 12 transmembrane domains and intracellular N and C terminals. It was cloned by Burant and colleagues in 1992. GLUT5 was expressed in Xenopus oocytes, and its substrate specificity and kinetic properties were determined using radiolabelled substrates. Northern and Western blotting demonstrated the presence of GLUT5 in human small intestine and testis. Further work by Davidson *et al* (1992) focused on the developmental expression of GLUT5 in the human and fetal small intestine[58]. GLUT5 mRNA levels increase with age, and are highest in the adult small intestine. In adults, GLUT5 was localized to the BBM by Western blotting. Immunohistochemical techniques confirmed this finding, and further localized GLUT5 to only the mature enterocytes populating the upper half of the villus. This luminal localization provided further support for the notion that GLUT5 played a role in the intestinal uptake of dietary sugars.

Rand *et al*, (1993) characterized the expression of GLUT5 in rats[49]. GLUT5 mRNA was detected in the small intestine, kidney and brain by Northern blotting, and in the small intestine, testis, adipose and skeletal muscle using *in situ* hybridization. In the intestine, a proximal-distal gradient was observed, with GLUT5 mRNA levels being higher in the proximal small intestine when compared to the distal small intestine. A distinct pattern of expression was seen along the crypt-villous axis, with mRNA being highest in midvillus region.

The functional domain of GLUT5 was investigated by Buchs *et al*[59]. In order to ensure proper transport and insertion into the membrane, GLUT5-GLUT3 chimeras were created, and included various combinations of the GLUT3 and GLUT5 peptides. These chimeric GLUTs were expressed in Xenopus oocytes. This enabled the researchers to conclude that the regions necessary for fructose transport lie between the amino terminus and the third transmembrane domain, and between the 5th and 11th transmembrane domain.

The response of GLUT5 to dietary sugars was investigated by Miyamoto *et al*[23]. In this study, they fed sugar-enriched diets (55% D-glucose, D-galactose, 3-0-methylglucose, D-fructose, D-mannose or D-xylose) to male Sprague Dawley rats for 5 d. Northern blotting on intestinal samples showed that GLUT5 mRNA was increased only by dietary D-fructose, and was unaffected by the other sugars (Table 3). This was consistent with the suggestion that GLUT5 was a high affinity fructose transporter. Subsequent work by David *et al* (1995) showed that in 16 d old rats, feeding fructose but not glucose increased fructose uptake[60]. Furthermore, while both fructose and sucrose feeding enhanced absorption in older (21-60 d old) animals, glucose alone had no effect.

An interesting study by Castello *et al* (1995) demonstrated that GLUT5 mRNA in rats followed a circadean rhythm, with a 12-fold increase in mRNA at the end of the light cycle as compared to early in the light cycle[61]. BBM GLUT5 protein followed a similar pattern, which is also observed for other small intestinal genes such as BBM SI and lactase[62]. Although this pattern was thought to be a reflection of rodent feeding patterns, Corpe *et al* (1996) found that gene expression is hard-wired, because GLUT5 is up-regulated prior to the onset of feeding, even in the absence of dietary fructose[63]. Shu *et al* (1998) noted that this circadean rhythm was not developed at the time of weaning, possibly because the feeding patterns of suckling rats do not follow the same adult nocturnal patterns[64]. This diurnal variation in adult animals needs to be carefully considered when designing experiments in which levels of GLUT5 are measured, by performing studies in the morning in the early post-prandial period.

The regulation of GLUT5 was studied by Mahraoui *et al* (1994) using Caco-2 cells[65]. Treatment of the cells with forskolin, which stimulates adenylate cyclase and raises intracellular cAMP levels, increased fructose uptake 2-fold, and increased GLUT5 protein and mRNA 5-fold and 7-fold, respectively. Matosin-Matekalo *et al* (1999) used Caco2 cells transfected with a GLUT5 promoter inserted up-stream of the luciferase reporter gene[66]. They found that a region of the GLUT5 promoter binds the thyroid hormone receptor/retinoid X receptor heterodimers, and that both triiodothyronine (T3) and glucose increase GLUT5 mRNA.

Helliwell *et al*[67] looked at the regulation of GLUT5 by a number of signals that have well-established roles in the regulation of sugar transport. Isolated loops of rat jejunum were perfused with activators and inhibitors of the ERK, p38 and PI3K pathways. The findings suggest that the p38 pathway stimulates fructose transport, while the ERK and the PI3K pathways had little effect on fructose transport (Figure 3). Extensive cross-talk occurs between the pathways. For example, inhibiting the ERK pathway with PD98059 increased the sensitivity to anisomysin, which stimulates the p38 pathway. The authors concluded that the three pathways have the potential to regulate fructose transport during the digestion and absorption of a meal. They suggested that future work should focus on

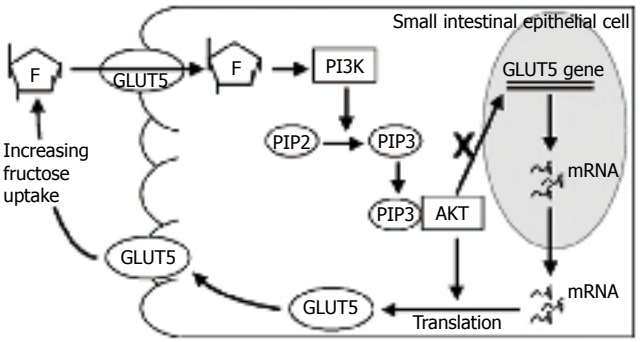

Figure 2 Proposed role of PI3K/Akt signalling pathway in the regulation of GLUT5 synthesis and trafficking (from Cui *et al*, 2005). Abbreviations: *F* = fructose, PIP3 = phosphatidylinositol-3, 4, 5-triphosphate, PIP2 = phosphatidylinositol-4, 5-biphosphate, PI3K = phosphatidylinositol 3-kinase.

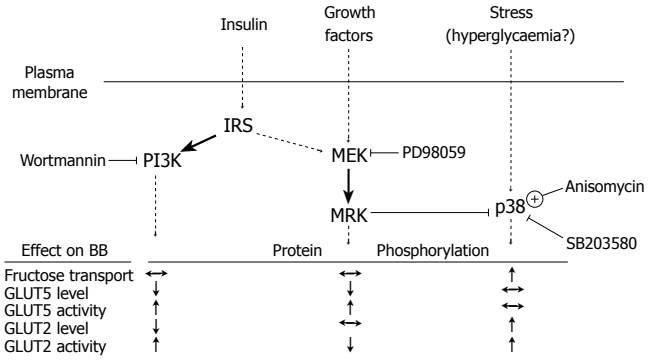

Figure 3 The regulation of BBM fructose transport by the PI3K, ERK and p38 MAPK signalling pathways (from Helliwell et al, 2000). Abbreviations: IRS=insulin regulatory subunit, ERK=extracellular regulated kinase, MEK=mitogen activated kinase kinases, PI3K=phosphatidylinositol 3-kinase, PD98059=ERK/MEK inhibitor, SB203580=p38 MAPK inhibitor, anisomycin=activator of p38 and jun kinase pathways.

determining the hormones that influence these pathways, and the molecular mechanisms that regulate the levels and activities of the sugar transporters.

Gouyon *et al*[68] used Caco-2 cells to investigate the mechanism by which fructose increases GLUT5 expression. Although both glucose and fructose increased the activity of the GLUT5 promoter, the effect of fructose was stronger and associated with higher cAMP concentrations. If cAMP signalling was blocked by a protein kinase A inhibitor, extensive GLUT5 mRNA degradation occurred, suggesting that the mRNA stability was influenced by PKA. A sugar response element was identified in the GLUT5 promoter. PABP-interacting protein 2, which represses translation[69,70], was identified as a component of GLUT5 3'-UTR RNA-protein complex, where it may act to destabilize transcripts. The differences between the effects of glucose and fructose on GLUT5 expression may be attributed to variations in their ability to increase cAMP levels, and to modulate the formation of protein complexes with GLUT5 3'-UTR.

Infection may also regulate fructose transport. Intravenous administration of Tumor necrosis factor-α (TNF-α) in rabbits significantly reduced jejunal fructose transport and GLUT5 protein[71]. This inhibition was related to the secretagogue effect of TNF-α, and both nitric oxide and prostaglandins were implicated in the inhibition of fructose uptake. Adaptive immunity also influences the expression of a number of developmentally regulated genes. In mice lacking in adaptive immunity (B cell deficient recombination-activating gene [RAG] mice), RNase protection assays demonstrated that GLUT5 was increased[72].

Recent advances have been made in understanding the signalling pathways involved in the regulation of GLUT5. Cui *et al*[73] have demonstrated that cAMP stimulates fructose transport in the neonatal rat intestine. Perfusing fructose (100mM) plus 8-bromo-cAMP in 22-d-old rats increased fructose uptake rates, while an inhibitor of adenylate cyclase abolished this effect. Despite the presence of two cAMP response elements in the human GLUT5 promoter region[65], GLUT5 mRNA was not affected by cAMP treatment. Interestingly, inhibitors of PKA did not prevent the fructose-associated increases in transport, suggesting that cAMP modulates fructose transport independent of PKA (Table 3).

Subsequent work by the same group has shown that fructose-induced increases in neonatal rat intestinal fructose uptake involve the PI3K/Akt signalling pathway[74]. In this study, PI3K inhibitors (wortmannin and LY94002) and an Akt inhibitor (SH-5) abolished the increase in fructose uptake, as well as the abundance of GLUT5 protein (but not mRNA) seen following fructose (100 mmol/L) perfusions in neonatal rats. Fructose perfusion increased phosphatidylinositol-3, 4, 5-triphosphate (PIP3), the product of PI3K, in the mid to upper regions of the villus, where most of the GLUT5 was located. The authors suggest that the PI3K/Akt pathway may be involved in the synthesis and/or recruitment of GLUT5 to the BBM in response to luminal fructose (Figure 2).

GLUT2

GLUT2 is a low affinity, high capacity facilitative transporter in the BLM that transports glucose, fructose, galactose and mannose[10,63,75-77]. It has 12 transmembrane domains, with intracellular N and C terminals. Using immunohistochemistry, Thorens *et al*[75] showed that GLUT2 expression increases as enterocytes migrate up from the crypt to the villous tip. Amino acid sequences in transmembrane segments 9-12 are primarily responsible for GLUT2's distinctive glucose affinity, whereas amino acid sequences in transmembrane segments 7-8 enable GLUT2 to transport fructose[78].

Luminal sugars[23,79] or vascular infusions of glucose or fructose[80,81] stimulate GLUT2 expression and activity. The response of GLUT2 to dietary sugars was investigated by Miyamoto *et al*[23]. In this study, they fed sugar-enriched diets to male Sprague Dawley rats for 5 d. GLUT2 mRNA was up-regulated by glucose, fructose and galactose. GLUT2 modulation required intracellular metabolism of the sugar, as it was unaffected by 3-O-methyglucose, a non-metabolized glucose analog.

In a study by Cui *et al*[82], the jejunum of 20-d-old anaesthetized rat pups was perfused with 100 mmol/L glucose or fructose. Increases in GLUT2 mRNA were

observed, and this effect was inhibited by actinomycin D, an inhibitor of transcription. Cycloheximide, an inhibitor of translation, did not block the enhanced expression of GLUT2 mRNA, suggesting that the synthesis of new proteins is not necessary for increases in GLUT2 mRNA. Because levels of GLUT2 mRNA and protein are tightly correlated, the regulation of GLUT2 may be transcriptional[83].

PASSIVE UPTAKE

For years there has been considered to be a "passive" component to sugar absorption. This traditional view has been challenged, with the suggestion that the kinetic characteristics of sugar uptake could also be described by a second high affinity, high capacity BBM transporter[83]. In order to better understand the new "GLUT2 trafficking model", we need first to consider the classic "passive permeation" model.

The fact that SGLT1 saturates at 30-50 mmol/L glucose was inconsistent with the observation that intestinal glucose absorption increases linearly with increases in luminal glucose concentrations up to several hundred millimolar[85]. This finding suggests the presence of two components: an active, phloridzin-sensitive component, and a phloridzin-insensitive, possibly passive component that does not appear to be saturable. Some studies have suggested that the "passive" component played a large role in glucose transport at high glucose concentrations, in some models contributing 3-5 times as much as the active component[86,87].

The passive component of glucose transport was characterized by Pappenheimer and Reiss (1987)[88]. The observation that high rates of water absorption accompany glucose absorption[89] provided a rationale for proposing that glucose in the intercellular spaces provided an osmotic force that resulted in bulk flow of nutrients. Pappenheimer and Reiss (1987) perfused isolated segments of hamster small intestine with 10-25 mmol/L glucose[88]. Structural studies using electron microscopy and freeze fracture analysis revealed large dilatations within junctions following glucose perfusion. They concluded that Na^+-coupled transport of solutes from the intestinal lumen to the cytosol of the enterocytes provides the driving force for the absorption of fluid and nutrients, and triggers the widening of intercellular junctions, thereby promoting the bulk absorption of nutrients by solvent drag. They calculated that the contribution of solvent drag exceeds that of active transport at luminal glucose concentrations greater than 250 mmol/L. Madara and Pappenheimer (1987) further demonstrated that the transport of glucose via SGLT1 caused dilatation of the tight junctions[90]. They concluded that passive glucose absorption is a result of paracellular solvent drag, and is indeed SGLT1 dependent. Therefore, like the more recent model suggested by Loo et al (2002)[39], these investigators suggest that the transport of water is SGLT1-dependent. However, this theory suggests the presence of a non-specific route, which could potentially allow passage of several solutes.

Ferraris and Diamond proposed an alternative theory, in which paracellular flow is negligible[91,92]. Based on the determination of up-dated kinetic constants for glucose absorption, and the determination of the usual free glucose concentrations in the intestinal lumen, they concluded that SGLT1 fully accounts for glucose absorption. Much of their work is based on studies examining long-term dietary adaptations, from which they concluded that BBM transporters are matched to dietary intake. Their model is supported by the findings of Lane *et al*[93], who demonstrated that paracellular flow in unanaesthetized dogs did not account for more than 2%-7% of total absorption.

Much of the controversy surrounding the role of the paracellular pathway stems from the discrepancies between the estimated concentrations of glucose in the intestinal lumen. Pappenheimer and Reiss[88] based their calculations on luminal glucose concentrations of 300 mmol/L, whereas Ferraris *et al*[92] did a detailed analysis of luminal glucose concentrations and concluded that physiological luminal values ranged from 0.2-48 mmol/L. Pappenheimer[94] used the rate of membrane hydrolysis of maltose to indirectly measure luminal glucose concentrations. They also point out that the techniques used by Ferraris *et al* (1990)[92], which involve glucose analysis of luminal contents, will underestimate the concentration found at the membrane following hydrolysis by disaccharidases. The actual physiological levels of glucose in the lumen remain a subject of debate.

The concept of more than one transport system for glucose was suggested by Malo[95]. Using human fetal and adult BBM vesicles, curvilinear Eadie-Hofstee plots and sodium activation curves were obtained when glucose concentrations were varied in the medium. These findings, coupled with determinations of phloretin-sensitive and -insensitive components, and the ability of the BBM vesicles to transport 3-O-methylglucose, suggested the presence of two transport systems: a high-affinity low-capacity system and a low-affinity high-capacity system[95,96]. This agrees with the observation that Na^+/glucose cotransport saturates at 30-50 mmol/L, yet absorption is linear from 50 mmol/L to several hundred mmol/L[85].

Although this concept was proposed many years ago, it was not until recently that interest in the area has re-emerged due to an alternative model of intestinal glucose transport proposed by George Kellett and his colleagues at the University of York, and by Edith Brot-Laroche and her colleagues at the University of Paris. Let us briefly explore this fascinating "voyage of discovery".

GLUT2 IN BBM

Several years ago, GLUT2 was detected in the BBM of enterocytes in diabetic animals, although at the time this was interpreted to be a pathological event[63]. More recently, Kellett and his colleagues proposed a model by which BBM SGLT1, in the presence of luminal glucose, promotes the rapid insertion of GLUT2 into the BBM via PKCβII and the MAP kinase-dependent signal transduction pathways[67,97,98]. PKCβII is located in the terminal web of mature enterocytes in the upper part of the intestinal villus[99]. Interestingly, these are the same cells that are responsible for glucose absorption.

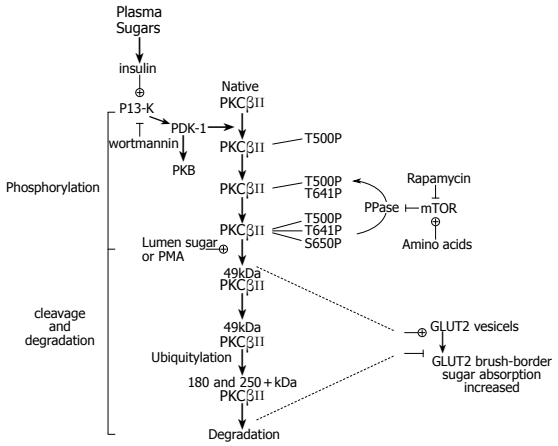

Figure 4 Potential signaling pathways for the regulation of GLUT2-mediated sugar absorption by insulin and amino acids through the control of PKC βII activity. (from Helliwell et al, 2003). Abbreviations: PMA= phorbol 12-myristate 13-acetate, PDK-1=protein-dependent kinase-1, PI3K=phosphatidylinositol-3-kinase, PKB=protein kinase B, mTOR=mammalian target of rapamycin, PKCβII=protein kinase C βII.

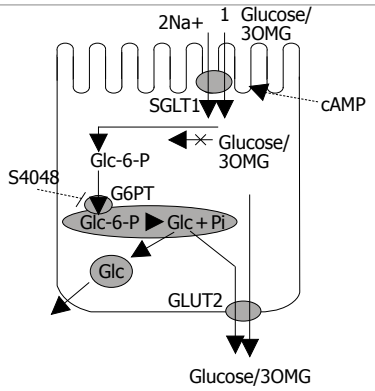

Figure 5 Proposed alternative mechanism for intestinal glucose transepithelial transport (from Stumpel et al, 2001). Abbreviations:Glc=glucose, Glc-6-P=glucose-6-phosphate, G6PT=glucose-6-phosphate translocase, 3-OMG=3-O-methylglucose, S4048=inhibitor of glucose-6-phosphate translocase, Pi=phosphate.

Using a luminal perfusion model, Kellett and coworkers measured the phloretin-insensitive (SGLT1) component and phloretin sensitive component (GLUT2) of glucose transport. They also showed using Western blotting that BBM GLUT2 increased 2.2 fold when the concentration of glucose in the perfusate increased from 0 to 100 mmol/L. Similarly, the BBM level of PKCβII increased with increasing glucose concentrations. This finding, coupled with the observation that PKCβII shows a saturation response and has a Km similar to that of SGLT1 (21-27 mmol/L), suggests that PKCβII is an important signal in the recruitment of GLUT2 to the BBM. PKCβII levels also correlate with levels of GLUT2 in the BBM, and this association offers further support for its role in the recruitment of GLUT2 to the BBM. The ability of the PKC inhibitor "chelerythrine" to block phorbol 12-myristate 13-acetate (PMA)-stimulated fructose transport and GLUT2 abundance in the BBM also supports this mode l[97].

Kellett's working hypothesis proposes that before a meal, when luminal concentrations of glucose are low, GLUT2 levels in the BBM are also low, which would minimize the escape of glucose from the cell (any glucose that did escape would be recycled by SGLT1, which can transport it against the glucose gradient). Once a meal is ingested and BBM enzymes hydrolyse disaccharides, luminal glucose concentrations increase. Glucose uptake via SGLT1 causes increases in enterocyte volume due to a rise in osmolarity (and the co-transport of water molecules by SGLT1), and may trigger the entry of Ca+, activating PKCβII and promoting the insertion of GLUT2 in the BBM. The involvement of SGLT1 in the recruitment of GLUT2 to the BBM agrees with observations that phloridzin (an SGLT1 inhibitor) fully blocks glucose uptake, and that patients with defective SGLT1 suffer from glucose-galactose malabsorption. Trafficking of GLUT2 is thought to be rapid, with a $t_{1/2}$ of less than 5 minutes. A rounding of the apical surface, due to a contraction of the peri-junctional actomyosin ring, allows luminal glucose to have increased access to the BBM enzymes and transporters. The authors recognize that there may also be an activation of the small amount of GLUT2 that is already present in the BBM.

Helliwell *et al*[67] investigated the role of several signalling pathways in intestinal fructose absorption. Using an *in vivo* perfusion model, they showed that fructose transport was mediated by both GLUT5 and GLUT2. Using PMA to stimulate a 4-fold stimulation of fructose transport, they saw a 4-fold increase in GLUT2 protein in the BBM that correlated with PKCβII activation. Only minor changes in GLUT5 levels were observed, suggesting that recruitment of GLUT2 to the BBM represents a mechanism by which absorptive capacity is matched to dietary intake.

Helliwell *et al*[100] also established a role for the PI-3K and the mTOR pathways in the phosphorylation, turnover and degradation of PKCβII. Using an *in vivo* perfusion model, they showed that inhibitors of these pathways (wortmannin and rapamycin, respectively) block GLUT2 trafficking to the BBM and inhibit sugar absorption. A role for insulin in the regulation of intestinal sugar absorption is suggested. In their model, they suggest that as sugar absorption increases, the plasma sugar concentration increases, stimulating the release of insulin, which activates PI 3-kinase, resulting in the phosphorylation of PKCβII (Figure 4).

They also proposed a model by which amino acids promote the formation of competent PKCβII by activating the mTOR pathway, which prevents dephosphorylation of PKCβII (Figure 4). Thus, the dynamic control of intestinal sugar absorption may be achieved by the rapid turnover and degradation of PKCβII.

Why haven't previous investigators been able to detect GLUT2 in the BBM? *In vivo* endogenous hormones and nutrients activate PKCβII. Kellett's group points out that the process of harvesting tissue for *in vitro* preparations causes the inactivation of PKCβII and the rapid trafficking of GLUT2 away from the BBM. This observation may help to explain why the passive component was more apparent in the *in vivo* studies, as compared to *in vitro* experiments. In order to minimize the loss of GLUT2 from the BBM, Kellett's group perform all stages of tissue harvesting and membrane vesicle preparations at 0-4℃

after perfusing the intestine with a sugar load.

Helliwell and Kellett [101] looked at perfusion conditions in order to determine if the so called passive component was SGLT1-dependent, as suggested by their work, or was SGLT1-independent, as suggested by earlier work by Debnam and Levin[102]. They concluded that the passive component is independent of the active component in high mechanical stress perfusions, suggesting that SGLT1-dependent recruitment of GLUT2 did not occur under these conditions. This may be related to the restrictions in blood flow and supply of endogenous nutrients and hormones caused by the high stress perfusions.

However, under conditions of low mechanical stress, inhibition of SGLT1 with phloridzin does decrease GLUT2 levels in the BBM. Clearly, the perfusion conditions affect the results of the experiment, and this may explain the discrepancies between various studies. Finally, Kellett and colleagues suggest that the term "facilitated" should be used rather than "passive" to more accurately describe the GLUT2 mediated component of sugar uptake.

The antibody used to detect GLUT2 is also critically important in being able to detect GLUT2 in the BBM. Currently, there are two commercially available antibodies that recognize GLUT2: one that recognizes the extracellular loop between transmembrane 1 and 2 (Biogenesis, Poole, England); and another that recognizes a portion of the C- terminus (Research Diagnostics, Flanders, NJ). The choice of antibody is important, as Au *et al* (2002) demonstrated that the biotinylation procedure they used to detect surface proteins interfered with the ability of the GLUT2 antibody to recognize the extracellular loop, forcing them to use the C-terminus antibody[103]. However, Thorens *et al*[103] were unable to detect GLUT2 in the BBM by immunohistochemistry using an antibody that recognizes the C-terminus[75]. In contrast, when using the antibody directed against the extracellular loop, Cheeseman's group was able to detect BBM GLUT2**.** In contrast, when using Western blotting on BBM vesicles, they were able to detect GLUT2 using either of the antibodies. Clearly, the choice of antibody depends on what method of detection is used.

Although earlier studies established a role for GLP-2 in modulating GLUT2 activity in the BLM[63,104], more recently Au *et al*[103] investigated the effect of GLP-2 on the transient expression of GLUT2 in the BBM. Using an *in vivo* perfusion model in rats, they showed that a one hour vascular infusion of GLP-2 (800 pM) doubled the rate of fructose absorption, and this enhanced absorption of fructose could be blocked by phloretin, an inhibitor of GLUT2. Immunohistochemistry localized GLUT2 to both the BBM and BLM, and identified a pool of transporter located just under the microvilli in the terminal web region. This raises the possibility that the cytoskeleton structure is involved in the insertion of GLUT2 into the BBM. Luminal glucose perfusion (50 mmol/L) or vascular GLP-2 infusion (800 pmol/L) increased GLUT2 in the BBM two-fold as determined by Western blotting of biotinylated surface proteins. Both Kellett and Cheeseman suggest that in addition to the insertion of GLUT2 in the BBM, the intrinsic activity of apical GLUT2 may also be regulated[103,105]. The concept of intrinsic activity and its regulation will be discussed in subsequent sections.

Gouyon *et al*[68] used confocal microscopy and immunofluorescence in mice to confirm the presence of GLUT2 in the BBM following five days of feeding a 65% sugar meal, or following an oral bolus of either fructose or glucose. Wild type and GLUT2 null mice were fed fructose, glucose or sucrose (65% glucose, fructose or sucrose)-rich diets for five days, or were fasted and then received a 40% fructose, glucose or sucrose bolus. The absence of GLUT2 did not significantly affect fructose absorption in animals fed a low-carbohydrate diet, suggesting that under these conditions GLUT5 is soley responsible for fructose uptake into the enterocyte.

In animals fed a high fructose diet, cytochalasin B (an inhibitor of GLUT2) inhibited fructose uptake 60% in wild type mice, whereas GLUT2 null mice were unaffected. A 40% reduction was observed in animals fed a high glucose diet. This suggests that under these conditions fructose enters the cell by both a cytochalasin B dependent process (GLUT2) and a cytochalasin B independent process (GLUT5). The trafficking of GLUT2 to the BBM may represent a mechanism by which sugar absorptive capacity is matched to dietary intake.

When GLUT2 null mice were challenged with oral fructose, transport was 60% lower than in wild type animals, indicating firstly that the absence of GLUT2 limited fructose uptake, and secondly that this could not be fully compensated for by GLUT5. Still, some compensatory changes were noted, as GLUT5 mRNA was found to be increased three-fold in the ileum, possibly indicating some fructose malabsorption in the GLUT2 null mice. Therefore, Gouyon's study demonstrates that while under control conditions GLUT5 is solely responsible for BBM fructose uptake, BBM GLUT2 may be responsible for 40%-60% of sugar uptake when the luminal sugar concentrations are high.

The role of AMP-activated kinase (AMPK), an intracellular energy sensor, in the regulation of intestinal sugar uptake has also been examined. Walker *et al*[106] demonstrated that the activation of AMPK resulted in the recruitment of GLUT2 to the BBM and a down-regulation of the energy-requiring SGLT1-mediated glucose uptake. The importance of this phenomenon, particularly in models of intestinal damage or stress, warrants further investigation.

The presence of GLUT2 in the BBM of humans has not been confirmed. Dyer *et al*[107] found GLUT2 expression was restricted to the BLM in humans, although critics of this work have speculated that this may be a result of the methods used to obtain and process biopsies, and the lack of feeding a high sugar diet or giving an oral sugar test prior to obtaining the biopsies[68].

In summary, the role of GLUT2 in the transport of sugars across the BBM remains controversial. The most recent data suggests that in specific situations, such as when luminal sugar concentrations are high, GLUT2 is recruited to the BBM and contributes to sugar uptake from the lumen. This may explain the discrepancies between studies and the inability of many researchers to detect GLUT2 in the BBM in their experimental models.

INTRINSIC ACTIVITY AND TRANSPORTER TRAFFICKING

A number of factors are involved in the regulation of intestinal sugar transport. These factors may modify sugar transport by altering the abundance of sugar transporters in the intestine. Alternatively, sugar transport may be regulated at an entirely different level. The intrinsic activity of the transporters (amount of substrate transported per unit of transporter protein) may be altered, in the absence of detectable changes in transporter abundance. Indeed, there has been a long history of reports of discrepancies between glucose uptake and the protein abundance of glucose transporters both in skeletal muscle[108], adipose[109] and in the intestine[67,103,110-114]. Changes in the intrinsic activity of glucose transporters have observed with hyperglycemia[110], diabetes[63], low luminal glucose concentrations[98] and following the activation of MAPK and PI3K[67]. The post-translational mechanism by which intrinsic activity is regulated is not known, but may involve phosphorylation or dephosphorylation of the transporter or the activation or inhibition of the transporter by a regulatory protein.

Kellett and his colleagues have shown that the PI3K pathway is involved in the modification of the intrinsic activity of GLUT2 and GLUT5[67]. Control of transport by the modulation of both the levels and activities of the transporters occurred as a result of extensive cross-talk between the extracellular signal-regulated kinase (ERK), p38, and phosphatidylinositol 3-kinase (PI 3-kinase) pathways. Activation of the p38 pathway stimulates fructose transport by increasing GLUT2 levels in the BBM, as well as increasing the intrinsic activity of GLUT2. In contrast, the ERK or PI 3-kinase pathways have regulatory effects on transporter trafficking and intrinsic activity, without having significant effects on fructose transport (Figure 3). However, these results are derived from independently modulating these pathways, when clearly there is extensive cross-talk. For example, when the ERK pathway is inhibited, fructose transport stimulated by the activation of the p38 pathway increases 50-fold, suggesting that the ERK pathway restrains the p38 pathway.

It is not known if PI3K/Akt modifies the intrinsic activity of SGLT1. However, a study by Alexander and Carey (2001) showed that orogastric IGF-1 treatment increased glucose uptake in piglets without increasing SGLT1 abundance, suggesting an effect on intrinsic activity of the transporter[115]. Inhibiting Akt blocked the increase in glucose uptake, possibly by modifying the activity of the transporter.

PI3K has also been implicated in the regulation of GLUT4 trafficking to the plasma membrane in adipocytes or muscle[108]. Despite this possibility, several studies have demonstrated that the trafficking of transporter protein to the BBM cannot fully explain changes in intestinal sugar uptake seen after IGF-1, GLP-2 or glucose administration [103,115,116]. Nevertheless, both alterations in trafficking and intrinsic activity may contribute to the changes seen in sugar uptake. Further work is required to further characterize the relative contributions of each of these mechanisms.

ALTERNATIVE THEORIES

The previously well-accepted role of GLUT2 as the sole BLM glucose transporter is also a subject of debate. The role of GLUT2 was originally based on it being immunolocalized to the BLM. However, this does not exclude the possibility of other basolateral transport pathways. Recently, GLUT2 null mice were developed, in which GLUT1 or GLUT2 was re-expressed in pancreatic β cells to enable survival. This was an important step in investigating the role of GLUT2 in sugar transport. In these animals, normal rates of glucose appearance in the tail vein blood were seen following an oral glucose load, suggesting that GLUT2 was not required for transepithelial glucose transport[117]. It is important to note that this paper has limitations, as the appearance of glucose in the tail vein is not a direct measure of intestinal sugar transport. Further work by Stumpel *et al*[118] using an isolated intestinal perfusion model demonstrated normal glucose transport kinetics despite a lack of GLUT2. This finding was noted under control conditions and following cAMP perfusion, which is known to increase glucose absorption via SGLT1[119]. Even with this accelerated apical uptake of glucose into the enterocyte, the basolateral transport of glucose did not appear to be rate-limiting.

Interestingly, sugar transport was dose-dependently inhibited by an agent that inhibits the glucose 6-phosphate translocase located in the endoplasmic reticulum (ER) membrane. Glucose 6-phosphate translocase transports glucose-6-phosphate from the cytosol into the lumen of the ER, where the active site of glucose-6-phosphatase is located. Furthermore, 3-O-methylglucose, which cannot be phosphorylated by the hexokinases, was not transported, despite the fact that it is a known substrate for both GLUT2 and SGLT1. Taken together, these findings suggest that a distinct pathway exists that involves glucose phosphorylation, transport to the ER, dephosphorylation, and release via a membrane-traffic based pathway (Figure 5). Interestingly, the expression of the glucose-6-phosphatase and the glucose-6-phosphate translocase, as determined by Northern blotting, were not increased in the GLUT2 null animals. This contrasts with the work of Gouyon *et al*[68], who used RT-PCR to demonstrate that GLUT2 null mice had increased mRNA expression of glucose-6-phosphatase.

Stumpel and colleagues[118] also noted that GLUT5 mRNA expression was increased in the GLUT2 null mice, while the expression of all other known GLUT transporters did not change. Human studies have demonstrated the presence of GLUT5 in the BLM of enterocytes[120]. The finding that fructose absorption was unaffected by GLUT2 status suggests that GLUT5 may have been present in the BLM, contributing to fructose release on the serosal surface of the enterocyte. However, the authors dismissed the possibility that fructose and glucose shared a common serosal transport system based on the observation that the release of glucose, but not fructose, was blocked by an inhibitor of the glucose 6-phosphate translocase.

Stumpel *et al*[118] also performed fructose perfusion experiments in GLUT2 null mice. The results showed

that intracellular fructose was not converted to glucose, further supporting the notion that this alternative pathway does not contribute to fructose efflux. The authors also discounted the possibility that the paracellular pathway significantly contributed to glucose absorption, as the SGLT1 inhibitor phloridzin greatly reduced glucose absorption. They concluded that a microsomal membrane traffic-based mechanism may be an important component of transepithelial glucose transport.

The investigators point out that the concept of a microsomal membrane-trafficking transport system is supported by the following observation: genes for glucose-6-phosphate translocase (G6PT1)[121] and glucose-6-phosphatase (G6PC)[122] are expressed in human intestinal cells, despite the fact that only minimal amounts of glycogen are found in jejunal biopsies[123]. Similarly, the high levels of hexokinase activity in intestinal cells [124] support the concept of an alternative transport system characterized by glucose phosphorylation and subsequent microsomal transport and trafficking.

Santer *et al*[125] re-evaluated the role of GLUT2 in intestinal sugar absorption in one patient with Fanconi Bickel syndrome (FBS). FBS is characterized by congenital GLUT2 deficiency. Oral glucose tolerance tests performed on this patient failed to demonstrate differences in breath hydrogen concentrations when compared to control subjects, indicating that sugar was not being malabsorbed, at least within the sensitivity limits of hydrogen breath testing. These findings also suggest that other mechanisms are in place to transport sugars across the basolateral membrane of enterocytes.

RECENT DISCOVERIES

The model of intestinal sugar transport is an ever-changing story. Recently, a new facilitative glucose transporter, GLUT7, has been cloned and characterized[126]. GLUT7 has a high affinity for glucose (Km = 0.3 mmol/L) and fructose (IC50 = 0.060 mmol/L), but not for galactose. GLUT7 mRNA is present in the human small intestine, colon, testis and prostate. GLUT7 protein was found in the intestine, mostly in the BBM. The transporter's high affinity led the researchers to speculate that it may be important in fructose absorption at the end of the meal, when concentrations of fructose in the intestinal lumen are low. The physiological relevance of GLUT7 is unknown, as it doesn't appear to compensate for the loss of SGLT1 in glucose-galactose malabsorption.

Tazawa *et al*[127] have also cloned SGLT4, a sodium-dependent sugar transporter found in the intestine, liver, and kidney. COS-7 cells expressing SGLT4 exhibited Na+-dependent α-methyl-D-glucopyranoside (AMG) transport activity (Km = 2.6 mmol/L), suggesting that SGLT4 is a low affinity transporter. Several sugars were able to inhibit AMG transport (D-mannose > D-glucose > D-fructose > D-galactose), suggesting that these sugars may also be substrates. However, only mannose was confirmed to be a substrate by studies demonstrating direct uptake of mannose into the cell. Because mannose is elevated in diabetes[128] and in the metabolic syndrome[128], the authors suggest that SGLT4 may be a potential therapeutic target for patients afflicted with these disorders. Further characterization of these novel intestinal transporters will add to understanding of intestinal sugar transport.

CONCLUSION

The process of intestinal sugar absorption remains a controversial topic. An increased understanding of this process will enable the development of better therapeutic strategies in conditions where the modulation of intestinal sugar transport could improve health. For example, reducing sugar absorption may be beneficial with regards to the treatment of diabetes or obesity. Conversely, stimulating sugar absorption may be desirable in patients with short bowel syndrome, or in malnourished elderly patients. Furthermore, the targeted delivery of drugs to tumour cells expressing glucose transporters is an exciting area of research that warrants further exploration.

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REVIEW

Helicobacter pylori eradication to prevent gastric cancer: Underlying molecular and cellular mechanisms

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Abstract

Numerous cellular and molecular events have been described in development of gastric cancer. In this article, we overviewed roles of *Helicobacter pylori (H pylori)* infection on some of the important events in gastric carcinogenesis and discussed whether these cellular and molecular events are reversible after cure of the infection. There are several bacterial components affecting gastric epithelial kinetics and promotion of gastric carcinogenesis. The bacterium also increases risks of genetic instability and mutations due to NO and other reactive oxygen species. Epigenetic silencing of tumor suppressor genes such as RUNX3 may alter the frequency of phenotype change of gastric glands to those with intestinal metaplasia. Host factors such as increased expression of growth factors, cytokines and COX-2 have been also reported in non-cancerous tissue in *H pylori*-positive subjects. It is noteworthy that most of the above phenomena are reversed after the cure of the infection. However, some of them including overexpression of COX-2 continue to exist and may increase risks for carcinogenesis in metaplastic or dysplastic mucosa even after successful *H pylori* eradication. Thus, *H pylori* eradication may not completely abolish the risk for gastric carcinogenesis. Efficiency of the cure of the infection in suppressing gastric cancer depends on the timing and the target population, and warrant further investigation.

Key words: Helicobacter; Cancer; Gastric acid; p53; Inflammation; Gastric atrophy; Intestinal metaplasia

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INTRODUCTION

Gastric cancer is one of the most common neoplasmas worldwide, accounting for over 870 000 new cases and over 650 000 deaths annually[1]. Mortality from gastric cancer is the second most in death from malignancies, following to lung cancer. With exceptions in countries that have developed screening programs for early diagnoses, most patients reach treatment with cancers already in advanced stages[2]. Since surgery can be palliative, and gastric cancers are largely resistant to chemotherapy and radiotherapy, advanced gastric cancer has a poor prognosis. Therefore gastric cancer still remains a major clinical challenge and a great public health concern.

Extensive epidemiologic studies have shown that *Helicobacter pylori (H pylori)* infection is a major risk factor for developing gastric cancer and its precursor lesions[3]. The bacterium affects more than 50% of the world popultion[4]. The risk of patients with *H pylori* infection developing gastric cancer is in the order of two- to six-fold according to most retrospective case-control and prospective epidemiologic studies[5]. Furthermore, some of the trials eradicating *H pylori* have shown that cure of the infection reduces development of gastric cancer in high risk populations[6-8]. Thus, a hope is emerging and growing that cure of the *H pylori* infection may reduce incidence of gastric cancer.

The goal of this review is to clarify whether eradication *H pylori* results in eradication of gastric cancer. To accomplish this, we will discuss what types of cellular and molecular events occur in the *H pylori*-infected gastric mucosa; what bacterial factors are involved in the process of gastric carcinogenesis; and what host factors are able or unable to be reversed after the cure of the infection.

CELLULAR BASIS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Histopathologic alterations

Chronic infection with *H pylori* causes active inflammation of gastric mucosa in majority of the population, although it is mostly asymptomatic. The bacterium also alters physiologic and histological behaviors of gastric mucosa, including hypochlorhydria, hyperchlorhydria, and changes in mucosal population of gastric epithelial cells that are specifically differentiated. In the hypochlorhydric population, hypergastrinemia occurs, while parietal cells do not respond to gastrin to produce acid, probably due to corpus inflammation. Apoptotic loss of superficial epithelial cells in the process of differentiation and migration in gastric glands increases[9], while proliferation of neck cells also increases possibly by some sort of compensation and by transactivation of growth stimuli in gastric mucosa[10]. In corpus mucosa, parietal cell population is also diminished in a long term, which is associated with alteration in population of other types of cells in each gland. Together with lowered density of glands partly due to inflammatory and edematous changes in subepithelial tissues, the above changes are known as gastric mucosal atrophy or atrophic gastritis. In addition, epithelial clones with incomplete and complete intestinal phenotypes emerge in the long-term process.

Currently, origins of the epithelial clones for the intestinal metaplasia have not yet been clearly determined. It is likely that epithelial clones for incomplete and complete intestinal metaplasia are developed from gastric epithelial cells by an activation of a series of genes including cdx-1/cdx-2[11-16]. In addition, bone marrow-derived adult somatic stem cells are involved in the regeneration of gastric glands, and may be another source of epithelial population[17]. Although our own study suggest that bone marrow-derived epithelial cells do not harbor permanently in a gastric gland, gastric adenocarcinomas are recently reported to be bone marrow-derived[18]. Stem cells in gastric glands locate neck region, whereas those in intestinal glands reside bottom region, a location completely different from the neck. Transdifferentiation of gastric gland cells to metaplastic cells remains an important question in gastric carcinogenesis.

Bacterial and/or host factors affecting the histologic alterations

Several pathogenic factors of *H pylori* have been demonstrated to be involved in the above alterations in gastric mucosa and the following development of gastric diseases.

Ammonia (NH_3), produced by *H pylori* urease, has been shown to cause acute gastric injury[19] in rats *in vivo* and to accelerate gastric epithelial cell death *in vitro*[19-21]. Chronic administration of NH_3, whose concentration is comparable to that found in *H pylori*-infected patients, increases epithelial cell replication and epithelial cell turnover, associated with accelerated epithelial cell death, cell exfoliation, preferential loss of parietal cells and gastric mucosal atrophy[22, 23]. The damaging effects of NH_3 on gastric mucosa are pH-dependent, while sodium hydroxide at the same pH does not cause significant mucosal injury[19]. Ammonia dissolves readily in water where it forms, and is in equilibrium with, ammonium ions (NH_4^+). With decreases in pH, NH_4^+ predominates, but increases in pH may materially increase levels of non-ionized NH_3[19]. On the other hand, *per os* administration with ammonium chloride (NH_4Cl) results in intragastric NH_4^+, and does not induce significant mucosal atrophy. Rather, NH_4Cl is reported to stimulate antral mucosa to increase gastrin release[24], which possibly induces gastric mucosal hypertrophy[25]. Therefore, not only *H pylori* but also gastric acid secretion of the host is an important determinant of gastric cell kinetics.

NH_3 also increases incidence and size of gastric adenocarcinomas in rats pretreated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)[26, 27]. Prior administration of NH_3 followed by MNNG does not increase incidence of gastric adenocarcinomas in rats, indicating that NH_3 may act as a promoter in the chemically-induced gastric carcinogenesis. Immunohistochemical analysis using bromo deoxy-uridine (BrdU) demonstrates that NH_3 increases cell replication in gastric tumors as well as non-cancerous tissues surrounding the tumors. Thus, NH_3 and the consequent host epithelial responses play important roles not only in increased cell proliferation in untransformed gastric mucosa but also in promotion of gastric cancer.

The other virulence factors including CagA and other cag pathogenicity island (PAI) proteins, VacA and adhesions have been considered to be involved in wide diversity of *H pylori*-related diseases. For an example, strains containing the cag PAI have been reported to trigger signaling cascades in gastric epithelial cells, resulting in NF-κB activation and other cellular responses. Furthermore, CagA, which can be injected into the host cells, is able to be phosphorylated in the host, and to alter epithelial morphology probably through signaling pathway similar to that of HGF/c-met[28-31]. Roles of phosphorylated CagA protein in gastric epithelium are under extensive investigation and reviewed elsewhere[32]. Since it is related to gastric inflammation, cag PAI may stimulate indirectly excessive production of reactive oxygen species, including nitric oxide, and lead to programmed cell death. Indeed, studies show conflicting results for an association between cag PAI and apoptosis[33, 34].

VacA reportedly induces gastric epithelial cell apoptosis[35, 36]. It is found that VacA also induces apoptosis of marophages and suppresses T-cell responses[37-40]. Shibayama *et al*[41] showed that γ-glutamyl transpeptidase induces apoptosis. Furthermore, several apoptotic mediators such as TNF-α, FAS-ligand, TRAILs and their receptors are reported to be upregulated[42-44]. Thus, proapoptotic factors from either the bacterium or the host appear to be involved in altered cell kinetics as well as disturbed immunologic surveillance in gastric mucosa. Once certain clones acquire the resistance from apoptotic or immunologic surveillance, they begin to grow to form clusters of neoplastic phenotypes.

MOLECULAR ALTERATIONS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Events promoting gastric carcinogenesis

Gastric cancer is divided into two histologic entities: 'in-

testinal-type' and 'diffuse-type'. These two types differ in epidemiology and clinical outcome. Molecular profiles are also distinct between these phenotypes[45-47], and actually consist of wide variety of alterations including mutations, loss of heterozygosity (LOH), and epigenetic changes of expression of unmutated genes(Table 1). It is not surprising that numerous reviews have been published regarding this topic[45-52], considering the size of population with gastric cancers or with *H pylori* infection. In diffuse-type gastric adenocarcinomas, DNA-repair errors, p16 suppression and cyclin E amplification occur frequently in early stages. In early stages of intestinal-type gastric adenocarcinomas, inactivation of APC due to LOH or mutation and nonfunctioning p53 frequently occur. Events due to changes in tumor microenvironments, *i.e.,* overexpression or transactivation of growth factors such as EGF-family growth factors (TGFα, EGF, HB-EGF, etc), insulin-like growth factors (IGF-1 and IGF-2), transforming growth factor-β, cytokines, and gastrin, also play important roles in phenotypic change in gastric epithelial cells [53, 54]. For an example, elevated gastrin may transactivate HB-EGF and its receptors, resulting in upregulation of mitogen-inducible cyclooxygenase (COX-2) and its products (prostaglandin E_2, etc)[55, 56].

Recently, COX-2[57-71] attracts attention of many oncologists and gastroenterologists. In fact, some epidemiologic studies have shown that a long-term NSAID-use results in significant reduction of incidence and mortality of digestive cancers including not only colon but also stomach[72, 73]. We have shown that the COX-2 overexpression alters cell kinetics, suppresses programmed cell death, induces invasive phenotypes, supports tumor angiogenesis and influences cell adhesion to endothelial cells[54, 70, 71, 74-79]. *H pylori* infection induces gastric COX-2 upregulation[71, 80-86], and cure of the infection reduces the COX-2 expression[70]. However, in mucosa with intestinal metaplasia, COX-2 is overexpressed even after the cure of the infection (Figure 1)[70]. Procarcinogenic effects of COX-2 on stomach could be only partially reversed by successful *H pylori* eradication. Similar findings were also observed in the case of expression of nitrotyrosine, a product of nitric oxide (NO), in precancerous gastric mucosa. Expression of nitrotyrosine is elevated in gastric mucosa in patients with *H pylori* gastritis, which is reversible after successful *H pylori* eradication. However, in gastric mucosa with intestinal metaplasia, nitrotyrosine continue to be overexpressed even after the cure of the *H pylori* infection, suggesting that NO and other reactive nitrogen species is highly produced in metaplastic lesions[70].

Mismatch repair deficiency

Microsatellite instability (MSI) is defined as the presence of replication errors in simple repetitive microsatellite sequences due to mismatch repair (MMR) deficiency[48]. It is classified as high-frequency (MSI-H), low-frequency (MSI-L) or stable (MSS)[87]. MSI has been recognized as one of the earliest changes in carcinogenesis and results in genomic instability. MSI is detected not only in gastric cancer but also in intestinal metaplasia from subjects both with and without gastric cancer[88], suggesting that MSI can be an early event in gastric carcinogenesis[89-91]. Further-

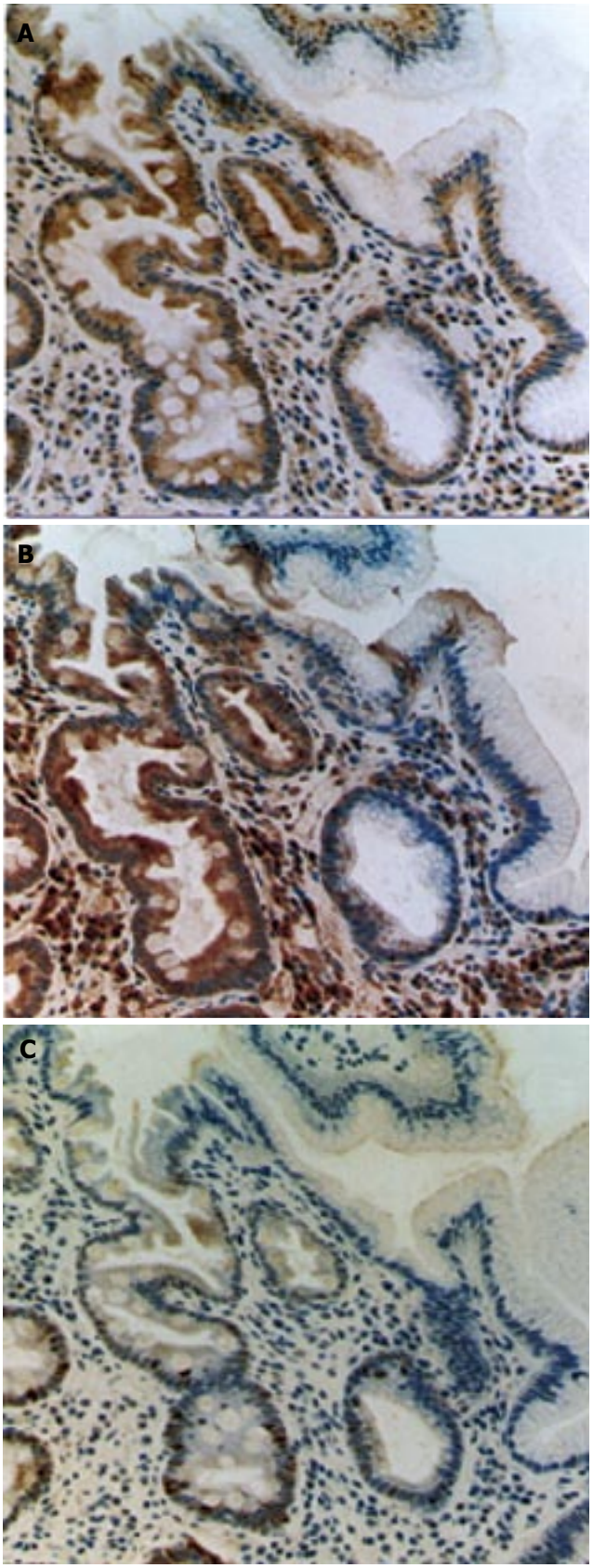

Figure 1 Expression of COX-2, nitrotyrosine and Ki-67 immunoreactivity in human gastric mucosa with intestinal metaplasia after cure of the H pylori infection. **A**: COX-2 immunostaining; **B**: nitrotyrosine immunostaining; **C**: Ki-67 immunostaining. The overexpression of COX-2 and nitrotyrosine, adduct of nitric oxide, are reported in gastric mucosa with *H pylori* infection[66, 68, 70, 71]. In these photographs, metaplastic gland with goblet cells (in the left side of each photograph) and non-metaplastic gastric glands (in the right side) are shown. COX-2 and nitrotyrosine immunoreactivities continue to exist in gastric mucosa with intestinal metaplasia after the successful *H pylori* eradication with PPI-triple therapy.

Table 1 Molecular alteration in the process of gastric carcinogenesis

Molecules	Major alterations	Comments	Category
p53	Mutation, LOH	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
APC	Mutation, LOH	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
DCC	LOH	Reported in intestinal-type adenocarcinomas. Related to cell adhesion	Tumor suppressor
CDH1	Mutation	Reported in diffuse-type adenocarcinomas.	Tumor suppressor
β-catenin	Mutation	Reported in intestinal-type adenocarcinomas.	Tumor suppressor
Fhit	LOH or deletion at chr. 3p14.2	Reported in diffuse-type and, in less frequency, intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
RUNX3	Hypermethylation	Related to TGF-β/SMAD signaling.	Tumor suppressor
K-ras	Mutation	Reported in intestinal-type adenocarcinomas. An element in signal transduction regulating cell proliferation, etc..	Oncogene
bcl-2	LOH	Reported in intestinal-type adenocarcinomas. Anti-apoptotic factor.	Oncogene
c-met	Amplification	Reported in diffuse-type and intestinal-type adenocarcinomas. The HGF receptor / tyrosine-kinase. Upregulation without mutation is also reported after mucosal injury.	Oncogene / Growth stimulus
c-erbB2	Amplification	Reported in intestinal-type adenocarcinomas. One of receptor-tyrosine kinases for EGF-family proteins.	Oncogene / Growth stimulus
Cyclin E	Amplification	Reported in diffuse-type and intestinal type adenocarciomas.	Cell cycle regulator
K-sam	Amplification	Reported in diffuse-type adenocarcinomas. One of bFGF receptor family proteins, FGFR2.	Oncogene
Mismatch repair (MMR) genes	Silencing due to hypermethylation	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions. A possible source of mutations of other genes involving gastric carcinogenesis.	Determinants of microsatelite instability (MSI)
MMR genes	Mutation	Reported in diffuse-type and intestinal-type adenocarcinomas. There are conflicting data suggesting that mucosa with intestinal metaplasia is prone to and resistant to MSI.	Determinants of MSI
EGFR	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Growth stimulus
EGF	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Growth stimulus
TGF-α	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions. Another EGF-family protein.	Growth stimulus
VEGF	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Angiogenic factor
iNOS	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions and mucosa with H. pylori.	Enzyme
COX-2	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions and mucosa with H. pylori. Cytokines and growth factors are possible inducer of COX-2.	Enzyme
ODC	Overexpression	Reported earlier in gastritis.	Enzyme
Telomerase	Activated	Enlongs telomere and prevents cell senescence.	Enzyme
CDXs	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as precancerous lesions. Is involved in intestinal metaplasia.	Transcription factor
Ets1	Overexpression	A transcription factor involving angiogenesis.	Transcription factor
NF-κB	Overexpression	A transcription factor regulating expression of proinflammatory cytokines, chemokines, iNOS and COX-2.	Transcription factor
Sp-1	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Transcription factor
SC-1	Overexpression	Reported in diffuse-type adenocarcinomas.	Apoptosis receptor
Fas/CD95	Overexpression	Reported in diffuse-type adenocarcinomas.	Apoptosis receptor
E-cadherin	Mutation	Reported in diffuse-type and intestinal-type adenocarcinomas.	Cell adhesion
CD44	Splicing variant	Reported in diffuse-type and intestinal-type adenocarcinomas.	Cell adhesion
Gastrin	Elevation in serum	Elevation of amidated gastrin is reported. Transactivates EGF-family proteins.	Gut hormone

more, hypermethylation of CpG islands in the promoter region of the hMLH1 gene is associated with decreased hMLH1 protein, and often occurs in gastric cancer cases with MSI-H, indicating that epigenetic inactivation of hMLH1 may underlie MSI[92]. MSI in gastric cancer is associated with antral tumors, intestinal-type differentiation, and a better prognosis. Cancer cases with MSI exhibit mutations in BAX, hMSH3, hMSH6, E2F-4, TGF-β receptor II, and IGF-R II, which have simple tandem repeat sequences within their coding regions[93-99]. *H pylori* infection and following gastric mucosal alteration are closely related to MSI[100-102]. In particular, Park *et al*[100] recently reported an immunohistochemical study demonstrating that DNA MMR protein expression (hMLH1 and hMSH2) decreases in patients with *H pylori* infection. Cure of the infection resulted in significant increases in the percentage of hMLH1 (76.60 ± 20.27, 84.82 ± 12.73, $P = 0.01$) and hMSH2 (82.36 ± 12.86, 88.11 ± 9.27, $P < 0.05$) positive epithelial cells[100], suggesting that the effects of *H pylori* on MSI are reversible at least in a part. On the other hand, MSI results in frame-shift mutations of hMSH3 and hMSH6, and loss of hMSH1 and hMSH2 functions, which may lead gastric

Table 2 "p53" mutation in gastric cancers of early stages and precancerous gastric lesions. In gastric cancers of early stages and precancer gastric lesions, LOH and splicing are merely reported. Abbreviations for mutation: Del: deletion; Ins: insertion; F/S: frame shift. Abbreviati for lesion: EGC: early gastric cancer; AD: adenoma, CA/AD: carcinoma in adenoma; D: dysplasia; IM: intestinal metaplasia; N: muc without dysplasia, IM or carcinoma. Data are collected from references 104, 105, 113-118. (Modified from Tsuji *et al*[119, 120])

First author	Year	Case	G:C→A:T	G:C→T:A	A:T→G:C	A:T→C:G	A:T→T:A	G or C	Del	Ins	F/S	Lesions
Yokozaki	1992	1			2	1	1	1				EGC
Tohdo	1993	5			3						1	AD or CA/AD
Uchino	1993	12	10			2		1				EGC
Correa	1994	8	4		3				1			D, IM, or N
Hongyo	1995	9	10					1	1	2		Cancer at stage I
Sakurai	1995	7	4									AD, CA/AD or EGC
Tamura	1995	1	1									AD
Tamura	1995	4	3	2								EGC
Ranzani	1995	18	13	1			1	1	1	2		EGC
Summary			45	3	8	3	2	3	4	4	1	
(%)			62	4	11	4	3	4	5	5	1	

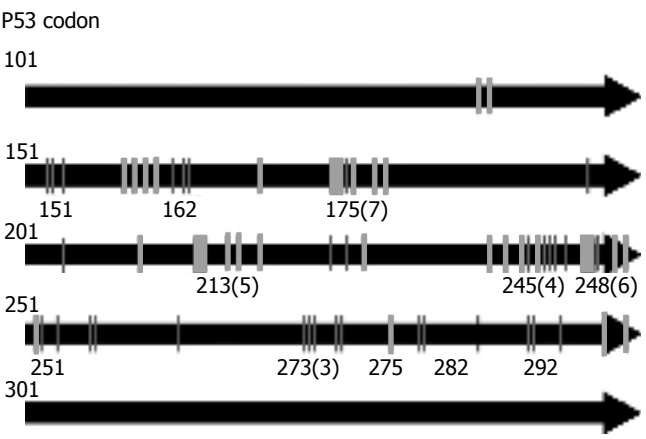

Figure 2 Location of point mutations of p53 in gastric cancers and premalignant lesions of the stomach. Horizontal lines mean codons of p53 gene. Thick and thin vertical lines respectively mean 5 and 1 mutations of the corresponding codon. Gray numbers indicate location of mutated codon followed by number of mutated cases in parentheses. As shown in this figure, codons 175, 213, 245, and 248 are preferably mutated in early stages of gastric cancer. Data are collected from references 104, 105, 113-118.

epithelial cells to further genetic instability that cannot be reverted by *H pylori* eradication. Therefore, the precise mechanism for *H pylori*-induced suppression on MMR protein has not yet been clarified and one of the important topics in *H pylori*-related gastric carcinogenesis.

Oncogenes

Certain EGF-like growth factors and their receptors are activated by membrane-type proteases called ADAMs (a disintegrin and metalloproteinase) following the stimulation including gastrin[56], endothelin and IL-8 that have G-protein coupled receptors[103]. IL-1β is also known to transactivate EGF-receptor via pathways dependent and independent of IL-8[103].

In addition, certain growth factors, their receptors and components of intracellular signaling have mutations or amplifications activating cell growth, inhibiting programmed cell death, and altering cell phenotypes. These oncogenes include HGF receptor (c-met), c-erbB2 (HER-2/neu), c-erbB3, K-sam, ras, c-myc and others, and have been reported to be mutated, amplified, or overexpressed in the process of gastric carcinogenesis[47, 52]. Once these oncogenes are mutated, it would be hardly possible for *H pylori* eradication to suppress oncogenes.

Tumor suppressor genes

Various tumor suppressor genes have been reported to be inactivated and involved in gastric carcinogenesis. For example, inactivation of p53 and p16 has been shown in both diffuse- and intestinal-type gastric cancers[52,104,105]. On the other hand, mutation of adenomatous polyposis coli (APC) gene occurs more often in intestinal-type gastric cancer. Another important tumor suppressor gene in intestinal-type gastric cancer is runt-related gene 3 (RUNX3) coding a subunit of polyomavirus enhancer binding protein 2[106-110], since expression of RUNX3 is greatly reduced in intestinal metaplasias in human stomachs[111] and Runx3-/- mouse gastric epithelial cells have a potential to differentiate into Cdx-2 positive intestinal type cells[112]. The product of the gene appears to interact with smad 2/3, which mediates TGF-β signaling pathway, and induces $p21^{WAF1/Cip1}$ expression.

Inactivation of these tumor suppressor genes includes, inactivating mutations, LOH, and epigenetic silencing. For example, hot spot mutations on CpG islands in p53 have been reported not only in gastric cancers at early stages but also in non-cancerous tissues with intestinal metaplasia [104, 105, 113-118]. In stomach, mutated p53 proteins are largely non-functioning and accumulate in the cells. Interestingly, p53 mutation frequently include G:C→A:T transition (Table 2, Figure 1)[119, 120], and NO is an important mutagen causing this type of mutation[120-122]. On the other hand, silencing of RUNX3 by promoter hypermethylation is frequently found in gastric cancers and in intestinal metaplasia. Although the silencing of tumor suppressor genes due to mutation may not be reversed, the epigenetic silencing may be reversed in methylation and demethylation processes. At present, there is no evidence indicating *H pylori per se* increases aberrant hypermethylation of tumor suppressor genes[123]: rather, Epstein-Barr virus-related gastric cancer is associated with a high frequency of DNA

hypermethylation, suggesting that viral oncogenesis might involve DNA hypermethylation with inactivation of tumor suppressor genes[124]. However, male gender, intestinal metaplasia and chronic inflammation with monocytic infiltration are strongly associated with increased methylation in non-cancerous gastric mucosa[123], and *H pylori* infection is one of the major causes of gastric inflammation. Thus, it remains an important question whether cure of the infection reduces the epigenetic alterations in tumor suppressor genes in non-transformed gastric epithelia.

Telomere and/or telomerase

Activation of telomerase that prevents shortening of telomeres during cell division may play an important role in immortalizing cells[47, 125-127]. In brief, telomeres cover the ends of chromosomes and are important in maintaining chromosomal integrity. In intestinal metaplasia, shortening of telomeres[52] as well as telomerase activation[127, 128] are observed, suggesting an important role in development of gastric cancer with intestinal type. Interestingly, it has been reported that *H pylori* infection reactivates telomerase[129, 130], and that cure of the infection appears to reduce telomerase activity[130]. Since clinical studies using human subjects may suffer from sampling errors, it remains an open question whether *H pylori* eradication reverses telomerase activation.

HOST GENETICS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Genetic predisposition affecting inflammation and acidity of stomach

Genetic predisposition plays an important role in developing gastric cancer. The most widely reported are IL1B and NAT1 polymorphisms[131-138]. The association of IL1B polymorphism and gastric carcinogenesis was hypothetically explained by El-Omar *et al*[134] to be a strong acid-inhibiting and proinflammatory capacity of the gene product. Indeed, gastric acid secretion is known to be suppressed by IL-1β, which is mediated by nitric oxide[139]. These genetic factors may have strong association with *H pylori* infection, since the bacterium induces production of interleukins, inflammation, and elevates intragastric pH, which may result in increase of xenobiotic products. On the other hand, IL-1β and IL-8 were recently reported to transactivate EGF-receptor *via* ADAM-10 activation[103]. IL-1β is also known to up-regulate COX-2 in gastric epithelium[140]. Therefore, the reason for the association of IL1B polymorphisms and the risk for gastric cancer remains an open question and may require further investigation.

Genetic predisposition possibly independent of acidity of stomach

Another example of the genetic predisposition is families of hereditary nonpolyposis colorectal cancer (HNPCC) kindred of which have an excess of gastric carcinoma; complete intestinal metaplasia and chronic atrophic gastritis restricted to the antrum[141-143]. Interestingly, HNPCC patients frequently have a mutation in one of two DNA mismatch repair genes, hMSH2 or hMLH1, and demonstrate MSI-H. As mentioned earlier, *H pylori* has an ability to decrease MMR activity. Several genetic predispositions in MSI may share the same mutations to those found in *H pylori*-induced carcinogenesis. In these cases, the bacterial infection has a potent impact on gastric carcinogenesis, since it could lower the MMR activity more than the hereditary predisposition alone.

Hereditary gastric cancer due to germline mutation of the E-cadherin has been reported[144], which is a risk factor possibly independent of *H pylori* infection.

DOES *H PYLORI* ERADICATION ERADICATE GASTRIC CANCER?

Unlike the typical adenoma-carcinoma sequence of colon, development of gastric cancer appears to be a complex process. Due to the complexity of molecular events of gastric carcinogenesis, factors discussed here do not cover every aspect of gastric carcinogenesis. Rather, we tried to overview some of the possible factors initiating, promoting and supporting the development of gastric cancer. By doing so, we discussed what types of risks exists in *H pylori* positive subjects and what extent of these risks could be withdrawn after the cure of the infection.

Certain bacterial factors affect gastric epithelial cells directly to support establishment and development of metaplastic or dysplastic clones. Successful *H pylori* eradication withdraws these bacterial factors and therefore lowers the promotional effects on tumor development. The bacterium also increases genetic instability and risks of mutation. Some host factors such as NO and other reactive oxygen species are induced by *H pylori* and increase risks of mutation. Although cure of the infection may reduce these risks leading to epithelial mutagenesis, it does not abolish the risk completely. Particularly, in gastric mucosa with intestinal metaplasia and other phenotypically altered tissues, increases in MSI and NO synthesis, as well as COX-2 overexpression are unaltered after the cure of the *H pylori* infection. Thus, *H pylori* eradication is an effective strategy in reducing the risk of gastric cancer; however, it is not efficient enough to eradicate gastric cancer. Prevention of the infection, *H pylori* immunization, *H pylori* eradication in the youth, selection of the high risk population, and alternative chemopreventive measures may be essential for optimal management of malignancy of the stomach.

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REVIEW

Interleukin-10 and chronic liver disease

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Abstract

Interleukin (IL)-10 is an important immunoregulatory cytokine produced by many cell populations. Numerous investigations suggest that IL-10 plays a major role in chronic liver diseases. IL-10 gene polymorphisms are possibly associated with liver disease susceptibility or severity. Recombinant human IL-10 has been produced and is currently tested in clinical trials. These trials may give new insights into the immunobiology of IL-10 and suggest that the IL-10/IL-10 receptor system may become a new therapeutic target.

Key words: Interleukin (IL)-10; Cytokine; Chronic liver diseases; Polymorphisms

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BIOLOGICAL CHARACTERISTICS AND REGULATION OF INTERLEUKIN-10

Interleukin-10 (IL-10) first described as a cytokine synthesis inhibitory factor for T lymphocytes produced by T helper 2 (Th_2) cell clones, can inhibit interferon (IFN)-γ synthesis in Th_1 cell clones[1]. The human IL-10 gene, a homodimer with a molecular mass of 37 ku, is located on chromosome 1 and encodes for 5 exons. Each monomer consists of 160 amino acids. X-ray crystal-structure-analysis showed the two identical intertwining polypeptide chains of 160 animo acids are rotated by 180° to each other, forming two domains in a V- shape structure, each containing six helices[2-4]. Murine and human IL-10 exhibits a homology of about 80%. Various cell populations produce IL-10 in the body, including T cell subsets (Th_2, Tc_2, Tr_1, *etc*), monocytes, and macrophages. IL-10 is produced also by various cell types in other organs, including the liver[5-6]. Also, the stress axis plays a significant role in regulating IL-10 expression *in vivo*. Inflammation of the central nervous system or indirect activation of the stress axis by endotoxemia/bacteremia triggers the release of catecholamines that up-regulate IL-10 production in macrophages, particularly in the liver[7-8]. Within the liver, production of IL-10 has been documented within hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and liver-associated lymphocytes[9]. These cells are stimulated to produce IL-10 through the cAMP/protein kinase A/CREB-1/ATF-1 signaling by several endogenous and exogenous factors such as stress, exdotoxin, tumor necrosis factor-α, catecholamines, and cAMP-elevating drugs. Recent data suggest that the p38 mitogen-activated kinase pathway also regulates the human IL-10 promoter via the activation of sp1 transcription factor[10]. IL-10 activity is mediated by its specific cell surface receptor-IL-10 receptor, which is expressed on a variety of cells, especially in immune cells[11]. Only a few copies of IL-10R are expressed on the surface of cells[12-13]. The expression is variable, but so far only a few regulating factors are known. IL-10R is composed of two different chains[14]. The interaction of hIL-10R with hIL-10 has been characterized recently and seems to be highly complex[15-16]. The IL-10Rβchain is essential for IL-10-mediated effects and CRFB4-deficient mice display the same phenotype as IL-10 deficient mice[17]. Only in cells expressing both IL-10R α and βchains, can the characteristic pattern of IL-10 signaling be observed[18]. The IL-10/IL-10R interaction activating the tyrosine kinases Jak1 and Tyk2, inhibiting the activity of NF-κB, results in transcriptional activation of several hundred genes[19]. The effects of IL-10 have been confirmed by experimental research in animals including IL-10 knockout mice[20] as well as by the effects of IL-10 observed in several inflammatory, autoimmune, and tumor models. IL-10 inhibits the ability of monocytes and macrophages to produce antigens to T cells[21-22] and monocytic production of IL-12. Inhibits proliferation and cytokine synthesis of $CD4^+$ T cells by exerting some direct effects of T cells, but does not exert potent direct inhibitory effects on $CD8^+$ T cells. IL-10 has various but weak stimulatory effects on B cells. IL-10 prevents apoptosis and enhances proliferation and differentiation of plasma cells as well as IgM synthesis, and inhibits the release of various chemokines by neutrophils. One of the most important properties of IL-10 is its anti-inflammatory action[23], which restrains the

immune response under various stimuli. Evidence of *in vivo* function of IL-10 indicates that inflammatory bowel disease is exacerated in the absence of IL-10.

EFFECTS OF IL-10 ON CHRONIC LIVER DISEASE AND LIVER FIBROSIS

Experimental data from animal models and clinical data from patients suggest that inflammation-associated cytokines including pro-inflammatory cytokines such as TNF-αand TGF-β, and anti-inflammatory cytokines such as IL-10 , are involved in the development of liver injury[24]. The effects of IL-10 have been observed in viral or autoimmune hepatitis, alcoholic liver disease, and animal models. Patients with a strong Th_1 response during acute HCV infection can clear the virus, while patients presenting with a Th_2 response (high levels of IL-10) evolve into chronicity[25]. In Con A-induced liver injury model[26-28], using a blocking IL-10 monoclonal antibody could lead to severe hepatic necrosis. On the other hand, administration of recombinant IL-10 in mice challenged with Con A could dramatically reduce secretion of pro-inflammatory cytokines, apoptosis of hepatocytes, hepatic neutrophil infiltrate and delay hepatic necrosis. In the model of liver injury induced by lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB) in D-galactosamine (GalN)-sensitized mice[29-31], treatment with IL-10 could markedly reduce serum transaminase activities in a dose-dependent manner and hemorrhagic liver damage in sensitized mice exposed to toxins. IL-10 also inhibits increases in serum TNF-alpha and IFN-gamma concentrations with the toxins. Treatment with IL-10 could significantly reduce TNF-alpha mRNA and IFN-gamma mRNA expression in the liver and spleen after administration of the toxins to sensitized mice. These findings suggest that IL-10 is capable of regulating hepatic injury *in vivo* mediated by T cells macrophages. Injury of the liver requires the participation of proinflammatory cytokines and chemokines, many of which are regulated by the transcription factor, nuclear factor κB (NFκB). Other data suggest that IL-10 protects against hepatic ischemia/reperfusion injury by suppressing NFκB activation and subsequent expression of proinflammatory mediators[32]. IL-10 has been shown to be beneficial in the setting of liver transplantation[33], treatment with IL-10 can increase allograft survival. Current studies demonstrate that IL-10 may protect against surgery-or trauma related organ injuries secondary to hepatic ischemia-reperfusion. In human alcoholic liver disease or in rats fed with alcohol, defective production of IL-10 might result in chronic liver disease, suggesting that IL-10 might be of therapeutic value for alcoholic hepatitis by decreasing hepatocyte death[34]. In the model of CCl_4-induced chronic liver injury, IL-10 deficient animals had a persistently increased inflammatory infiltrate, and developed a more extensive fibrosis than the animals able to produce IL-10, indicating that IL-10 is involved in the control of fibrogenesis[35-37]. Several studies indicate that IL-10 might play an important role in antifibrogenesis during CCl_4-induced hepatic fibrogenesis[38-39]. Hepatic stellate cells (HSCs) are involved in liver fibrogenesis since, *in vitro* experiments have shown that HSCs express IL-10 receptor and produce IL-10[40-42]. In highly purified preparations of rat HSCs, messenger RNA (mRNA) for IL-10 can be detected by reverse-transcription polymerase chain reaction (RT-PCR). Long-term incubation of unstimulated mouse HSCs secrete IL-10 protein as detected by immunoblotting and specific enzyme-linked immunosorbent assay (ELISA). IL-10 protein is undetectable by immunohistochemistry in mouse HSCs during the first 3 d of culture. The percentage of IL-10-positive cells increases to 45% on d 7 and 100% on d 14, and IL-10 continues its expression in long-term culture of up to 120 d. These data indicate that IL-10 plays an important role in liver fibrosis by suppressing the function of HSC and promoting apoptosis of HSC[43-45]. IL-10 has a direct effect on the production of collagen and collagenases, modulates remodeling of the extracellular matrix[46-47], and indirectly limits the fibrogeneic response by controlling $TGF\beta_1$ secretion.

IL-10 GENE POLYMORPHISMS IN CHRONIC LIVER DISEASE

Genetic markers in cytokine genes are widely used in studies of immune-mediated diseases to determine disease susceptibility and severity[48]. In recent years, increasing attention has been paid to the role of cytokine levels in inflammatory and immune response., which may account for some of the heterogeneity observed in the outcome of chronic liver diseases, such as HBV and HCV infection, alcoholic and autoimmune hepatic disease. Possible linkage of IL-10 promoter haplotypes to disease susceptibility or severity has been reported[49]. The IL-10 promoter is highly polymorphie with two informative microsatellites, IL-10G and IL-10R. Single nucleotide polymorphisms (SNPs) in the promoter form SNP combinations (ATA, ACC, GCC) associated with differential IL-10 expression[50]. There are several lines of evidence that ATA haplotype in the IL-10 gene promoter is relevant to a genetically low capacity for IL-10 production, whereas GCC haplotype is identified as a high IL-10-producing phenotype, suggesting that the difference in disease progression of patients results from the inheritance of the IL-10 gene promoter polymorphisms. The influence of cytokine genotypes either on different clinical features of liver disease or in the response to antiviral therapy has been evaluated in several studies. Since inadequate expression of IL-10 seems to be of pathophysiological relevance in several diseases and the expression levels seem to have a genetic background. Increased serum levels of IL-10 are often observed in chronic HCV infection and inheritance of the interleukin-10 -1082 G/G may be associated with susceptibility to chronic hepatitis C infection and resistance to combined antiviral therapy[51-53], suggesting that chronic HCV infection patients with the haplotype conferring a high production of IL-10 have a lower rate of response to interferon therapy. IL-10 promoter allelic frequencies of T and A at positions -819 and -592, as well as the frequencies of ATA haplotype at positions -1082/-819/-592, are significantly higher in asymptomatic carriers than in patients with progressive chronic liver disease, suggesting that patients with haplotype

conferring a high production of IL-10 develop chronic progressive liver disease, while patients with a lower production of IL-10 tend to be asymptomatic carriers[54, 55]. Possession of the A allele at position -627 in the IL-10 promoter (low IL-10 expression) is associated with an increased risk of advanced liver disease in heavy drinkers[56, 57].

Genetic association analysis has revealed that one of the IL-10 haplotypes, IL10-ht2 (-1082A/-819T/-592C/+117T) is strongly associated with hepatocellular carcinoma (HCC) occurrence in a dose-dependent manner[58]. The frequency of susceptible IL10-ht2 is much higher in HCC patients and significantly increased in order of susceptibility to HBV progression from chronic to cirrhosis and HCC. In addition, the onset age of HCC is also accelerated among chronic hepatitis B patients who carry IL10-ht2. Increased IL-10 production mediated by IL10-ht2 suggests that up-regulation of IL-10 accelerates progression of chronic HBV infection, to HCC.

APPLICATION OF IL-10 AS A THERAPEUTIC AGENT

The promising results from IL-10 applied to several inflammatory diseases in experimental models induce the interest in clinical application of IL-10. So far human recombinant IL-10 has been tested in healthy volunteers, patients with Crohn's disease, rheumatoid arthritis, psoriasis, hepatitis C and HIV infection[59]. In phase I clinical trials, safety, tolerance, pharmacokinetics, pharmacodynamics, immunological and hematological effects of single or multiple doses of IL-10 administered by intravenous (i.v.) or subcutaneous (s.c.) route have been investigated in healthy volunteers[60]. IL-10 is well tolerated without serious side effects at the dose of 25 μg/kg and mild to moderate flu-like symptoms are observed in a fraction of recipients at the doses of 100 μg/kg.

Single i.v. or s.c. of IL-10 results in transient dose-dependent changes in white blood cell population, including increase of total white blood cells and neutrophils, lymphocytopenia and monocytosis as well as decrease in platelet counts are observed[61]. Following i.v. administration, IL-10 serum levels initially decline rapidly but yields a less steep terminal phase. IL-10 is cleared mainly through the kidneys as indicated by the increased $t_{1/2}$ and AUC of IL-10 in patients with moderate to severe renal insufficiency. Taken together, IL-10 application induces a number of immunological changes and is well tolerated[62]. IL-10 treatment does not result in significantly higher remission rate or clinical improvement for Crohn's disease compared with placebo treatment[63]. IL-10 can prevent postoperative recurrence of Chorine's disease but the clinical results are unsatisfactory[64]. The data from rheumatoid arthritis patients are rather discouraging, showing only marginal activity of the drug[65]. For psoriasis, IL-10 is likely to have antipsoriatic activity[66].

IL-10 is able to express antifibrotic properties in experimental models of liver cirrhosis[59]. It has been postulated that *in vivo* administration of IL-10 to patients with HCV infection may shift the intrahepatic immunologic balance away from Th1 cytokine predominance, thus exerting its anti-inflammatory and subsequent antifibrotic effect[67]. It was reported that long-term therapy with interleukin-10 decreases hepatic inflammatory activity and fibrosis, but leads to increased HCV viral levels[68].

IL-10 increases the susceptibility to infections due to its immunosuppressive activity and inhibition of bactericidal activity[69]. In the future, it may be used to target the delivery of IL-10 to avoid systemic side effects and low biodisponibility. IL-10 could be delivered locally with an adenovirus in the liver[70], suggesting that anti-inflammatory cytokines may have a future in the treatment of liver injury and the prevention of its complications.

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ESOPHAGEAL CANCER

Association of smoking, alcohol drinking and dietary factors with esophageal cancer in high- and low-risk areas of Jiangsu Province, China

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Abstract

AIM: To study the main environmental and lifestyle factors that account for the regional differences in esophageal cancer (EC) risk in low- and high-risk areas of Jiangsu Province, China.

METHODS: Since 2003, a population-based case-control study has been conducted simultaneously in low-risk (Ganyu County) and high-risk (Dafeng County) areas of Jiangsu Province, China. Using identical protocols and pre-tested standardized questionnaire, following written informed consent, eligible subjects were inquired about their detail information on potential determinants of EC, including demographic information, socio-economic status, living conditions, disease history, family cancer history, smoking, alcohol drinking, dietary habits, frequency, amount of food intake, etc. Conditional logistic regression with maximum likelihood estimation was used to obtain Odds ratio (OR) and 95 % confidence interval (95% CI), after adjustment for potential confounders.

RESULTS: In the preliminary analysis of the ongoing study, we recruited 291 pairs of cases and controls in Dafeng and 240 pairs of cases and controls in Ganyu, respectively. In both low-risk and high-risk areas, EC was inversely associated with socio-economic status, such as level of education, past economic status and body mass index. However, this disease was more frequent among those who had a family history of cancer or encountered misfortune in the past 10 years. EC was also more frequent among smokers, alcohol drinkers and fast eaters. Furthermore, there was a geographic variation of the associations between smoking, alcohol drinking and EC risk despite the similar prevalence of these risk factors in both low-risk and high-risk areas. The dose-response relationship of smoking and smoking related variables, such as age of the first smoking, duration and amount were apparent only in high-risk areas. On the contrary, a dose-response relationship on the effect of alcohol drinking on EC was observed only in low-risk areas.

CONCLUSION: The environmental risk factors, together with genetic factors and gene-environmental interactions might be the main reason for this high-risk gradient in Jiangsu Province, China.

Key words: Esophageal cacner; Case-control study; Smoking; Alcohol drinking; Dietary factors

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INTRODUCTION

Esophageal cancer (EC) is the sixth most common cause of cancer mortality worldwide. The incidence of this disease shows a striking geographic variation in the world; a 20-fold variation is observed between high-risk China and low-risk western Africa[1]. Jiangsu Province, southeastern China is one of the highest EC incidence areas with a mortality rate of 30.0/100 000 between 1990-1992, which was significantly higher than the national average of 17.0/100 000[2,3]. EC has been the third leading cause of cancer mortality in Jiangsu Province since the 1970s[3]. Although the mortality rates of EC are high in most counties

of Jiangsu, national surveys conducted in the 1970s and 1990s have shown that rates differ considerably between different counties within the province, despite their similar geographic characteristics and socioeconomic status[3,4].

Comprehensive studies on the etiology and carcinogenesis of EC in high-risk areas have been carried out during the past decades. Epidemiological evidence suggests that the independent risk factors, tobacco smoking and alcohol drinking, are strongly associated with EC risk and have approximately multiplicative joint effects[5-7]. Dietary factors were also found to play an important role in the development of EC. Increased risk of EC was found to be associated with low intake of raw vegetables and fresh fruits, a deficiency in vitamins or protective antioxidants (e.g. Vitamin C and E, β-carotene, and selenium), high intake of carcinogens (frequent consumption of pickled vegetables and fungi toxins) and thermal injuries (fast eating speed for hot drinks and soups)[8-10]. While the contributory factors of EC are the high consumption of tobacco and alcoholic beverages in Western countries, the causative factors of EC in high-risk areas of China are nutrition deficiency, N-nitrosamines, fungi toxins and genetic factors[11].

Although numerous epidemiological studies have been conducted to explore the associations between environmental, lifestyle, dietary factors and the risk of esophageal cancer, few studies have been conducted to compare the association between risk factors and EC in apparently similar areas with a high risk gradient. Thus, a population-based case-control study has been conducted since 2003 in both low and high-risk areas of Jiangsu Province, China to study main environmental and lifestyle factors that account for regional differences in EC risk.

This paper reports the preliminary results on the independent and joint effects of smoking, alcohol drinking and dietary factors on EC risk and compares their associations with EC in both high-risk and low-risk areas.

MATERIALS AND METHODS

Study area

This study has been conducted in Dafeng and Ganyu counties since late 2003. Both counties are less developed coastal areas in northern Jiangsu Province, China, with 0.7 million and 1.1 million inhabitants, respectively. Dafeng is a high cancer incidence area and has a much higher mortality rate of EC than Ganyu. From 1996 to 2002, the yearly average age-adjusted mortality rate of EC in Dafeng was 36/100 000, whereas Ganyu had a considerably lower age-adjusted EC mortality rate of 24/100 000 during the same period ($P < 0.01$)[12].

Selection of cases and controls

Cases Newly diagnosed patients with primary esophageal cancer were recruited using data from regional cancer registry agencies. The cancer registry agencies in both counties were established in the late 1990s and are connected to the local Center for Disease Control and Prevention (CDC). All cases were coded according to the International Classification of Diseases, tenth revision (ICD-10, code C15.0 to C15.9). Secondary and recurrent cancers were excluded. All cases were restricted to local inhabitants of the two counties who have lived in either area for at least 5 years. In 2004, 45% and 72% of all newly registered EC cases were recruited and interviewed in Dafeng and Ganyu, respectively. The comparatively low response rate in Dafeng was partly due to the low involvement of local hospitals during the beginning of the study. A small number of cases were also unwilling to participate. Presently, the response rate in Dafeng is much higher. A system of rapid case recognition was used in the study. All regional hospitals were required by the local health authorities to report new EC patients shortly after diagnosis. As the cancer registry agencies are connected to the local CDC, the field investigators from the local CDC were able to identify and interview most patients within one month after their diagnosis. Of all the EC cases in Dafeng, 46% were histologically confirmed, 40% and 13% were diagnosed by endoscopy and radiology, respectively. In Ganyu, 30% of EC cases were histologically confirmed, 50% and 16% were diagnosed by endoscopy and radiology, respectively.

Controls Cases and controls were individually matched and derived from the same county. The criteria for the eligibility of controls were established as: controls had to be the same gender and within 5 years of age as the case, had to have lived in the area for at least 5 years, and had to have had good physical and mental health to answer questions reliably. Controls were randomly selected by a computer from the demographic database of the general population in the county police station. Local interviewers were responsible for locating and interviewing controls. If a selected control refused to participate, a replacement was found using the same recruitment criteria. The response rate of controls in both Dafeng and Ganyu was around 70%. Till March 2005, more than 400 EC cases were recruited in each county. However, the control recruitment rate is slower than the rate of case identification; thus only 291 and 240 pairs of cases and their matched controls were used in this analysis.

Data collection

Identical protocols and pre-tested standardized questionnaires were used. Data collection included a written informed consent, a face-to-face interview, a physical examination, and a 5 ml blood sample taken by professional interviewers from the local CDC in both counties. The questionnaire elicited detailed information on potential determinants of EC, including demographic information, socio-economic status, living conditions, disease history, family cancer history (any malignant neoplasm in first-degree relatives), smoking, alcohol drinking, dietary habits, and frequency and amount of food intake.

In our study, never-smokers were defined as having smoked fewer than 100 cigarettes in their lifetime. Current smokers and drinkers were defined as those who had the habit during the time of interview or those who stopped the habit because of health problems within one year of the date of interview. The dietary questionnaire used in this study included 90 food items. For each food item, the amount and frequency of consumption over the past year

were inquired. For cases, the amount and frequency of consumption referred to the year prior to the onset of the disease. In the final analysis, foods were categorized into several major groups: staple foods, preserved foods, meat, fish, eggs, soybean, and fruits and vegetables.

An anthropometric measurement and physical examination also took place at the time of interview to evaluate the subject's health status. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (i.e., kg/m^2). BMI was grouped into quartiles according to the Chinese national standard (underweight: <18.5, normal: 18.5-23.9, overweight: 24~27.9, obesity: ⩾28)[13].

Statistical analysis

Data were double entered using Epidata 2.1b and cleaned and analyzed using SAS v8.2. Chi-square and Student *t*-tests were used to compare the distribution of relevant factors among the control groups between the two counties. Conditional logistic regression with maximum likelihood estimation of parameters was applied for both univariate and multivariate analyses. This was done by transforming each matched pair into a single observation, where the explanatory variable value was the difference between the corresponding values for the case-control pair [14]. Continuous variables such as income level and amount of food intake were divided into quartiles based on the frequency distribution among control groups.

The strength of the association was quantified as odds ratio (OR) obtained from conditional logistic regression. Statistical significance was set at 0.05, and accordingly, 95% confidence interval (CI) around the OR was used to address precision.

RESULTS

Subject characteristics

There were 291 pairs of cases and controls (200 male and 91 female pairs) in Dafeng and 240 pairs (181 male and 59 female) in Ganyu, respectively. By design, cases and controls had similar distributions in terms of gender and age in both two counties (Table 1). The differences in the distribution of the above-mentioned variables between the two counties were examined by comparing the two control groups. The proportion of patients who were older than 70 years of age and the proportion of illiteracy in Ganyu were higher than that of Dafeng ($P=0.002$ and $P<0.001$, respectively). There was also a geographic variation between the two counties between the proportion of BMI ($P=0.014$), occurrences of misfortunes such as fire disasters, lost of family members, divorces, etc in the past 10 years ($P=0.008$) and family history of cancer ($P<0.001$). On the other hand, there was no significant difference in past economic status, ever-smoking and ever-alcohol drinking habits between the two counties.

Socio-economic status

EC occurred less in higher socio-economic groups characterized by high levels of education and high economic status in both counties (Table 2). On the contrary, low levels of education, low economic status, family history of cancer in first-degree relatives (Dafeng OR: 1.53, Ganyu OR: 2.07), and occurrences of misfortune in the past 10 years (Dafeng OR: 1.26, Ganyu OR: 1.64) increased the risk of developing EC in both areas. In Dafeng, when compared to the lowest quartile (underweight people) of BMI, the second quartile (normal weight people, OR = 0.45) and the third quartile (overweight people, OR = 0.26) significantly showed a reduced risk of EC; whereas the OR increased in the highest quartile (obese people, OR = 0.49). A similar association between BMI and EC risk was also found in Ganyu, although the trend was not significant.

Tobacco smoking and alcohol drinking

Consistent smoking elevated the risk of developing EC in both counties (Table 3). In Dafeng, former smokers and current smokers have a 1.93- and 2.42- fold higher risk of developing EC than never-smokers. In Ganyu former smoking and current smoking also increased the risk of developing EC (OR = 1.28 and 2.36 respectively). We found in Dafeng that smoking at an earlier age (for trend $P=0.016$), long durations of smoking (for trend $P=0.006$), and large amounts of cigarettes per day (for trend $P=0.029$) were significantly associated with increased EC risk, with an apparent dose-response relationship. However, these associations were not significant in Ganyu.

In Ganyu, subjects who had drunk alcohol tended to have a higher risk of EC (OR = 1.71, 95% CI: 1.02-2.88). Moreover, drinking at an early age (for trend $P=0.012$) and long durations of drinking (for trend $P=0.061$) showed an increasing association with EC (Table 4). A high consumption of pure ethanol per week, 10 years ago, slightly elevated the risk of EC, but no significant dose-response relationship was found. We did not find any significant association between alcohol drinking and EC in Dafeng, despite its similar alcohol drinking prevalence as Ganyu. The joint effects by smoking and alcohol drinking were also explored in both counties, but no significant interaction was observed either in Dafeng ($P=0.900$) or in Ganyu ($P=0.870$).

Dietary factors

After adjusting for potential confounders in both Dafeng and Ganyu, subjects with fast eating speeds showed an increased risk of developing EC (Dafeng: OR=4.01; Ganyu: OR=3.09). On the other hand, high food temperatures, the possibility of being exposed to grain fungi pollution, and frequent intake of fresh garlic did not influence EC risk significantly (Table 5).

With regard to the consumption of major food groups, high consumptions of fish and seafood products significantly elevated the risk of developing EC in Dafeng (for trend $P=0.024$). Staple foods, preserved foods, fruits and vegetables, and soybean, however, were not apparently associated with EC risk in either county.

DISCUSSION

This population-based case-control study, conducted in high and low-risk areas of Jiangsu Province, China demonstrated an association between tobacco smoking, alcohol drinking, dietary factors and EC. These

Table 1 Characteristics among EC cases and controls in Dafeng and Ganyu[1] *n* (%)

Characteristics	Dafeng (high-risk)		Ganyu (low-risk)		*P* value[2]
	Case (*n* = 291)	Control (*n* = 291)	Control (*n* = 240)	Case (*n* = 240)	
Gender: Male	200 (68.7)	200 (68.7)	181 (75.4)	181 (75.4)	
Female	91 (31.3)	91 (31.3)	59 (24.6)	59 (24.6)	0.088
Age (yr) Mean±SD	64.8 ± 8.6	64.6 ± 8.9	65.4 ± 10.3	65.6 ± 10.4	
<50	14 (4.8)	17 (5.8)	19 (7.9)	17 (7.1)	
50-59	61 (30.0)	59 (20.3)	48 (20.0)	51 (21.3)	
60-69	137(47.1)	137 (47.1)	78 (32.5)	76 (31.7)	0.002
70-79	71 (24.4)	69 (23.7)	77 (32.1)	78 (32.5)	
⩾80	8 (2.8)	9 (3.1)	18 (7.5)	18 (7.5)	
Level of education					
Illiterate	156 (53.6)	130 (44.7)	138 (57.7)	164 (68.6)	
Primary school	95 (32.7)	119 (40.9)	63 (26.4)	54 (22.6)	<0.001
Secondary school & above	40 (13.7)	42 (14.4)	38 (15.9)	21 (8.8)	
Past economic status (By separate cut-off points)					
Median (CNY/yr)	1250	1500	1000	775	
1 (lowest)	97 (33.5)	55 (19.0)	38 (16.2)	47 (20.3)	
2	68 (23.5)	64 (22.1)	71 (30.2)	87 (37.5)	
3	73 (25.2)	96 (33.1)	71 (30.2)	59 (25.4)	0.235
4 (highest)	52 (17.9)	75 (25.9)	55 (23.4)	39 (16.8)	
Smoking status[3]					
Neversmoker	92 (31.6)	122 (41.9)	95 (39.6)	82 (34.2)	
Former-smoker	71 (24.4)	64 (22.0)	19 (7.9)	17 (7.1)	0.067
Current smoker	128 (44.0)	105 (36.1)	126 (52.5)	141 (58.7)	
Alcohol drinking status[4]					
Never drinker	175 (60.1)	181 (62.2)	143 (59.6)	131 (54.6)	
Former drinker	5 (1.7)	7 (2.4)	7 (2.9)	7 (2.9)	0.076
Current drinker	111 (38.1)	103 (35.4)	90 (37.5)	102 (42.5)	
Encountered misfortune in past10 yr:					
No					
Yes	54 (18.6)	41 (14.2)	33 (14.0)	54 (23.3)	
	237 (81.4)	248 (85.8)	203 (86.0)	178 (76.7)	0.008
History of family cancer					
No	112 (38.5)	86 (29.6)	16 (6.7)	29 (12.1)	
Yes	179 (61.5)	205 (70.5)	224 (93.3)	211 (87.9)	<0.001
Body mass index					
<18.5	60 (20.8)	27 (9.3)	18 (7.6)	31 (13.4)	
18.5-23.9	182 (63.0)	192 (66.2)	161 (67.9)	155 (67.1)	0.014
23.9-27.9	34 (11.8)	60 (20.7)	46 (19.4)	28 (12.1)	
⩾28	13 (4.5)	11 (3.8)	12 (5.1)	17 (7.4)	

[1] Some strata do not match the total because of missing values; [2] The *P*-value for comparing the distribution of factors between the two counties; [3] Never-smokers and ever-smokers were used for comparing the smoking habits between the two counties; [4] Because of the few numbers of former drinkers in both two counties, alcohol drinking status was categorized to never-drinkers and ever-drinkers for the comparison between the two counties and following analyses.

associations were compared separately in the two regions which had similar socioeconomic status and geographic characteristics. From our awareness, this is the first comparative population-based case-control study conducted in low-risk and high-risk areas simultaneously to compare the different associations of risk factors and EC in similar areas with high-risk gradients. Like other epidemiological researches, our study showed that EC was inversely associated with socioeconomic status, such as level of education and income. However this disease was more frequent among subjects with smoking and alcohol drinking habits and unhealthy dietary factors. Furthermore, a geographic variation of some associations was observed between the low-risk and high-risk areas. Smoking elevated the risk of EC in both areas concordantly, but the dose-response relationship of smoking and smoking related variables (age of first smoking, duration and amount) was apparent only in the high-risk area. On the contrary, the effect of alcohol drinking on EC and a dose-response relationship was only observed in the low-risk area.

Supporting previous studies, the risk of EC was inversely associated with socioeconomic status in the present study[15,16]. People with higher levels of education and better financial situations tend to have a lower risk of developing EC due to good living conditions and better health care access. Increased risk was found in people who had encountered misfortune in the past 10 years (Dafeng OR: 1.26, Ganyu OR: 1.64), or had a history of family cancer in first-degree relatives (Dafeng OR: 1.53, Ganyu OR: 1.57). These results were consistent in both high-risk and low-risk areas.

Associations between body mass index (BMI) and EC have been explored in several studies. Chow *et al*[17] reported a tendency towards a decreasing risk of esophageal squamous cell carcinoma with increasing BMI. Engeland found that low BMI increased the risk

Table 2 OR[1] and 95% CI of socioeconomic status in EC of Dafeng and Ganyu

Socioeconomic status	Dafeng (high-risk)	Ganyu (low-risk)
Level of education		
Illiterate	1.00 (Referent)	1.00 (Referent)
Primary school	0.54 (0.35-0.84)	0.58 (0.33-1.03)
Secondary school & above	0.74 (0.39-1.41)	0.42 (0.21-0.83)
P value for trend	0.08	0.008
Past economic status		
1 (lowest)	1.00 (Referent)	1.00 (Referent)
2	0.67 (0.43-1.06)	1.03 (0.61-1.75)
3	0.44 (0.28-0.68)	0.76 (0.44-1.31)
4 (highest)	0.39 (0.23-0.65)	0.73 (0.41-1.28)
P value for trend	<0.001	0.024
Encountered misfortune in past 10 yr		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.26 (0.80-1.98)	1.64 (0.98-2.73)
History of family cancer		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.53 (1.06-2.19)	2.07 (1.03-4.17)
Body mass index		
<18.5	1.00 (Referent)	1.00 (Referent)
18.5-23.9	0.45 (0.26-0.76)	0.50 (0.28-0.90)
23.9-27.9	0.26 (0.13-0.50)	0.36 (0.17-0.75)
⩾28	0.49 (0.18-1.33)	0.80 (0.33-1.98)
P value for trend	0.002	0.376

[1] Matched by age and gender, further adjusted for education level and past economic status (quartile).

Table 3 OR[1] and 95% CI of tobacco smoking in EC of Dafeng and Ganyu

Tobacco smoking	Dafeng (high-risk)	Ganyu (low-risk)
Smoking status[2]		
Former smoker	1.93 (0.91-4.08)	1.28 (0.28-5.83)
Current smoker	2.42 (1.28-4.56)	2.36 (0.89-6.26)
P value for trend	0.005	0.07
Age at first smoke[2]		
<20	2.02 (0.93-4.38)	1.60 (0.31-7.90)
20-34	2.32 (1.15-4.67)	2.25 (0.80-6.35)
⩾35	1.80 (0.62-5.24)	0.98 (0.29-3.24)
P value for trend	0.016	0.249
Duration of smoking (yr)[2]		
1-29	1.61 (0.67-3.86)	1.44 (0.46-4.42)
30-49	2.65 (1.28-5.49)	2.04 (0.60-6.92)
⩾50	2.04 (0.78-5.35)	1.98 (0.43-9.11)
P value for trend	0.009	0.194
Amount of smoking (Cig/d)[2]		
1-9	1.36 (0.50-3.74)	1.12 (0.27-4.68)
10-19	2.21 (1.01-4.80)	1.56 (0.42-5.78)
⩾20	2.04 (1.00-4.18)	0.91 (0.32-2.61)
P value for trend	0.015	0.915
Total consumption of cigarettes[2]		
1 (lowest)	1.40 (0.61-3.21)	0.96 (032-2.82)
2	2.55 (1.06-6.14)	3.50 (0.37-32.8)
3	1.88 (0.79-4.49)	1.94 (0.25-14.7)
4 (highest)	1.81 (0.57-5.74)	0.74 (0.19-2.81)
P value for trend	0.029	0.959

[1] Matched by age and gender, further adjusted for level of education, past economic status (group) and alcohol drinking; [2] Never-smokers were used as the reference group.

of esophageal squamous cell carcinoma, while high BMI increased the risk of esophageal adenocarcinoma. In general, lowest BMI had the highest risk of EC[18]. Our study found similar results in both low-risk and high-risk areas. The risk of developing EC was significantly lower in normal and overweight groups when compared to the underweight group. However, the OR was high in the obese group. An increased risk of esophageal adenocarcinoma among obese persons has been explained by a dose-dependent association between increasing BMI and the risk of gastro-esophageal reflux symptoms, as observed by Nilsson *et al*[19].

Table 4 OR[1] and 95% CI of alcohol drinking in EC of Dafeng and Ganyu

Alcohol drinking	Dafeng (high-risk)	Ganyu (low-risk)
Alcohol drinking		
Never	1.00 (Referent)	1.00 (Referent)
Ever	1.01 (0.70-1.46)	1.71 (1.02-2.88)
P value	0.964	0.043
Age of first drink[2]		
<20	0.83 (0.44-1.58)	2.59 (1.03-6.50)
20-34	1.23 (0.79-1.91)	1.95 (1.08-3.53)
⩾35	0.81 (0.48-1.35)	1.18 (0.56-2.47)
P value for trend	0.815	0.012
Duration of drinking (yr)[2]		
1-24	0.96 (0.56-1.59)	1.28 (0.58-2.79)
25-34	0.89 (0.48-1.64)	1.48 (0.75-2.94)
35-44	1.57 (0.92-2.70)	1.47 (0.71-3.01)
⩾45	0.77 (0.43-1.40)	1.88 (0.95-3.75)
P value for trend	0.834	0.061
Alcohol consumption 10 years ago[2] (pure ethanol mL/wk)		
1-249	0.87 (0.49-1.54)	0.79 (0.36-1.74)
250-499	1.06 (0.60-1.89)	0.61 (0.30-1.25)
500-749	0.97 (0.52-1.79)	1.63 (0.77-3.43)
⩾750	1.10 (0.63-1.93)	1.27 (0.71-2.28)
P value for trend	0.74	0.223

[1] Matched by age and gender, further adjusted for level of education, past economic status (group) and tobacco smoking; [2] Never-drinkers were used as the reference group.

In conformity with other epidemiological studies shown in Western countries and some areas of Asia and Africa[20-22], increased risks of EC among former smoking and current smoking subjects were observed in both areas of our study. Tobacco smoke contains over 3000 constituents including 30 carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and N-nitrosamines. The metabolites of these carcinogens may lead to gene mutation and cancer[23]. Age of first smoke, duration and dosage of tobacco use were also strongly associated with an elevated risk of developing EC in Dafeng, with an apparent dose-response relationship. Although Ganyu had a similar smoking prevalence, these time and dosage dependent results were not statistically significant in this area.

Several studies have reported a strong correlation between EC and alcohol abuse[24,25]. Alcoholic beverages also contain carcinogens and other compounds and may facilitate the absorption of esophageal mucosal cells and make them more susceptible to chemical carcinogens[26]. On the contrary of tobacco smoking, the positive association between alcohol drinking and EC was only found in Ganyu in our study (OR=1.71). Several studies

Table 5 OR and 95% CI of dietary habits, food consumption in EC of Dafeng and Ganyu

Dietary factor	County	Category				*P* value for trend
		1 (lowest)	2	3	4 (highest)	
Food temperature[1]	Dafeng	1	0.51 (0.24-1.09)	-	-	0.08
(1-Normal; 2- Hot)	Ganyu	1	1.14 (0.55-2.41)	-	-	0.714
Eating speed[1]	Dafeng	1	4.01 (1.87-8.62)	-	-	<0.001
(1-Normal; 2-Fast)	Ganyu	1	3.09 (1.24-7.70)	-	-	0.015
Self reported grain fungi pollution[1]	Dafeng	1	2.27 (0.79-6.54)	-	-	0.131
(1-Likely; 2-Not likely)	Ganyu	1	1.18 (0.45-3.11)	-	-	0.741
Fresh garlic/wk[2,3]	Dafeng	1	0.64 (0.26-1.60)	-	-	0.337
	Ganyu	1	1.17 (0.57-2.41)	-	-	0.664
Staple foods[1]	Dafeng	1	0.45 (0.19-1.10)	0.54 (0.21-1.38)	0.73 (0.26-2.04)	0.474
	Ganyu	1	0.98 (0.10-9.77)	0.45 (0.05-4.38)	0.54 (0.05-4.34)	0.324
Meat[4]	Dafeng	1	0.73 (0.29-1.85)	1.66 (0.68-4.10)	1.93 (0.64-5.77)	0.16
	Ganyu	1	0.54 (0.12-2.42)	1.17 (0.28-4.92)	0.65 (0.11-3.67)	0.305
Fish and seafood products[4]	Dafeng	1	1.14 (0.64-2.03)	2.11 (1.12-3.96)	1.91 (1.00-3.64)	0.023
	Ganyu	1	0.98 (0.42-2.28)	0.64 (0.28-1.44)	1.04 (0.46-2.33)	0.794
Eggs[4]	Dafeng	1	0.53 (0.20-1.44)	1.23 (0.54-2.80)	1.99 (0.72-5.49)	0.146
	Ganyu	1	0.69 (0.28-1.73)	0.30 (0.10-1.10)	0.95 (0.41-2.22)	0.936
Soybean[4,5]	Dafeng	1	1.81 (0.88-3.74)	-	-	0.11
	Ganyu	1	1.31 (0.37-4.59)	-	-	0.677
Preserved foods[4]	Dafeng	1	0.26(0.09-0.75)	0.49 (0.16-1.46)	0.94 (0.37-2.36)	0.635
	Ganyu	1	1.05 (0.37-2.97)	0.56 (0.21-1.48)	1.21 (0.46-3.20)	0.932
Vegetables[4]	Dafeng	1	1.26 (0.50-3.16)	0.94 (0.39-2.30)	1.37 (0.49-3.83)	0.72
	Ganyu	1	0.34 (0.08-1.54)	0.80 (0.20-3.18)	0.76 (0.15-3.72)	0.889
Fruits[2]	Dafeng	1	1.02 (0.42-2.47)	0.42 (0.16-1.12)	1.23 (0.51-2. 98)	0.802
	Ganyu	1	1.61 (0.68-3.80)	1.13 (0.43-2.95)	1.17 (0.41-3.37)	0.746

[1] Matched by age and gender, further adjusted for level of education, past economic status (group), smoking, alcohol drinking, BMI group, cancer family history, eating speed, food temperature and self-reported grain fungi pollution; [2] Matched by age and gender, further adjusted for education level, past economic status (group), smoking, alcohol drinking, BMI group, eating speed and family history of cancer; [3] Less than 3 times per week=1, 3 times per week and above=2; [4] Matched by age and gender, further adjusted for level of education, past economic status (group), smoking, alcohol drinking, BMI group, cancer family history, eating speed and food temperature; [5] Categorized by median among controls.

have reported a linear relationship between an overall daily ethanol consumption and EC risk[27,28]. However, in our study only the age of initial drinking and years of alcohol drinking were found to be associated with EC risk in Ganyu. No clear relationship between daily alcohol consumption and EC was found.

The interaction between tobacco smoking and alcohol drinking has been studied in many researches. It has been suggested that alcohol and tobacco interact in a multiplicative way[29,30]. In a large scale study, Castellsagué reported that the risk of EC in the highest joint level of alcohol and cigarette smoking increased 50.85-fold and 35.34-fold among men and women[7]. However the joint effect of smoking and alcohol was not found to be statistically significant in the high-risk area and the low-risk area in our study. The link between smoking, alcohol and EC in China are not as apparent as in Western countries. Several previous studies conducted in other high-risk areas of Jiangsu, China either did not find any relation or found only a weak association between smoking, alcohol drinking and EC[31,32].

Dietary factors are thought to play an important role in the pathogenesis of EC. Some epidemiological studies have suggested that the risk of EC is inversely associated with a higher intake of fruits and vegetables[10,33], while a detrimental effect was observed among high intake of certain types of meat, butter and saturated fatty acids[34]. Increased risk was related to N-nitrosamine compounds (mainly from preserved foods), foods contamination by fungus and the presence of toxins. Some unhealthy dietary habits such as fast eating speeds, consumption of hot foods and soups can cause the injury of esophageal mucosa and render the mucosa more susceptible to carcinogens.

An increased OR was found among fast eating subjects in both areas of this study (Dafeng OR: 4.01, Ganyu OR: 3.09). However, the associations between high food temperatures, the possibility of fungi pollution of grain, frequent intake of fresh garlic and EC was not statistically significant. In the food group analysis, after adjusting for potential confounders, we did not find any significant association between major food consumption and EC risk in either area. A positive association of fish and seafood product intake in Dafeng was found (for trend $P=0.024$). Fish is a rich dietary source of n-3 fatty acids. It has been reported that this long chain of fatty acid can suppress mutation, inhibit cell growth, and enhance cell apoptosis, thus reducing the risk of developing cancer[35]. The contradicting results found in our study of the increased risk found in the association between fish consumption and EC in Dafeng was probably due to water contamination or other unidentified confounders. However, this hypothesis needs to be further clarified and studied. Moreover, it may be more reasonable to study food composition and micronutrients in our future analysis rather than to use individual foods or food groups[36].

Ganyu has a high proportion of ageing and illiterate residents. The economic status of residents in Ganyu is also lower than Dafeng, although the difference is not significant (Table 1). As disease is more prevalent

among ageing populations and the level of education and economic situation are inversely associated with the risk of developing EC, it can be expected that the two counties would have a far higher risk gradient if they had a similar distribution of age and socioeconomic related factors.

As mentioned above, a heterogeneous association between smoking, alcohol drinking and EC was observed in the low- and high-risk areas in our study, despite their similar geographic characteristics and general socioeconomic statuses. A malignant tumor is the result of a series of DNA alterations in a single cell, which leads to a loss of normal functioning. A large number of gene coding for enzymes and receptors are involved in xenobiotic metabolism, with many of them showing polymorphisms. Many molecular epidemiological studies have proved that polymorphisms in activation and detoxification enzymes can interact with environmental carcinogens. It has been reported that GSTM1 null carriers may be especially susceptible to the action of tobacco with regards to EC[32], while inactive the ALDH2 genotype increases the risk of EC in alcoholics[37]. Genetic polymorphisms can interact with dietary factors. For example, cruciferous vegetables can inhibit the metabolic activation of phase I enzymes and induce the detoxification of carcinogens via phase II enzymes[38]. The polymorphism of one gene may also have an effect on other genes. Gene-gene interactions between GSTM1 0/0 and CYP1A1 and CYP1A2 enzyme induction have been observed in smokers[39]. Another example is that individuals with CYP1A1 Ile/Val alleles have greater CYP1A2 activity than those with wild type CYP1A1[40]. Furthermore, it has been suggested that genes can influence individual behaviours such as smoking, alcohol drinking and excess calorie intake, thereby having the potential to affect cancer risk[41].

Both environmental factors and human genes can show considerable regional variability. The variation in these factors together with their separate and joint effects ultimately determine the risk of cancer in different regions and may be the main reason for the large EC risk gradient between the counties in Jiangsu Province. Unfortunately scientific evidence on genetic polymorphisms, gene-environmental and gene-gene interactions remains inconsistent and inconclusive because of low statistical power and few candidate genes in previous studies. Moreover, no study has ever been conducted to compare the association between gene-environmental interaction and EC risk in apparently similar areas with a high risk gradient. Therefore, our future study will focus on genetic polymorphisms and their interactions with different environmental, lifestyle and dietary factors in the etiology of EC in high and low-risk areas, with a sufficient sample size and candidate genes.

Our present population-based case-control study has some limitations. Differences in the etiological factors between esophageal adenocarcinoma and squamous cell carcinoma may exist. Because of the low histological examination rate in China, it is difficult to differentiate between the subtypes of EC in a population-based study. Additionally, most risk factors in our study are based on self-reported data and may be subject to recall bias. Moreover, the relationship between BMI and EC was examined by using height and weight measurements obtained at the time of interview. Some cases might have begun to lose weight at an earlier time because of the disease. This factor could also have caused bias in our study.

In summary, the present study demonstrated the association between smoking, alcohol drinking, dietary factors and EC risk in the low-risk and high-risk areas of Jiangsu Province, China. Heterogeneous effects of smoking and alcohol drinking were found between the two areas, despite their similar geographic characteristics and general socioeconomic status. The variation in environmental risk factors, together with gene-environment and gene-gene interactions may be the main reason for these heterogeneous associations and may contribute to the large risk gradient of EC mortality in Jiangsu Province, China.

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GASTRIC CANCER

Effects of tachyplesin and n-sodium butyrate on proliferation and gene expression of human gastric adenocarcinoma cell line BGC-823

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Abstract

AIM: To investigate the effects of tachyplesin and n-sodium butyrate on proliferation and gene expression of human gastric adenocarcinoma cell line BGC-823.

METHODS: Effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells were determined with trypan blue dye exclusion test and HE staining. Effects of tachyplesin and n-sodium butyrate on cell cycle were detected by flow cytometry. Protein levels of c-erbB-2, c-myc, p53 and p16 were examined by immunocytochemistry.

RESULTS: The inhibiting effects were similar after 2.0 mg/L tachyplesin and 2.0 mmol/L n-sodium butyrate treatment, the inhibitory rate of cellular growth was 62.66% and 60.19% respectively, and the respective maximum mitotic index was decreased by 49.35% and 51.69% respectively. Tachyplesin and n-sodium butyrate treatment could markedly increase the proportion of cells at G_0/G_1 phase and decrease the proportion at S phase. The expression levels of oncogene c-erbB-2, c-myc, and mtp53 proteins were down-regulated while the expression level of tumor suppressor gene p16 protein was up-regulated after the treatment with tachyplesin or n-sodium butyrate. The effects of 1.0 mg/L tachyplesin in combination with 1.0 mmol/L n-sodium butyrate were obviously superior to their individual treatment in changing cell cycle distribution and expression of c-erbB-2, c-myc, mtp53 and p16 protein. The inhibitory rate of cellular growth of BGC-823 cells after combination treatment was 62.29% and the maximum mitotic index was decreased by 51.95%.

CONCLUSION: Tachyplesin as a differentiation inducer of tumor cells has similar effects as n-sodium butyrate on proliferation of tumor cells, expression of correlative oncogene and tumor suppressor gene. It also has a synergistic effect on differentiation of tumor cells.

Key words: Tachyplesin; n-sodium butyrate; Gastric adenocarcinoma cell; Cell differentiation

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INTRODUCTION

Tachyplesin, isolated from acid extracts of hemocytes of Chinese horseshoe crab (*Tachypleus tridentatus*), a sort of marine arthropod known as "live fossil", is a low-molecular-weight polypeptide [1]. This bioactive component has antitumor effect and induces tumor cell differentiation [2]. We used the small molecule polar compound n-sodium butyrate[3] as a parallel control to compare the effects on proliferation, cell cycle and expression of correlative oncogene and tumor suppressor gene of BGC-823 cells treated with tachyplesin, n-sodium butyrate and their combination. This research could provide information for further identification of antitumor low-molecular-weight crude bioactive peptides such as tachyplesin that can modulate the proliferation and differentiation of tumor cells.

MATERIALS AND METHODS

Tachyplesin isolation and extraction

Tachyplesin was isolated from acid extracts of Chinese horseshoe crab *(Tachypleus tridentatus)* hemocytes as previously described[4]. The crude extract was separated by SephadexG-50, CM-sepharose CL-6B column chromatography.

Cell culture

BGC-823 cells were cultured in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum,100 units/mL penicillin,100 mg/L streptomycin and 50 mg/L kanamycin at 37℃ in atmosphere containing 50 mL/L CO_2. BGC-823 cells were treated with culture medium containing inducers after seeded for 24 h.

Inducing treatment

The powder of tachyplesin obtained from separation, purification and lyophilization was dissolved in D-Hank's solution to prepare 100 mg/L concentrated solution. The mother liquor was prepared for solution of a given concentration with culture medium. N-sodium butyrate purchased from Sigma Co., was dissolved in appropriate concentration of D-Hank's solution to prepare 200 mmol/L concentrated solution. The concentrations of the three treatment solutions were as follows: 2.0 mg/L tachyplesin-treatment (Ta), 2.0 mmol/L n-sodium butyrate-treatment (Tb), and 1.0 mg/L tachyplesin + 1.0 mmol/L n-sodium butyrate for the combination treatment (Ta+Tb). The experimental groups were treated with the three reagents after medium was changed, while the control group was cultured continuously with fresh culture medium for future use.

Determination of cell growth curve

BGC-823 cells were collected in logarithmic phase, then suspension of BGC-823 cells was made in 5.0×10^4 cells/mL. The cells were seeded into 15 mL culture flasks with 2 mL per flask. After seeded for 24 h, the experimental groups were treated with the reagents containing different kinds of differentiation-induced gradients while the control group was cultured continuously in fresh culture medium. During the first seven days, untreated or treated cells were harvested from three flasks everyday, and the viable cells were counted by the trypan blue dye exclusion test to get average value. The similar results were found in triplicate experiments, the growth curve was derived from one of the results.

Determination of cell mitotic index

BGC-823 cells (5.0×10^4 mL) were seeded in to bottles containing little penicillin with cover slips. Treatments were performed after the cells were seeded for 24 h. During the first seven days, the cover slips were removed from two bottles of the untreated or treated cells everyday, fixed in Bouin-Hollande fixative, and stained with Hematoxylin-Eosin (HE). The mitotic cells in 1000 cells on each cover slip were counted, and the mitotic index curve was drawn.

Determination of cell cycle

BGC-823 cells were collected respectively from the treated groups and the control group after digested and centrifuged at 1000 r/min for 5 min. All the cells collected were rinsed three times with D-Hank's solution. The cells grown on cover slips were fixed in 75% pre-cooled ethanol at 4 ℃ overnight, centrifuged and resuspended in 100 mg/L RNase at 37℃ for 30 min. Then 50 mg/L propidium iodide was added into the suspended cells at 4 ℃ in dark for 30 min. The cell cycle was analyzed by flow cytometry (Bacton-Dickson Co.) and the data were analyzed by Cell FIT cell cycle analysis software（Version2.01.2）.

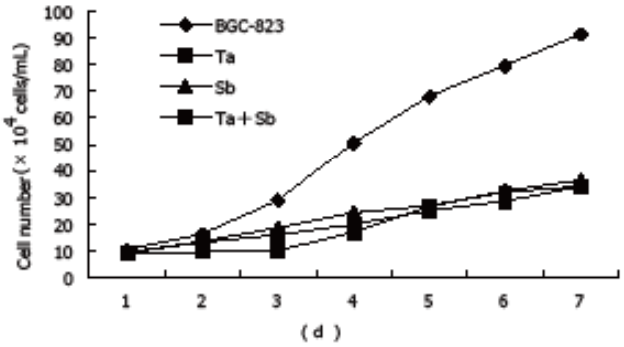

Figure 1 Cell growth inhibition in BGC-823 cells after tachyplesin, n-sodium butyrate and their combination treatment (×10^4 cells/mL).

Immunohistochemical analysis

BGC-823 cells (5.0×10^4/mL) and cells treated with different inducers were seeded into bottles containing little penicillin with cover slips for 36 h respectively. The cells grown on cover slips were rinsed with D-Hank's solution at 37℃. The changes in c-erbB-2, c-myc , p53 and p16 expression of treated and untreated BGC-823 cells were determined by SABC immunohistochemical assay. The reagent kit (Wuhan Boster Bioengineering Corporation) was used to determine the expression of these genes. PBS was used to take the place of primary antibody as the negative controls and positive specimens were used as positive controls.

RESULTS

Effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells

The cell growth curve determination showed that the proliferation of untreated BGC-823 cells was very fast. The cell number increased to 91.29×10^4/mL on the seventh day which was 18.26 times of that of the original 5.0×10^4/mL on the first day, with a doubling time of 45.82 h. However, after treated with tachyplesin or n-sodium butyrate, the growth rate of BGC-823 cells was inhibited. After treatment with 2.0 mg/L tachyplesin, the number of cells was 34.09×10^4/mL which was 6.8 times of that of the original number on the 7th day, with the doubling time prolonged to 75.7 h and the growth inhibitory rate increased to 62.77%. After treatment with 2.0 μg/mL n-sodium butyrate, the number of cells was 36.34×10^4/mL which was 7.27 times of that of the original number on the 7th day, the doubling time was 67.10 h and the growth inhibitory rate was 60.19%. After treatment with 1.0 mg/L tachyplesin + 1.0 mmol/L n-sodium butyrate, the number of cells was 33.87×10^4/mL on the 7th day, the doubling time was 69.75 h and the growth inhibitory rate was 62.29% (Figure 1).

The cell mitotic index determination showed that BGC-823 cells had vigorous proliferation capability, which reached to the divided peak on the fourth day and the maximum mitotic index was 38.5‰. However the mitotic

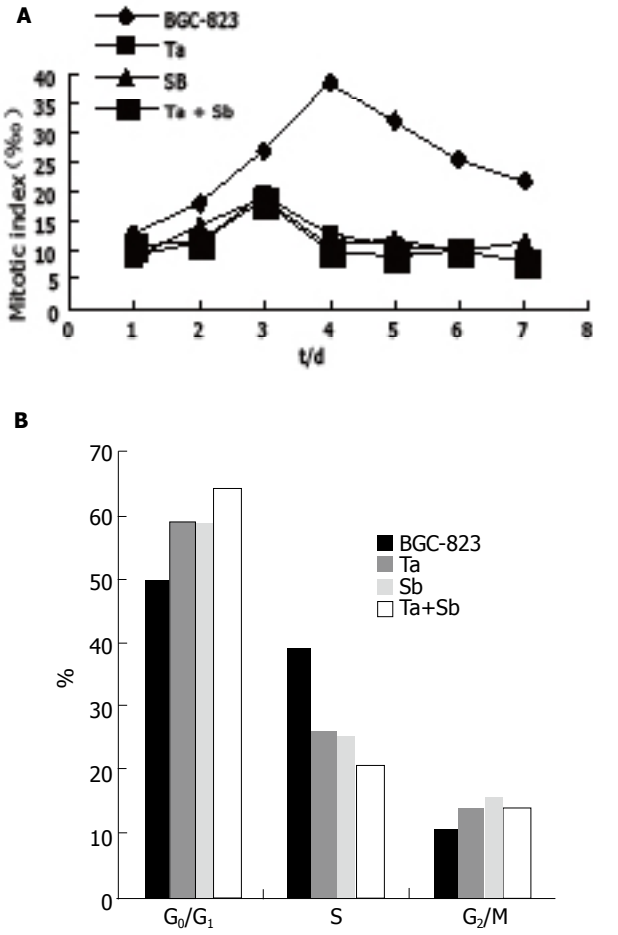

Figure 2 Influence of tachyplesin, n-sodium butyrate and their combination on mitotic index (**A**) and cell cycle (**B**) of BGC-823 cells.

index of cells treated with tachyplesin or n-sodium butyrate or their combination was only 19.5‰, 18.6‰ and 18.5‰ respectively at the divided peak, and declined by 49.35%, 51.69% and 51.95% respectively. The divided peak occurred on the third day after inducing treatment (Figure 2A).

Effects of tachyplesin and n-sodium butyrate on cell cycle of BGC-823 cells

Cell cycle of BGC-823 cells was analyzed by flow cytometry. The results showed that the cell cycle distribution of BGC-823 cells changed obviously when the cells were treated with tachyplesin or n-sodium butyrate. The proportion of untreated cells was 50.7% in G_0/G_1 phase, 39.1% in S phase and 10.2% in G_2+M phase. However, the proportion of cells at G_0/G_1 phase was 59.5%, 26.3% at S phase and 14.2% at G_2+M phase after treated with 2.0 mg/L tachyplesin. Similar changes occured in cells treated with 2.0 mmol/L n-sodium butyrate r the proportion of cells in G_0/G_1 phase was 59.2%, 25.2% in S phase and 15.6% in G_2+M phase. Meanwhile, the proportion of BGC-823 cells increased from 50.7% to 64.5% in G_0/G_1 phase and decreased from 39.1% to 20.9% in S phase(Table 1). The proportion of cells in G2+M phase increased from 10.2% to 14.6%(Figure 2B).

Table 1 Influence of tachyplesin, n-sodium butyrate and their combination on cell cycle of BGC-823 cells (%)

Group	G_0/G_1	S	G_2/M
BGC-823	50.7	39.1	10.2
Ta	59.5	26.3	14.2
Sb	59.2	25.2	15.6
Ta+Sb	64.5	20.9	14.6

Effects of tachyplesin and n-sodium butyrate on expression of oncogene and tumor suppressor gene of BGC-823 cells

Immunocytochemistry showed that c-erbB-2 protein level in the control group was high. The dark brown-yellow granules were mainly detected in cytoplasm and membrane of BGC-823 cells. But in nuclei, the protein level was low, while a few unevenly-distributed buff granules could be detected (Figure 3A). After treated with tachyplesin, the positive rate of c-erbB-2 expression in BGC-823 cells decreased. The brown-yellow particles were mainly detected in cytoplasm around nuclear membrane but a few in nuclei (Figure 3B). After treated with n-sodium butyrate, relatively bigger brown-yellow granules were mainly detected in nuclei and cytoplasm around the nuclei but a few around the cell membrane(Figure 3C). After treated with the combination of the two inducers, the immunocytochemistry signal became weaker than after treated with n-sodium butyrate alone. The smooth and evenly-distributed brown-yellow granules were mainly detected in nucleoli and nuclear membrane of the cells (Figure 3D).

Immunocytochemistry showed that the c-MYC protein level in the control group was high, the brown granules were mainly detected in nuclei and cytoplasm around the nuclei of BGC-823 cells (Figure 3E). After treated with tachyplesin, the level of c-MYC protein in BGC-823 cells decreased. The light brown-yellow granules were mainly detected in karyoplasms around the nuclear membrane, while in nuclei, the protein level was very low (Figure 3F). After treated with n-sodium butyrate, the brown particles were mainly detected in nucleoli and cytoplasm, while in nuclei of the cells, the signal was almost negative (Figure 3G). After treated with combination of the two inducers, the signal became weaker than after treated with n-sodium butyrate alone. The evenly-distributed brown-yellow granules were mainly detected in nucleoli and cytoplasm while in nuclei of the cells, the signal could be scarcely detected (Figure 3H).

Immunocytochemistry showed that mtP53 protein level in the control group was high. The dark brown-yellow granules were mainly detected in nuclei, while a few granules were detected in cytoplasm of BGC-823 cells. Their distributing was irregular(Figure 3I). After treated with tachyplesin, the rate of positive expression of mtP53 in BGC-823 cells decreased. The light brown-yellow granules were mainly detected in cytoplasm, while in nuclei of the cells the expression was negative (Figure 3J). After treated with n-sodium butyrate, the light brownish-red granules

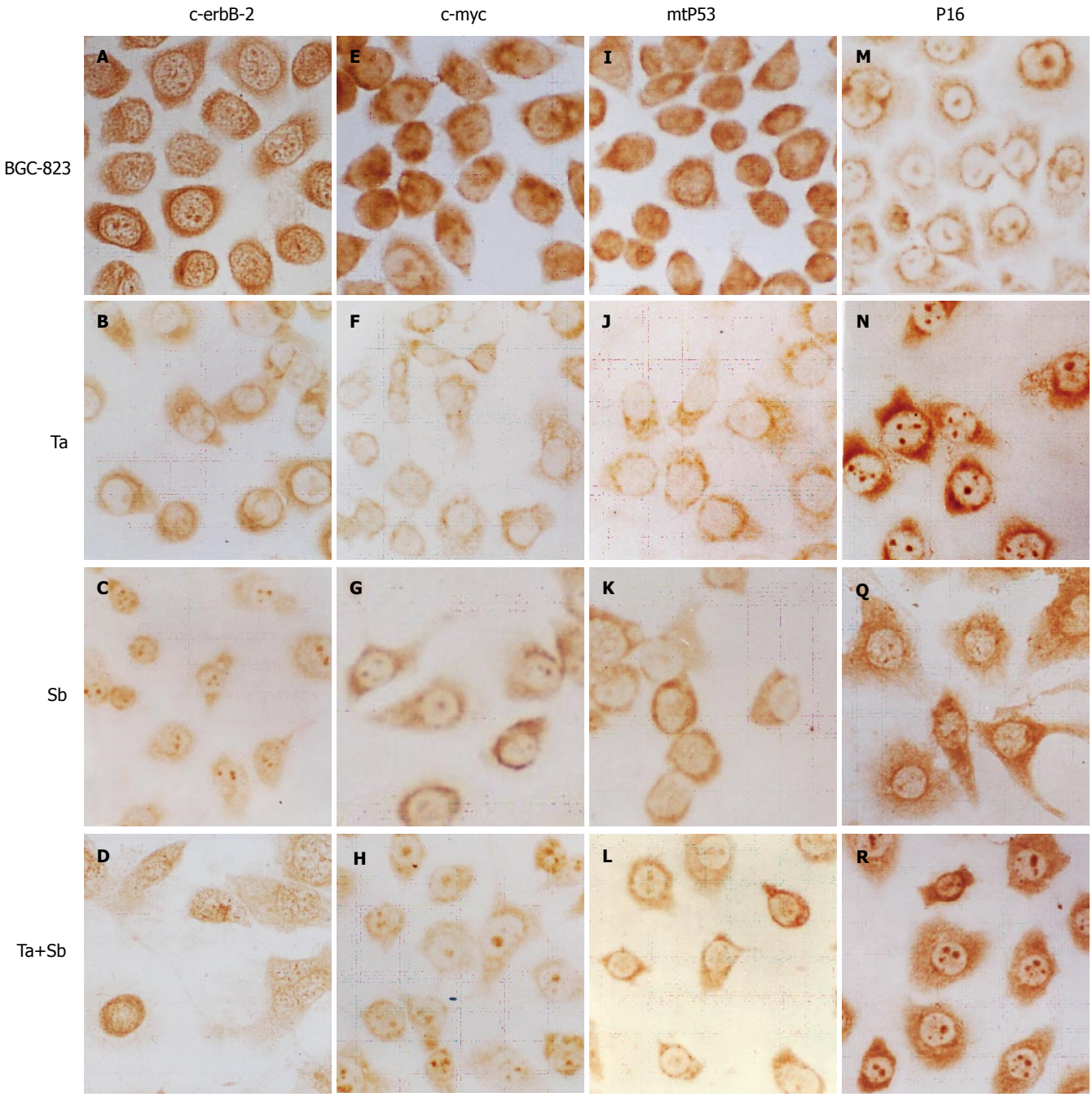

Figure 3 Expression of c-erbB-2 (A-D), c-myc (E-H), p53 (I-L), and p16 (M-R) in BGC-823 cells, tachyplesin-treated cells, n-sodium butyrate-treated cells, and tachyplesin + n-sodium butyrate-treated cells. ×536

were mainly detected in cytoplasm, while in nuclei of the cells, the mtP53 expression decreased greatly, and the signal became almost negative(Figure 3K). After treated with combination of the two inducers, the immunocytochemistry signal as a whole became weaker than after treated with n-sodium butyrate alone. The light brown granules were mainly detected in cytoplasm and could be hardly detected in nuclei of the cells (Figure 3L).

Immunocytochemistry showed that P16 protein level in the control group was low. The brown-yellow granules were detected in nucleoli and cytoplasm around the nuclear membrane of BGC-823 cells. The signal in cytoplasm and cell membrane was very weak (Figure 3M). However, after treated with tachyplesin, the P16 protein level in BGC-823 cells became very high. The evenly-distributed brown granules were detected in cytoplasm (Figure 3N). After treated with n-sodium butyrate, the P16 protein level became high. The brown granules were mainly detected in cytoplasm in a spare and dispersed manner. Some smaller granules were detected in marginal area of cytoplasm and protuberance of cells (Figure 3Q). After treated with combination of the two inducers, the P16 protein level became very high. The evenly-distributed brown granules were mainly detected in nucleoli and cytoplasm. The protein level in the combination treatment group was higher than in the group treated with n-sodium butyrate alone and the signal in nucleoli

became very strong (Figure 3R).

DISCUSSION

Sine continual division and constant proliferation are important characteristics of tumor cells, the proliferation of tumor cells is one of the significant indexes in identifying exogenous inducers of differentiation [5]. In our study, the cell growth curve, mitotic index and cell cycle indicated that BGC-823 cells had vigorous proliferation capability, the doubling time was 45.82 h, the maximum mitotic index was 38.5%, and the proportion of cells was 50.7% in G_0/G_1 phase and 39.1% in S phase. However the doubling time of cells treated with tachyplesin or n-sodium butyrate was 75.7 h and 67.1 h respectively, the rate of cell growth inhibition was 62.66% and 60.19% respectively, the maximum mitotic index decreased to 12.6% and 10.9% respectively, the proportion of cells in G_0/G_1 phase was 59.5% and 59.2% respectively, while the proportion of BGC-823 cells in S phase decreased to 26.3% and 25.2% respectively. The results demonstrated that the inhibitory effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells were significant. n-sodium butyrate, a small molecule polar compound, has been widely used in inducing differentiation of tumor cells [3, 6, 7]. The effects of n-sodium butyrate on cell growth and cell cycle are coincident with the reports about the anti-proliferative effects of n-sodium butyrate on the cell cycle arrest of human gastric, colonic and endometrial carcinoma cell lines [6-9]. Our experiment showed that the effects of tachyplesin and n-sodium butyrate on cell growth and cell cycle of BGC-823 were similar, indicating that tachyplesin has identical effects on inhibiting proliferation as chemical inducers of cancer cells.

The changes in expression of oncogenes and tumor suppressor genes play a role in cell carcinogenesis and reversal movement, amplification and inactivation of some associated genes such as c-erbB-2, c-myc, p53, p16 [10-11] are a main index in differentiation of human gastric carcinoma cells [11]. In our study, the levels of c-erbB2, c-myc, mtp53 proteins were high but p16 expression was low in untreated BGC-823 cells. However, after treated with tachyplesin or n-sodium butyrate, the level of c-erbB-2, c-myc, mtp53 proteins decreased and p16 protein expression increased significantly in BGC-823 cells, demonstrating that both tachyplesin and n-sodium butyrate can influence the expression of oncogenes and tumor suppressor genes. Previous studies showed that n-sodium butyrate could down-regulate the expression of oncogenes (c-erbB-2, c-myc, p53) and up-regulate the expression of tumor suppressor genes (p16) in endometrial and colonic carcinoma and melanoma[7,8,12,13], confirming that n-sodium butyrate has obvious effects on differentiation of BGC-823 cells. Our results also showed that tachyplesin had the same effect on up-regulating the expression of tumor suppressor genes and down-regulating the expression of oncogenes as n-sodium butyrate in BGC-823 cells.

The combination of different inducers may have a synergistic effect on differentiation and reduce toxicity and side effects by cutting down the dosage. It is not only an important subject in the field of differentiation, but also has a positive impact on the clinical application of different therapies for cancer [14]. Our study showed that at the concentration of 1.0 g tachyplesin + 1.0 mmol/L n-sodium butyrate, the growth inhibitory rate was 62.29%, the mitotic index decreased to 18.5‰ at the divided peak and the proportion of cells in G_0/G_1 phase increased to 64.5% and decreased to 20.9% in S phase. Immunocytochemistry also showed that the levels of c-erbB-2, c-myc, p53 proteins in BGC-823 cells treated with the combination were lower and the level of p16 protein was higher.

In conclusion, tachyplesin has synergistic effects with n-sodium butyrate and can be used in treatment of cancer.

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Assessment of metastatic liver disease in patients with primary extrahepatic tumors by contrast-enhanced sonography versus CT and MRI

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Abstract

AIM: To evaluate contrast-enhanced ultrasonography (CEUS) using SonoVue® in the detection of liver metastases in patients with known extrahepatic primary tumors versus the combined gold standard comprising CT, MRI and clinical/histological data.

METHODS: It is an international multicenter study, and there were 12 centres and 125 patients (64 males, 61 females, aged 59 ± 11 years) involved, with 102 patients per protocol. Primary tumors were colorectal in 35%, breast in 27%, pancreatic in 17% and others in 21%. CEUS using SonoVue® was employed with a low-mechanical-index technique and contrast-specific software using Siemens Elegra, Philips HDI 5000 and Acuson Sequoia; continuous scanning for at least five minutes.

RESULTS: CEUS with SonoVue® increased significantly the number of focal liver lesions detected versus unenhanced sonography. In 31.4% of the patients, more lesions were found after contrast enhancement. The total numbers of lesions detected were comparable with CEUS (55), triple-phase spiral CT (61) and MRI with a liver-specific contrast agent (53). Accuracy of detection of metastatic disease (i.e. at least one metastatic lesion) was significantly higher for CEUS (91.2%) than for unenhanced sonography (81.4%) and was similar to that of triple-phase spiral CT (89.2%). In 53 patients whose CEUS examination was negative, a follow-up examination 3-6 mo later confirmed the absence of metastatic lesions in 50 patients (94.4%).

CONCLUSION: CEUS is proved to be reliable in the detection of liver metastases in patients with known extrahepatic primary tumors and suspected liver lesions.

Key words: CT; MRI; Metastasis; Detection; SonoVue®

Dietrich CF, Kratzer W, Strobel D, Danse E, Fessl R, Bunk A, Vossas U, Hauenstein K, Koch W, Blank W, Oudkerk M, Hahn D, Greis C. Assessment of metastatic liver disease in patients with primary extrahepatic tumors by contrast-enhanced sonography versus CT and MRI. *World J Gastroenterol* 2006; 12(11): 1699-1705

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INTRODUCTION

The continuous routine follow-up of cancer patients requires an easily available, reliable and cost-effective diagnostic method for the detection of liver metastases. Sonography is a widely used method for the detection of liver lesions, but is generally regarded as inferior to contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI). The detection of liver

lesions with acoustic properties similar to those of the surrounding normal liver parenchyma has always been the significant limitation of grey scale (B-mode) imaging. To improve the detection of focal liver lesions, ultrasound imaging must also provide information on vascularity, exploiting the differences in blood flow between normal and pathological tissue. The concept of contrast-enhancing agents is not new, being derived from (bolus) dynamic computed tomography (CT) and magnetic resonance imaging (MRI).

Recent advances in contrast-enhanced techniques using high-mechanical-index imaging with Levovist® have improved the detection rate to a level similar to that attained using CT and MRI. This is clear from several, mostly single-centre, studies[1-5]. Owing to the high spatial resolution of sonography, CEUS may also detect subcentimetre metastases.

But there are still some important limitations since contrast-enhanced high-mechanical-index techniques must be performed intermittently and the examination technique is therefore difficult. It is expected that real-time scanning over the whole enhancement period of approximately three to five minutes using more stable contrast agents such as SonoVue® will facilitate routine use, making the examination easier and more reliable[6].

The purpose of this prospective international multicenter study using a real-time technique was to assess the ability of low-mechanical-index contrast-enhanced ultrasound (CEUS) to detect liver metastases in the presence of a known primary tumor versus a combined gold standard.

MATERIALS AND METHODS

The rationale of this study was to evaluate the diagnostic value of dynamic contrast sonography for the assessment of liver metastases versus established reference methods (CT and MRI) in combination with all clinical data except ultrasound techniques as a combined gold standard.

Study design

The study was a prospective, multicenter, open-label, intraindividual comparison. The new diagnostic procedure, dynamic contrast-enhanced ultrasound (CEUS), was compared with conventional sonography (without assessment of vascularity using contrast enhancement) and contrast-enhanced triple-phase CT, the method most commonly used in routine diagnosis. Furthermore, contrast-enhanced MRI was performed and the results from other diagnostic tests (biopsy, clinical data, etc.) were collected where available, to define the final diagnosis using the combined gold standard.

Patients

Between August 2001 and June 2002, 131 patients were enrolled at 12 European centres (see list of contributing centres). Included were male and female patients with known extrahepatic primary tumors and an indication for diagnostic assessment of possible liver metastases. Exclusion criteria were age < 18 years, pregnant or lactating women, known allergies to ingredients of the contrast agent, unstable medical conditions impairing the diagnostic procedure or contraindications to such a procedure, insufficient sonographic window for liver examination and participation in another investigational study.

Signed informed consent was obtained from all study patients before enrollment. The study protocol was approved by the ethics committee of the Landesärztekammer Baden-Württemberg and the local ethics committee of each centre. The study complied with Good Clinical Practice and the Declaration of Helsinki.

Methods and procedures

Ultrasonography

Ultrasound examinations were performed using a high-end sonographic scanner [Siemens Elegra (Ensemble Contrast Imaging), Acuson Sequoia (Coherent Contrast Imaging), Philips ATL HDI 5000 (Pulse Inversion Harmonic Contrast Imaging] with contrast-enhanced imaging software as indicated in square brackets. The contrast examinations were performed with low insonation power (low-MI imaging, mean MI = 0.22), to avoid destruction of microbubbles. Optimized pre-settings were provided for each type of machine, adjusting the imaging parameters to the contrast agent used.

BR1 (SonoVue®, Bracco International) was used as ultrasound contrast agent. BR 1 contains microbubbles of sulphur hexafluoride gas surrounded by a flexible phospholipid shell, allowing contrast-specific imaging at low insonation power. Owing to the size of the SonoVue® microbubbles (mean 2.5 μm), this contrast agent remains (as a so-called blood pool agent) within the vascular system, unlike current X-ray and MRI contrast agents which spread into the interstitial fluid. Thus contrast wash-in and wash-out can be assessed continuously during the whole enhancement phase.

Using a 20-gauge needle, BR1 was injected as an intravenous bolus of 2.4 mL (1 mL/second) into the cubital vein, followed by a 3-10 mL saline bolus for flushing. Additional injections of 4.8 mL (up to 3 contrast injections) were given, if required, to allow optimization of the procedure. There was an interval of at least 6 min between each injection of SonoVue®. To allow contrast clearance of the previous contrast injection the bubbles were destroyed by using high insonation power.

Prior to injection of the contrast agent (native) and throughout contrast enhancement (arterial and portal-venous phase), the entire liver tissue was examined by conventional B-mode ultrasonography as recently described[2,7]. Unenhanced and contrast-enhanced examinations (including the native and arterial and portal-venous enhancement phases) were evaluated separately.

All examinations were documented on S-VHS videotapes and some examinations were additionally digitally stored on magnetic-optical discs (MOD).

Computed tomography (CT)

Standard triple-phase spiral CT examinations were performed and evaluated in the radiology departments or associated radiology units of each centre. The CT examinations include native, arterial and portal-venous phase scans, using bolus injections of 123 mL (mean,

Table 1 Demographic data of the study population

Parameter	Mean	Range	
Age (yr)	59 ± 11	22-82	
Weight (kg)	76 ± 13	45-115	
Height (cm)	170 ± 8.9	152-192	
Sex			51.2 % male, 48.8 % female
Race			99.2 % white, 0.08 % Asian

Table 2 Nature of primary tumor

Parameter	Mean	Range
Primary tumor	*n*	%
Colorectal tumor	44	35.2
Breast tumor	27	21.6
Pancreas tumor	17	13.6
Bronchial tumor	7	5.6
Gastric tumor	6	4.8
Renal tumor	3	2.4
Endocrine gastrointestinal tumor	2	1.6
Melanoma	2	1.6
Others	23	18.4
In 6 patients, several primary tumors were present		

range 100-370 mL, in one patient 60 mL) iodinated contrast agent (300-350 mg/mL). In most cases a multislice scanner was used. Examinations were performed with a slice thickness ⩽ 5 mm (in two patients 8 mm).

Magnetic resonance imaging (MRI)

Standard MRI examinations were performed and evaluated in the radiology departments or associated radiology units of each centre. The MRI examinations included native and liver-specific late-phase scans with T1 and T2 weighted images obtained by SE, TSE and/or GE sequences, using a liver-specific contrast agent (in 18 patients only dynamic phase with a Gd agent). Slice thickness was ⩽ 6 mm (in 10 patients up to 10 mm).

Final reference diagnosis

The final reference diagnosis was defined by combining all available information from imaging (CT and MRI examinations) plus additional information from histology (17), surgery (8) and other clinical examinations (4). Thus the final reference diagnosis includes all information available at the end of the diagnostic evaluation, with the exception of the results from the ultrasound examination (being the test method).

Follow-up examination

Patients with negative findings at the initial examination (i.e. no metastatic lesions detected in the liver) were asked to come back for a follow-up examination 3-6 mo after the initial examination, either US, CT or MRI. This follow-up examination was used as an additional reference standard for patients with negative initial contrast-enhanced sonography, to assess the predictive clinical value of contrast-enhanced sonography.

Safety and tolerability of the ultrasound contrast agent

All adverse events occurring during the examination and a 2 h post-examination observation period were collected and listed, irrespective of a causal relationship. Adverse events were assessed with regard to severity and causal relationship.

Statistical analysis

Unenhanced and contrast-enhanced sonographies were compared by calculating the percentage difference with the two-sided 95% confidence interval. A difference of 10% between methods was defined a priori as clinically significant. The assessment of contrast-enhanced sonography invariably included native (representing tissue) as well as contrast-enhanced sequences (representing vascularity), in parallel to the assessment of CT and MRI. This reflects clinical reality, where vascularity information is always assessed in combination with tissue information. Contrast sequences are performed as a supplement to native baseline sequences, not as an alternative.

For all methods sensitivity, specificity, accuracy, and negative and positive predictive values were calculated together with the respective 95% confidence intervals, using the combined final reference diagnosis as gold standard. Thus CT, MRI, histology, clinical data, etc, but not ultrasound were part of the gold standard. This could introduce a bias in favour of CT and MRI (for example, if sonography showed a small metastasis but all other CT + MRI did not, sonography was assessed as false positive), especially in cases where invasive confirmation was impossible for ethical reasons (6 lesions in 3 patients).

For the assessment of lesion numbers only patients having at most 8 lesions were considered, since in cases with a very high number of lesions the result is more indicative of the counting efforts and moreover there is no real clinical relevance. For the assessment of the presence of metastatic disease a patient was rated as positive if at least one lesion classifiable (on the basis of characteristic features mainly of the perfusion pattern, e.g. lack of portal-venous enhancement) as metastasis could be identified. Owing to the inclusion criteria, all patients had a current or previous primary nonhepatic tumor. The comparison of the methods included all patients for whom valid results from both methods were available, irrespective of the number of lesions. The assessment of follow-up data included all patients having negative metastatic disease at the initial examination (no metastases found with CEUS) and having follow-up data available.

As statistical tests the Wilcoxon signed rank test (comparison of lesion numbers) and the McNemar test (assessment of metastatic disease) were used. For the comparison of CEUS *vs* CT the test was performed as a two-sided test (testing equivalence) and as a one-sided test (testing non-inferiority of CEUS *vs* CT).

RESULTS

Study population

Epidemiological data are summarized in Table 1. All 131 patients enrolled had a primary extrahepatic tumor (Table 2). 125 of the 131 patients received an adequate dose of

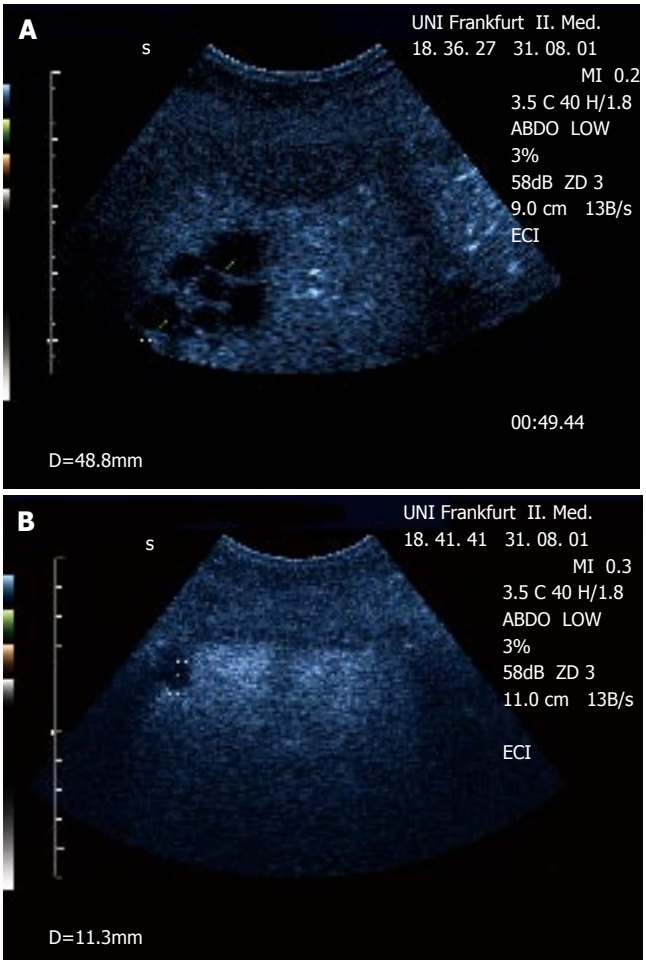

Figure 1 Demonstration of a focal (multicystic) lesion in a patient with cervix carcinoma using contrast-enhanced ultrasound (CEUS). **A**: The lesions can be delineated in the portal-venous phase as 'black spots' lacking portal-venous enhancement within normally enhanced liver tissue. **B**: An additional small lesion next to the diaphragm (not visible in native B-mode) was detected by CEUS but not with CT. Biopsy confirmed metastatic disease.

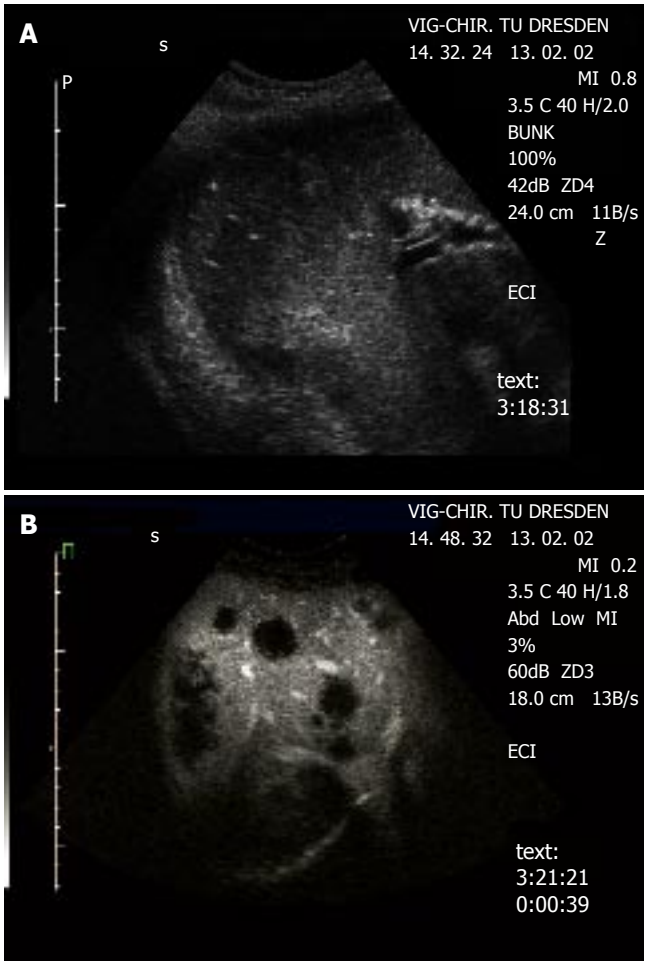

Figure 2 Detection of metastases in a patient with colorectal carcinoma **A**: Native B-mode sonography revealed 3 metastases (segment 6/7) in agreement with CT, MRI revealed 4 metastases (segment 6/7 and 4). **B**: Contrast-enhanced sonography identified diffuse metastatic disease in both liver lobes. The metastatic lesions are clearly delineated in the portal-venous phase as 'black spots', due to the lack of portal-venous blood supply.

the study medication (ultrasound contrast agent) and were considered eligible. 102 patients had no relevant protocol violations (per protocol population) and were used for the efficacy analysis. The reasons for exclusion from the primary efficacy analysis were (multiple instances possible): reference examination outside the stipulated time window of +/- 14 d (11 patients), reference examination missing or incomplete (8 patients), and inadequate visualization of the entire liver (5 patients).

The examination of the liver was performed as part of the initial staging in 63 patients (50.4%), as follow-up examination in 52 patients (41.6%), for presurgical diagnosis in 11 patients (8.8%) and for other purposes (abdominal pain, recurrent tumor staging, postsurgical assessment) in 3 patients (2.4%) (multiple reasons possible, percentage related to $n = 125$).

62 patients had negative contrast-enhanced sonography, i.e. no metastatic lesions were found at the initial examination. In 53 of these 62 patients, a follow-up examination was performed 3-6 mo after the initial examination and the absence of metastatic lesions was assessed additionally versus the information from follow-up.

Number of metastatic lesions

The metastatic lesions could be identified most clearly in the portal-venous phase, as lesions lacking portal-venous enhancement surrounded by normal liver tissue (Figure 1 and 2). Lack of portal-venous enhancement during CEUS occurred in 171 of 186 metastatic lesions (91.9%). In 60 of 186 lesions (32.3%) peripheral arterial enhancement could be detected (Figure 3). The number of lesions was assessable in 74 patients having fewer than 8 focal liver lesions. In 36 of these patients no metastatic lesion could be detected with any of the 3 imaging modalities (CEUS, CT, MRI). In the remaining 38 patients the number of metastatic lesions detected was 55 (CEUS), 61 (CT) and 53 (MRI). A comparison of the number of lesions per patient revealed that in 50 patients (67.6%) the same number of metastases was found with CEUS and CT. In 16 patients (21.6%) CT found more metastases, whereas in 8 patients (10.8%) CEUS found more metastases. However, since the histological gold standard was not available for every lesion, the rates of true and false positive lesions cannot be reliably defined. The differences between the numbers of metastases detected were not statistically significant (Wil-

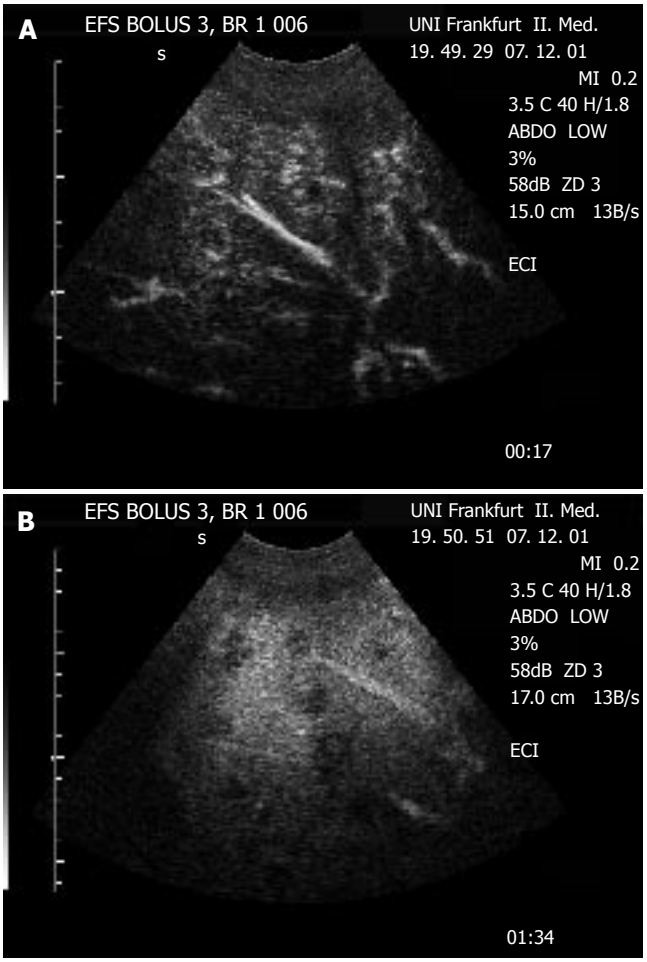

Figure 3 Detection of multiple metastatic lesions in the liver of a patient with sarcoma. **A**: In contrast-enhanced sonography, the lesions show a periperal enhancement in the arterial phase ("rim sign"), indicating the arterial blood supply to the peripheral proliferation zone of this metastatic lesions from a hypervascular tumor. **B**: During portal-venous phase, the lesions show lack of portal-venous enhancement ('black spot'), indication the absence of portal-venous blood flow typical for malignant liver lesions.

coxon signed rank test: $P = 0.28$ and $P = 0.95$ for CEUS versus CT and CEUS versus MRT, respectively). Thus with all 3 imaging modalities a comparable number of metastatic lesions could be found.

Assessment of metastatic disease

Contrast-enhanced ultrasound (CEUS) versus conventional (unenhanced) ultrasound On a patient basis, the detection of metastatic disease (i.e. of liver metastases irrespective of the number) was assessed and compared with the combined gold standard. With the use of contrast enhancement, the number of correctly classified patients rose from 83 out of 102 (native US) to 93 out of 102 (CEUS), showing clear superiority of contrast-enhanced sonography over unenhanced sono-graphy (Table 3). The superiority of CEUS was statistically significant (McNemar test: $P < 0.01$).

Contrast-enhanced ultrasound (CEUS) versus CT The correctly diagnosed number of patients with metastatic disease was 91 out of 102 patients in the case of triple-phase CT, compared with the 93 out of 102 patients who were correctly diagnosed using CEUS (Table 4). Thus contrast-enhanced ultrasound and spiral CT showed comparable accuracy for the detection of metastatic liver disease. The slight difference in favour of contrast-enhanced ultrasound was not statistically significant. The McNemar test revealed a two-sided 95% confidence interval [-6.2%; +10.1%],not clearly exceeding the stipulated 10% equivalence range.In comparison to the final reference diagnosis the sensitivi-ties were 84.6%, 88.5% and 92.3% and the respective specificities were 78.0%, 94.0% and 89.2% (unenhanced sonography, contrast-enhanced sonography and CT). Thus the accuracy for the detection of metastatic liver disease rose from 81.4% to 91.2% on use of contrast-enhancement for sonography, compared with 89.2% for spiral CT. Of the three methods, contrast-enhanced ultrasound showed the best specificity and accuracy for the detection/exclusion of metastatic liver disease.

Table 3 Patients with correct diagnosis (existence of metastatic disease) with unenhanced sonography and contrast-enhanced sonography (CEUS)

		CEUS Correct diagnosis		
		Yes	No	
Unenhanced sonography correct diagnosis	Yes	82	1	83
	No	11	8	19
		93	9	102

Table 4 Patients with correct diagnosis (existence of metastatic disease) with contrast-enhanced sonography (CEUS) and triple-phase spiral CT

		CEUS Correct diagnosis		
		Yes	No	
CT correct diagnosis	Yes	83	8	91
	No	10	1	11
		93	9	102

Contrast-enhanced ultrasound (CEUS) versus follow-up examination Of the 62 patients with a negative diagnosis (metastatic disease) on contrast-enhanced sonography, 53 had a follow-up examination 3-6 months after the initial examination. In 47 patients (88.7%) no additional lesions could be found at the follow-up examination. In 3 patients (5.7%) new lesions were found, which turned out not to be metastatic. Only 3 patients (5.7%) showed new metastatic lesions at the follow-up examination which were not diagnosed at the initial CEUS examination. One of these patients also showed no lesion on initial CT and MRI, so that this metastasis initially could not be detected by any of the 3 imaging modalities. In the other 2 patients, CEUS initially detected 5 lesions and 1 lesion respectively, but these lesions were not classified as metastatic and so, based on CEUS alone, the initial diagnosis was false negative. In the first patient (with 5 lesions), CT identified 1 of these lesions as metastasis

with peripheral portal-venous enhancement and MRI with SPIO particles identified 2 lesions as metastases. At follow-up, 3 lesions proved to be metastatic lesions. In the other patient (with 1 lesion), all methods (CT, MRI and CEUS) initially classified the lesion as nonmetastatic, and only at follow-up were 3 metastatic lesions identified. However, even versus the 3-6 mo follow-up, CEUS showed 94.4% correct assessment for nonexistence of metastatic liver disease, demonstrating the suitability of contrast-enhanced ultrasound for follow-up examinations of patients with primary extrahepatic tumors.

Safety and tolerability of the ultrasound contrast agent

The patients received 1-3 bolus injections of 2.4 or 4.8 mL SonoVue®, with a total dose of 2.4 mL (11.2%), 4.8 mL (12.8%), 7.2 mL (41.6%), 9.6 mL (5.6%) or 12.0 mL (28.8%) per patient. Only one adverse event - dry mouth of mild intensity - was reported. Thus the overall adverse event rate per patient in this study was 0.8%.

DISCUSSION

Brightness(B)-mode ultrasonography is highly sensitive and specific in characterizing cysts and calcifications, leading to a definitive diagnosis, but shows several limitations in patients with primary and secondary liver tumors. In addition, some focal lesions have the same echogenicity of normal liver parenchyma leading to false negative findings. To improve the detection of focal liver lesions, ultrasound imaging must also provide information on vascularity, exploiting the differences in blood flow between normal and pathological tissue.

It was recently shown that CEUS using contrast-specific nonlinear high-mechanical-index imaging techniques improves the detection rate of liver metastases in comparison with B-mode ultrasound, achieving a detection rate similar to that reported for computed tomography and magnetic resonance imaging techniques[1,2,4,5,8-9] In these recently published detection studies mainly using Levovist® in the portal venous and liver-specific late phase in patients with known malignancies, additional lesions could be found in 30-55% of patients. Additionally, it was shown that examination techniques employing Levovist® allow differentiation of histologically proven benign and malignant liver lesions. In 79 patients with histologically proven malignant liver lesions and in 95 patients with benign liver lesions it was shown that hypoechoic contrast enhancement in the portal venous or late phase as a predictive sign of malignancy had 100% sensitivity in patients mostly without underlying liver disease. Homogeneous Levovist® enhancement in the portal venous and late phase had 93% specificity as an indicator of benign disease[7]. It should be noted that there were no false negative findings in patients without underlying parenchymal liver disease. Furthermore, a lower interobserver variability was found in contrast-enhanced sonography than in baseline ultrasonography[10]. But there are still some important limitations since contrast-enhanced high-mechanical-index techniques must be performed intermittently and the examination technique is therefore difficult.

The present study employed a new-generation contrast medium (SonoVue®) allowing real-time imaging, providing similar information to the more difficult intermittent imaging technique using Levovist®. This method proved useful in routine application and is easy to learn. Contrast-enhanced sonography using low-mechanical-index techniques with SonoVue® has also proved useful intraoperatively[11] and in conjunction with 3D techniques[12] and additionally may differentiate between adenoma and focal nodular hyperplasia[13]. It was shown that these techniques gave a statistically significant improvement in the accuracy of detection of metastatic disease versus unenhanced sonography. In comparison with baseline ultrasound the number of metastatic lesions increased with a sensitivity comparable to contrast-enhanced triple-phase CT. In two thirds of patients the same numbers of metastatic lesions were found with contrast-enhanced sonography and CT. In the remaining patients, sometimes CT and sometimes sonography found more lesions, with no significant superiority of one method. Since contrast-enhanced sonography was not part of the gold standard this may introduce a possible bias in the calculation of accuracy. There were two patients in whom CEUS found a lesion but CT and MRI did not. One patient (female, 54 years, with breast cancer) had a 29 mm lesion and the other (female, 67 years, with pancreatic cancer) had an 11 mm lesion, which were both non-enhancing in the portal-venous phase. However, owing to the definition of the gold standard both were rated as false positive for CEUS, since no biopsy was obtained for clarification. This illustrates the limitation of such studies comparing imaging methods, since there is no absolute non-invasive gold standard. Additionally, contrast-enhanced sonography has the best specificity and accuracy for detection or exclusion of metastatic disease when compared with unenhanced sonography and triple-phase spiral CT. In 11% of the patients the existence of lesions could be ruled out, indicating the high specificity of contrast-enhanced sonography.

Monitoring of adverse events was mandatory and performed in all patients and demonstrated no relevant reaction, leading to an excellent tolerability of the ultrasound contrast agent.

In conclusion, contrast-enhanced ultrasound in the portal venous and late phase following injection of SonoVue® considerably improves the detection of liver tumors compared with conventional B-mode sonography and is therefore a suitable method for the follow-up of patients with primary extrahepatic cancer.

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VIRAL HEPATITIS

Protocol liver biopsies in long-term management of patients transplanted for hepatitis B-related liver disease

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Abstract

AIM: To evaluate the long-term histological outcome of patients transplanted for HBV-related liver disease and given HBIg prophylaxis indefinitely after LT.

METHODS: Forty-two consecutive patients transplanted for hepatitis B were prospectively studied. HBsAg, HBV-DNA and liver function tests were evaluated in the serum 3, 6 and 12 mo after LT and then yearly. LB was obtained 6 and 12 mo after LT and yearly thereafter. Chronic hepatitis (CH) B after LT was classified as minimal, mild, moderate or severe.

RESULTS: HBV recurred in 7/42 (16.6%) patients after 6-96 mo of follow-up. A hundred and eighty-seven LB were evaluated. Four of 7 patients with graft reinfection, all with unknown HBV DNA status before LT, developed cirrhosis at 12-36 mo of follow-up. Of the 122 LB obtained from 28 HBsAg+/HCV- recipients with no HBV recurrence after LT, all biopsies were completely normal in only 2 patients (7.1%), minimal/non-specific changes were observed in 18 (64.2%), and at least 1 biopsy showed CH in the remaining 8 (28.5%). Twenty-nine LB obtained from 7 patients transplanted for HBV-HCV cirrhosis and remaining HBsAg- after LT revealed recurrent CH-C. Actuarial survival was similar in patients with HBsAg+ or HBsAg- liver diseases.

CONCLUSION: Though protocol biopsies may enable the detection of graft dysfunction at an early stage, the risk of progression and the clinical significance of these findings remains to be determined.

Key words: Liver transplantation; Hepatitis B virus; Liver biopsy; Anti-HBs Immunoglobulins

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INTRODUCTION

Hepatitis B virus (HBV)-related liver disease is a common indication for liver transplantation (LT)[1,2]. Before the advent of effective prophylaxis, the rate of HBV recurrence after LT was reportedly more than 90% at one year in viremic patients and around 30% in those who were negative for serum HBV-DNA at the time of LT[3]. The majority of patients with recurrent infection develop progressive liver damage resulting in graft and patient survival rates being reduced to around 50% at 2 years after LT[4,5]. The prevention of HBV recurrence is based on long-term treatment with hepatitis B immunoglobulin (HBIg), with or without lamivudine[1,6-9,10-15]. The major drawback of using HBIg is its high cost, while the efficacy of lamivudine is limited by the onset of drug resistance[13,14,16-18].

The most reliable way to diagnose and establish the severity of any liver disease recurrence is by histological evaluation of the graft. Serial liver biopsies (LB) may identify subclinical histological changes in LT recipients with normal biochemical data and are useful in assessing disease progression during long-term follow-up[19]. At most centers, liver biopsies are performed in patients with graft dysfunction due to HBV reinfection. There is limited experience of protocol biopsies in patients after LT for HBV. The aim of the present study was to evaluate the usefulness of protocol liver biopsies in assessing long-term histological outcome in patients on indefinite HBIg

Table 1 Pre-transplant HBV-DNA in the study population and outcome after liver transplantation

Indication	*n*	HBV DNA		Total recurrence
		Negative	Unknown	
Cirrhosis	38	34	4	7
HBV	19	16	3	5
HBV-HDV	12	11	1	2
HBV-HCV	7	7		
Fulminant hepatitis	4		4	

Subgroups of HBV patients (with HDV/HCV coinfection and fulminant hepatitis) showing pre-transplant HBV-DNA status and disease recurrence.

prophylaxis following LT for hepatitis B.

MATERIALS AND METHODS

Study population

From November 1990 to December 2000, 246 adults underwent 268 LT at our center, and 55 (22.3%) of them were hepatitis B surface antigen (HBsAg)-positive. Among the patients with HBV infection, 45 (81.8%) had cirrhosis and 10 (18.2%) had fulminant hepatic failure (FHF). Eight patients with cirrhosis also had hepatocellular carcinoma (HCC), 6 were diagnosed pre-LT and 2 were incidental findings. HBV DNA detection methods became available at our center in August 1991. Since then, patients with HBV-related cirrhosis have only been listed for LT if their serum HBV DNA was negative. HBV DNA was unavailable for 5 cirrhotic patients transplanted before August 1991 and for all patients with FHF. Hepatitis delta virus (HDV) antibodies (anti-HDV IgG) were positive in 14 patients (25.4%), hepatitis C virus (HCV) antibodies in 7 (12.7%). Three patients (5.4%) had a history of alcohol abuse but had all abstained for at least 6 mo before LT. Eleven patients who died within 6 mo of LT (5 with cirrhosis and 6 with FHF) and 2 patients with a follow-up of <6 mo (both with cirrhosis) were excluded from the analysis.

Liver tests and serological/virological tests for HBV, HCV and HDV were obtained for all patients 6 and 12 mo after LT and yearly thereafter. Enzyme immunoassays were used to detect HBsAg (ELISA II Abbott Diagnostics, North Chicago, IL), anti-HDV (ELISA II Abbott Diagnostics) and anti-HCV (ELISA II-III, Ortho Diagnostics Raritan, NJ). Samples positive for anti-HCV were confirmed by recombinant immunoblotting assays (RIBA II, Ortho Diagnostics Raritan, NJ). Serum HCV RNA was investigated by RT-PCR and HBV DNA by chemiluminescence (Digene Hybrid Capture System) in serum samples and by PCR in liver tissue using primers from the conserved region of the surface gene of HBV[20].

Histological analysis

Liver biopsies were performed using a modified Menghini needle (16-17 gauge). Informed consent for liver biopsy was obtained from all patients. Hematoxylin and eosin, periodic acid-Schiff (PAS), van Gieson, reticulin and iron stains were available for all biopsy samples. Immunohistological staining was done for HBsAg and HBcAg.

Chronic viral hepatitis was defined as minimal, mild, moderate or severe according to Scheuer and Desmet[21-23]. Recurrent hepatitis C was graded according to Ishak's classification[24]. Acute and chronic rejection was classified according to Snover's classification[25]. Minimal and not-otherwise-specified changes were defined according to Pappo's classification[26].

HBIg prophylaxis

Patients received 10 000 IU of iv HBIg (VENBIG, Hardis) during the anhepatic phase of LT, daily from days 1 to 7 and weekly for 4 wk following LT. Long-term immunoprophylaxis consisted of weekly doses of 1600 IU of im HBIg (IMMUNOHBS, Hardis) indefinitely. Serum anti-HBs concentrations were investigated weekly before hospital discharge, at 3, 6 and 12 mo after LT and yearly thereafter. Target anti-HBs titers were >400 IU/L throughout the follow-up. HBIg therapy was discontinued in patients who became HBsAg-positive following LT.

Immunosuppression

Immunosuppressive therapy was a combination of cyclosporine or tacrolimus with corticosteroids. Azathioprine or mycophenolate mofetil were added in patients with serum creatinine >200 mol/L. No anti-lymphocyte antibody induction therapy was used. Hist-ologically-proven acute cellular rejection was treated with one or two courses of 1 g/d of iv methylprednisolone for 3 consecutive days.

Antiviral therapy

Lamivudine therapy (100 mg/d) was indicated in patients with detectable serum HBV DNA following LT, with or without reappearance of HBsAg.

Recurrent HBV infection and related liver disease

Recurrent HBV infection was defined as the detection of HBsAg in the serum at any time after LT.

Statistical analysis

The Kaplan-Meier method was used to calculate actuarial survivals and the log-rank test for comparisons between groups.

RESULTS

The study population included 42 patients transplanted for hepatitis B with a mean follow-up of 96 mo.

The overall rate of HBV recurrence was 16.6% (7/42 patients): 18.4% in patients with cirrhosis (7/38) and 0% in cases of FHF (0/4) (Table 1). Among the 38 patients with cirrhosis, the HBV recurrence rates varied according to etiology, pre-LT HBV DNA status and presence of HCC. Graft reinfection occurred in 5/19 patients with pure HBV-cirrhosis (26%), 2/12 patients with HBV/HDV cirrhosis (16.6%) and none of 7 with HBV/HCV-cirrhosis (Table 1). HBV DNA before LT was unavailable in 4 patients, who all developed recurrent infection - as opposed

to only 3/34 (8.8%) with negative HBV DNA ($P=0.013$). Finally, HBV recurred in 2/7 patients (28.5%) with HCC (1 with unknown pre-LT HBV DNA) and 5/31 (16.1%) without HCC: among the 4 patients with unknown pre-LT HBV DNA status, 2 died of recurrent HBV 17 and 44 mo after LT, 1 developed graft cirrhosis after 2 years and 1 had moderate chronic hepatitis with a stable clinical course; the remaining 3 patients with HBV recurrence had undetectable HBV DNA prior to LT and became HBsAg-positive 12, 12 and 14 mo after LT. In the first of these 3 patients, HBV recurrence at 12 mo coincided with de novo HCV infection. Although histology revealed mild chronic hepatitis, it was difficult to differentiate the role of HBV and HCV infection in allograft injury. Protocol liver biopsies showed cirrhosis 3, 4 and 5 years after LT. Now, 9 years after LT, without lamivudine treat-ment (which was not indicated because transaminases always remained within the normal range), the patient has compensated cirrhosis.

The second patient, though non-replicating at the time of LT, had intermittently positive HBV-DNA while on the waiting list. At the time of HBV recurrence, 12 mo after LT, liver biopsy showed mild chronic hepatitis. Lamivudine led to serum HBV DNA clearance after 2 mo of treatment. Repeated liver biopsies 2 and 3 years after LT showed stable, mild chronic hepatitis with focal cytoplasmic HBsAg on immunohistochemistry.

The third patient, who had HDV coinfection, developed mild chronic hepatitis 14 mo after LT and was treated with lamivudine.

Six of 7 patients with HBV-HCV coinfection developed histologically-proven recurrent HCV 12 to 48 mo after LT.

Liver HBV DNA, obtained from the first 11 transplanted patients, was positive in 1 patient who had recurrent positive serum HBsAg.

HBIg administration was well tolerated in all patients and no episodes of immunocomplex syndrome were observed. Mean anti-HBs titers 5 years after LT were 713±314 mIU/mL.

Only 2 of the 7 patients with recurrent HBV received lamivudine treatment because it was unavailable in 1991 and 1992 when the other 5 developed graft reinfection. Both patients were cleared of serum HBV-DNA after 6 mo of lamivudine therapy; they are both still taking the treatment with no evidence of any development of the escape mutants.

Actuarial survival was similar among patients with HBsAg-positive or HBsAg-negative liver disease (Figure 1), while it was significantly lower ($P=0.009$) in patients who had LT for fulminant HBV hepatitis than in those transplanted for HBV cirrhosis, HBV/HDV cirrhosis or HBV/HCV cirrhosis. Moreover, it was not significantly different in patients transplanted for HBV cirrhosis with vs without HCC, despite a decreasing trend in survival 100 mo after LT in patients with HCC (70% *vs* 88%).

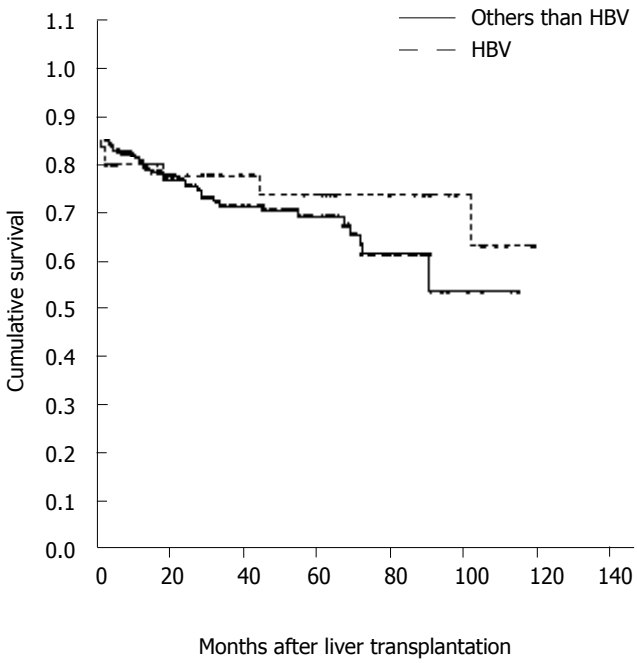

Figure 1 The figure shows the rate of cumulative survival in patients transplanted for HBV-related liver disease (cirrhosis plus fulminant hepatic failure) compared to the survival of patients transplanted for liver diseases of different etiologies. Outlined bar: survival in patients after liver transplantation fot HBV-related liver disease. Plain bar: survival in patients after liver transplantation for non-HBV-related liver disease. The difference is not statistically significant (Kaplan Meier Survival Analysis).

Histopathological features

The results of the 187 protocol biopsies performed were analyzed depending on the patients' HBV and HCV status after LT. A hundred and fifty-eight biopsies were obtained from the 35 HCV-negative recipients (mean 4.5 biopsies per patient; range 1-10) during the 6-96 months of follow-up: 36 from 7 patients with recurrent HBV (5.1 per patient; range 2-10) and 122 from 28 patients with no HBV recurrence (4.3 per patient; range 1-11).

Among the 6 HBsAg+/anti-HCV- patients (there were originally 7, but 1 became anti-HCV+ a year after LT and was consequently included in the HBsAg+/anti-HCV+ group), two had signs of mild chronic hepatitis in all biopsies (follow-up 6-24 mo), and one had at least 1 biopsy showing moderate chronic hepatitis (follow-up 6-96 mo). The other 3 patients, all with HBV-DNA unavailable before LT, developed cirrhosis (two at 12 mo and one at 24) (Figure 2).

As for the 28 HBsAg-/anti-HCV- patients (follow-up for 6-96 mo), biopsies were normal in 2 (7.1%), intermittent, mild inflammatory changes (follow-up for 6-96 mo) were observed in 18 (64.2%), at least 1 biopsy showed mild chronic hepatitis (follow-up for 6-84 mo) in 6 (21.4%) and at least 1 biopsy revealed moderate chronic hepatitis (follow-up for 6-84 mo) in 2 (7.1%). None of the patients in this group had severe chronic hepatitis or cirrhosis. Only 3 patients were HBeAg+ and due to this small number, no biochemical or histological correlations between HBeAg+ and anti-HBeAg+ patients were performed.

The histological features of the 28 patients without HBV recurrence are shown in Table 2. Twenty-nine biopsies in all were obtained from the 7 HCV+/HBsAg- patients during 6-60 mo of follow-up (4.1 biopsies per patient; range 2-6). All 7 patients developed chronic hepatitis with

Table 2 Histological features in anti-HCV negative patients, transplanted for HBV-related liver cirrhosis with no HBV recurrence

	6 mo	1 yr	2 yr	3 yr	4 yr	5 yr	6 yr	7 yr	8 yr
n	28	26	19	16	12	11	8	8	4
No. of biopsies/total biopsies (%)									
Negative	5/26 (19.2%)	9/23 (39.1%)	4/19 (21.0%)	6/14 (42.8%)	6/12 (50.0%)	3/10 (30.0%)	4/8 (50.0%)	4/8 (50.0%)	1/2 (50.0%)
Minimal, not otherwise specified changes	19/26 (73.0%)	8/23 (39.7%)	11/19 (57.8%)	6/14 (42.8%)	5/12 (41.6%)	6/10 (60.0%)	2/83/81/2 (25.0%)	(37.5%)	(50.0%)
Mild chronic hepatitis	2/26 (7.6%)	6/23 (26.0%)	4/19 (21.0%)	2/14 (14.2%)	- -	- -	1/8 (12.5%)		
Moderate chronic hepatitis	-	-	-	-	1/12 (8.3%)	1/10 (10.0%)	1/8 (12.5%)	1/8 (12.5%)	
Severe chronic hepatitis	-	-	-	-	-	-	-	-	-
Cirrhosis	-	-	-	-	-	-	-	-	

Histological features in 122 liver biopsies performed in 28 anti-HCV negative patients transplanted for HBV-related liver disease with no HBV recurrence. The number of patients considered declined year by year and the total number of liver biopsies performed each year is less than the number of patients per year.

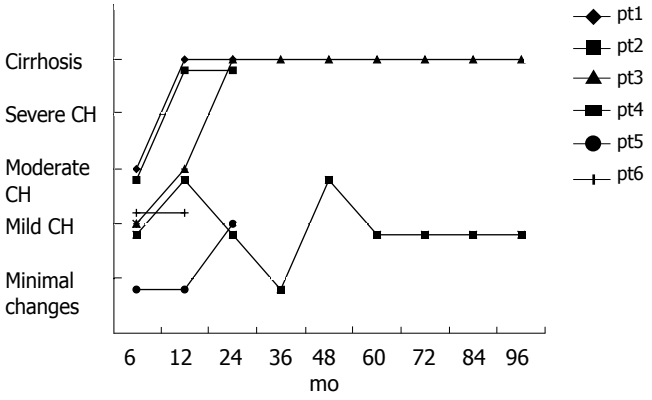

Figure 2 The figure shows the histological damage progression in the 6 anti-HCV negative patients with HBV recurrence.

Table 3 Staging and grading of liver damage in patients transplanted for HBV- and HCV-related liver cirrhosis

Pts	Stage	Grade	Months after transplantation
1	1	4	36
2	2	4	48
3	1	4	24
4	2	10	12
5	0	2	36
6	2	5	12
7	1	2	12

The worst grades and stages were in the 7 HBV/HCV coinfected patients. Staging and grading are based on Ishak's classification [24].

fibrosis 12 to 48 mo after LT (Table 3). The patient with recurrent HBsAg+, who also became anti-HCV positive 1 year after LT, developed mild chronic hepatitis a year after transplantation and cirrhosis 3 years after LT.

Immunohistochemistry was performed on 106/187 (56.7%) liver biopsies. Focal HBcAg positivity was seen in 4/106 (3.7%) biopsies, obtained from 4 HBsAg negative patients, all of them HBV-DNA negative at the time of the biopsy and offering no clue as to the clinical relevance of this finding. Focal HBsAg positivity was seen in only 1 patient with recurrent HBV.

Acute cellular rejection was histologically confirmed in 11/42 patients (26.1%), with a total of 14 episodes (0.33 episodes/patient), 1 to 6 mo after LT; 8 patients had one episode of rejection and 3 patients had two. None of the patients developed steroid-resistant or chronic rejection.

DISCUSSION

HBV-related liver disease is now a common indication for liver transplantation[1,27,28], since graft and patient survival rates are comparable with those of patients transplanted for other conditions[29]. Although perioperative mortality was high in this study, it was unrelated to recurrent HBV infection. Previous studies have shown that the outcome of LT is worse in patients with fulminant HBV hepatitis[30,31] and fulminant non-HBV liver failure[32], as confirmed by our findings. The survival rate was similar, however, between HBsAg positive and negative patients transplanted at our center.

The overall HBV recurrence rate in this series (16.6%) was low and similar to the rate reported in other studies using long-term HBIg monotherapy[14,33-36]. When the analysis was restricted to the cirrhotic cases with negative pre-transplant HBV-DNA, the recurrence rate was even lower (8.8%). These good results confirm that patients without HBV replication before LT and given long-term immunoglobulin prophylaxis are at low risk of recurrent infection, as amply reported elsewhere[5,37-41]. Our study also shows that iv infusions of HBIg are not required beyond the perioperative period; putatively protective levels of anti-HBs (>400 IU) can be obtained using the im route, as reported elsewhere[42-45].

In our series, the rate of HBV recurrence was similar in HBsAg-positive patients with and without HDV coinfection, unlike the situation observed in previous publications[28,46-49], where HDV coinfection was associated with

a lower rate of HBV recurrence and improved survival. Only 2 of our patients with HBV/HDV-cirrhosis developed recurrence, however, and one of them had unknown (but probably positive) pretransplant HBV-DNA.

Similarly, although HBV recurred more frequently in cirrhotic patients with HCC, as in previous publications [50-52], the difference was not statistically significant. In addition, one of the 2 HCC patients with HBV recurrence was intermittently positive for HBV-DNA, while the other had unknown (but probably positive) HBV-DNA pre-LT.

The strength of this study lies mainly in the performance of long-term per-protocol serial liver biopsies, the clinical utility of which is still not clear (though the value of protocol biopsies has recently been confirmed[19,53-55]). The International Liver Transplantation Society Expert Panel Consensus Conference on liver transplantation for hepatitis C considered per protocol biopsies essential: all anti-HCV+ liver transplant recipients should undergo annual liver biopsy to determine histological progression and provide additional data on the natural history of the disease[56].

Among the 28 patients treated with HBIg who remained HBsAg and anti-HCV-negative after LT, only 7% had a completely normal histology up to 3 years after LT. Although the majority of abnormal biopsies showed only minimal inflammatory changes, almost 30% fulfilled the criteria for the diagnosis of chronic hepatitis, as reported elsewhere[26,55]. The features of mild or moderate chronic hepatitis were seen in nearly 1/3 of our cases.

Protocol biopsies obtained from the 7 HBsAg-negative recipients with post-LT HCV re-infection showed recurrent hepatitis C with variable degrees of inflammation and fibrosis. It is worth noting, however, that none of the patients progressed beyond stage 2 fibrosis after a mean follow-up of 36 mo, suggesting that the HBIg preparation used may contain HCV neutralizing antibodies that may attenuate the severity of recurrence, as reported by Feray *et al*[57], but this is a controversial issue because no other papers have confirmed these data since 1990. HBcAg expression was found in the liver tissue of a minority of biopsies from HBsAg-negative recipients, but it was focal and transient. In addition, the finding of HBV-DNA in liver tissue correlated with serum HBV-DNA positivity. Despite the limited number of cases studied, we feel confident in saying that serum assay is clinically useful, whereas tissue HBV DNA should not be investigated because it is expensive and time-consuming. We did not assess HBV genotypes, but it has been recently reported that genotype C seems to be associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B[58]. As previously reported, we observed a low rate of acute rejection among patients transplanted for hepatitis B[59-60]. This may be an indirect consequence, at least in part, of the long-term administration of human polyclonal immunoglobulins, which have immunosuppressive properties[59].

In short, this study shows that 25% of patients transplanted for hepatitis B and remaining HBsAg-negative with HBIg prophylaxis develop histological features of mild or moderate chronic hepatitis despite having normal liver test results and negative virological markers[26,55]. These cases will require additional follow-up to estimate the risk of disease progression and further clarify the cause of liver damage. Our findings confirm that normal liver function test results cannot guarantee a healthy graft. The performance of protocol liver biopsies may enable the identification of early histological anomalies potentially capable of progressing to significant liver damage[19,53], but this needs further confirmation.

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Inter-observer variability in histopathological assessment of liver biopsies taken in a pediatric open label therapeutic program for chronic HBV infection treatment

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Abstract

AIM: To our knowledge, the inter-observer variability of the liver biopsy findings in HBV-infected children have not been studied as yet. Hence, we aimed to compare different pathologist's assessment of grading and staging in liver biopsies obtained from children prior to interferon treatment.

METHODS: We collected 920 biopsies from 11 medical centers. The biopsies were independently reviewed by 6 pathologists from academic centers who assessed Batts-Ludwig score for grading and staging. Satisfactory agreement among observers was defined as at least 60% of observers having the same opinion. Satisfactory dispersion between maximal and minimal score for the same biopsy specimen was defined as a maximum 1 point.

RESULTS: Satisfactory inter-observer agreement for grading was obtained in 51.6% and for staging in 75.7% of biopsies. Satisfactory dispersion for grading scores was observed in 44.5% and for staging in 72.7% of cases.

CONCLUSION: Our study demonstrates that: (1) pathologists differ in their assessment of grading and staging of liver biopsies; (2) inter-observer variability for staging is lower than that for grading; and (3) regardless of the inter-observer variability of assessments, the majority of children with chronic HBV infection have mild to moderate inflammation and mild to moderate fibrosis.

Key words: Grading; Staging; Type B Hepatitis; Children

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INTRODUCTION

Many authors believe that a liver biopsy is a gold standard in hepatology[1]. However, there is considerable confusion in terminology and the methods of liver biopsy evaluation are still under discussion. These are the reasons why many different scoring systems for liver histological evaluation were proposed. The first scoring system developed by Knodell *et al*[2] has been used in clinical trials since 1981. This classification differentiated necroinflammatory changes and fibrotic liver damage. However, both features were combined in one histological activity index. This was the reason why the Knodell classification was replaced by other systems with clear differentiation between grading of inflammation and staging of fibrosis[3-6]. Nowadays, the scoring systems are widely used by pathologists and clinicians. It often happens that the grading and staging scores replace the descriptive evaluation of biopsy slides. In this situation, one can raise the question: which grading/staging classification is a true gold standard for a liver biopsy assessment? The other issues that can be discussed are results reproducibility and inter-observer variability of the grading and staging scores for the same biopsy. These problems are vital for the clinicians who receive the biopsy reports. However, they are not frequently addressed in the literature.

Table1 The inter-observers agreement definitions

Agreement rate Number of equal assessment/number of observations	Description	Percentage of agreement (%)
6/6, 5/5, 4/4	All observers gave the same score	100
3/4, 5/6, 4/5,	One observer scored differently than others	75-85
4/6, 3/5	Two observers scored differently than others	60-66
3/6. 2/4	Agreement of half of observers	50
2/6, 2/5, 1/4	Agreement of less than half of observers	25-40

We met the problem of liver biopsy interpretation when we analyzed the results of a nationwide program for interferon treatment of chronic HBV infection in children, conducted in Poland between 1993 and 1999. Twenty-six centers from all over the country participated in this program and 3700 children received treatment there. All centers used their local laboratory facilities, but the entry criteria and treatment regime (interferon 3 MU TIW for 20 wk) were the same. The data collected in the local centers was entered into CRF and sent for central analysis. The results published for 1688 children showed 51.5% of HBe clearance at one year after interferon discontinuation. The response was better in younger children and there was a positive correlation with ALT activity[7]. This observation clearly illustrates the problems with liver biopsy result interpretation that are often met by physicians who review the biopsy reports provided by different pathologists.

The liver biopsy specimens could be easily stored in paraffin blocks and as microscopical slides and used for microscopic evaluation many times. These gave us the opportunity to return to the biopsy slides taken from children with chronic HBV infection, treated with interferon in a Polish therapeutic program, and to re-evaluate them according to selected grading and staging classification. Therefore, we asked all participating centers to provide liver biopsy slides for central review. We wanted to see whether the liver biopsies obtained as part of the routine procedure were representative and whether the quality of slides used for diagnosis was satisfactory. We selected scoring system[6] which was used to evaluate the grading and staging of liver damage, and also performed inter-observer variability analysis. The biopsy results were compared with a patient's clinical and serological data to evaluate the role of liver biopsy in the decision-making process regarding children with chronic HBV infection.

The aim of this study was to present the inter-observer variability of liver biopsy assessment performed according to the Batts and Ludwig grading/staging system[6]. The other questions raised in our project will be discussed in further publications.

MATERIALS AND METHODS

The liver biopsy slides taken from 920 children were provided by 11 centers. The number of slides per one biopsy ranged from 1 to 6 (mean 2.36). Hematoxylin-eosin staining were available for all biopsies and for most of them Azan or PAS stains were available as well.

Six independent pathologists, from academic centers, participated in the study. All of them have been collaborating with hepatology units. Before the study was started, the consensus meeting had been organized and Bats-Ludwig liver biopsy assessment scale was chosen for the study as it is widely used in Poland and has a recommendation of Polish Association of the Study of the Liver. Small sample of biopsy slides was reviewed by every pathologist at this consensus meeting and methods of biopsy assessment and results recording were discussed.

The biopsies were divided into sets, which were circulated among observers. The original identification of the biopsies was withheld and pathologists had no additional clinical data on any particular biopsy. Each observer was asked to assess representativity (size of the sample and number of portal zones) and the technical accuracy (fixation and staining) of the slides and to score grading and staging. The assessments were recorded in standard CRF and stored in a central database. For each biopsy, at least four observers' assessments were obtained.

We analyzed the score distribution for every observer and the agreement rate between different observers for the same biopsy (Table 1). Agreement of 60-66% of observers was selected as the desired minimal level of inter-observer agreement.

For each biopsy, the dispersion between maximal and minimal score for grading and staging was calculated. Satisfactory dispersion between maximal and minimal score was defined as being not greater than 1 point.

All biopsies were taken as part of a routine diagnostic procedure and the parents were asked to sign the consent for liver biopsy. This project of biopsy retrospective assessment received the approval from the Ethics Committee of Children's Health Memorial Institute in Warsaw (decision No 185/KE/2000).

RESULTS

Biopsy representativity and technical accuracy assessment

A total of 5 295 opinions on biopsy specimen representativity were available. In 83.9% of the reports, the observers stated that the specimen was representative of the tissue and could be used for grading and staging evaluation. However, the rate of representative specimens varied among different observers from 65.9% to 93.3% (Figure 1). The agreement of at least 60% of observers was reached in 97% of cases (Figure 2).

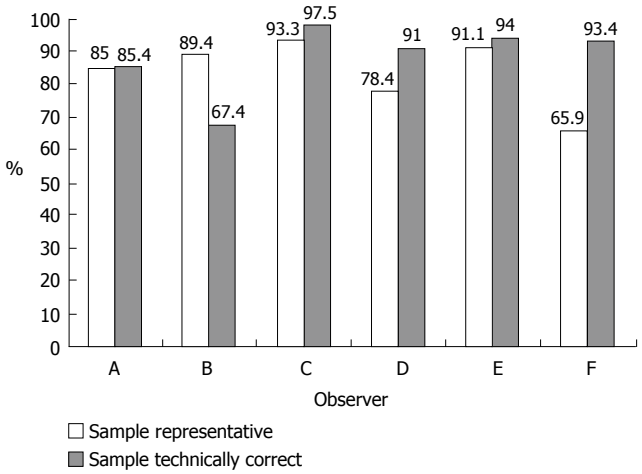

Figure 1 Observers' opinions on representativity and technical accuracy of liver biopsy specimens.

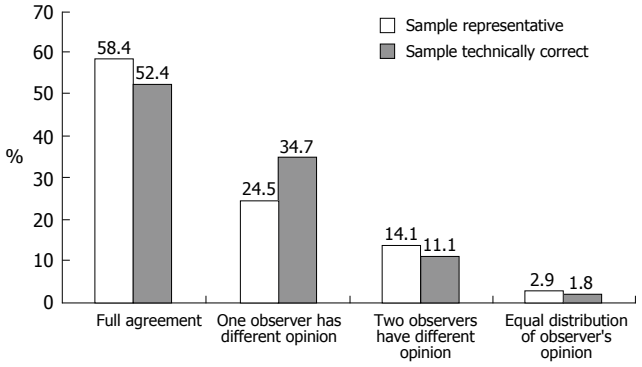

Figure 2 Inter-observer agreement in the assessment of liver biopsy specimen representativity and technical accuracy of the slides.

The opinions on technical accuracy of the biopsy slides were available in 5 296 reports and 88.1% of them showed that the slides were technically correct. The individual observers differed in their opinion on technical accuracy of the biopsy by between 67.4% and 97.5% (Figure 1). The desired level of agreement was reached in over 98% of cases (Figure 2).

Grading assessment

Four or more observers' assessments of biopsy grading were available for 844 biopsies and a total of 4 900 reports were analyzed. The frequency of different scoring varied among observers (Figure 3). The smallest differences in grading score frequencies among observers were noted for a score of 0 (0.3-17.2%, mean frequency for all observers: 8.1%) and 4 (0-7.8%, mean: 4.1%). The largest differences were noted for a score of 1 (13-50%, mean for all observers: 31%) and 3 (4-35.9%, mean: 22%).

The grading assessment agreement of more than 60% of the observers was obtained in 51.6% of cases, but full agreement of all observers reached in only 3.1% of cases. For 13.4% of biopsies, a large difference in observers' opinions was noted (Figure 4)

Different observers' grading score dispersion for the same biopsy equal or lower than 1 point was noted in 44.5% of cases and a dispersion of 2 points was noted in 47.3%. Substantial score dispersion among observers was noted in 8.2% of the cases (Figure 5).

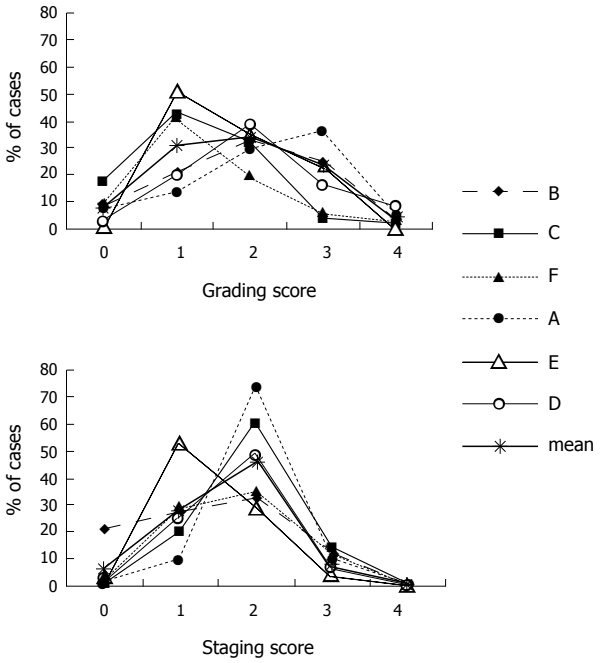

Figure 3 The frequency of different grading and staging scores for individual observer and the mean score frequency for all observers.

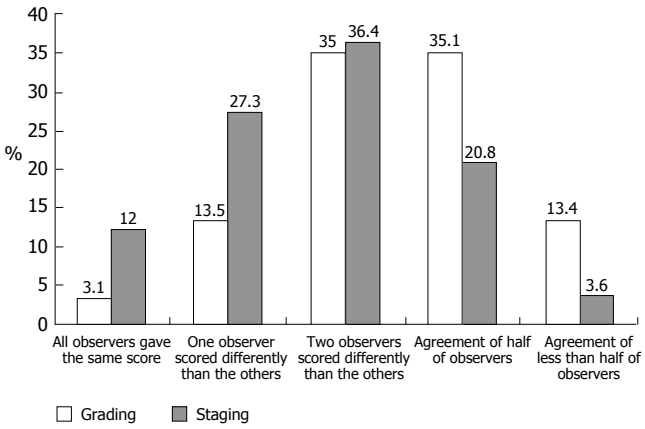

Figure 4 The agreement between different observers' grading and staging score for the same liver biopsy.

Staging assessment

Four or more observers' staging assessments were available for 843 biopsies and a total of 4 895 reports were analyzed. The frequency of different scoring varied among observers (Figure 3). The smallest differences in staging score frequency among observers were noted for a score of 4 (0-0.76%, mean frequency for all observers: 0.4%). The largest differences in score frequency among individual observers were noted for a score of 1 (9.6-52.7%, mean for all observers: 27.1%) and a score of 2 (28.4-72%, mean for all observers: 46.4%).

The staging assessment agreement of more than 60% of the observers was obtained in 75.7% of cases and

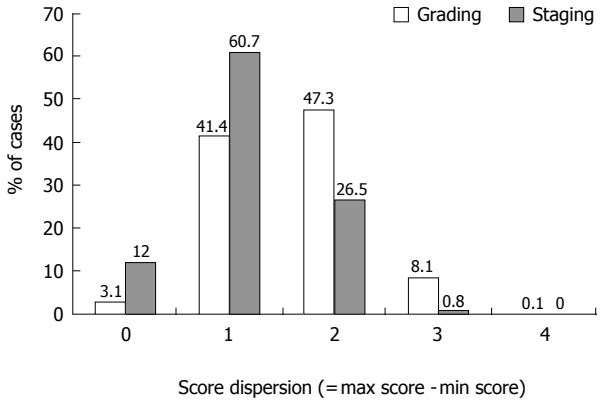

Figure 5 Grading and staging dispersion of assessments among different observers (maximal score - minimal score for the same biopsy).

full agreement of all observers reached in 12% of cases. Substantial differences in observers' opinion were noted in only 3.6% of biopsies (Figure 4).

Different observers' staging score dispersion for the same biopsy equal or lower than 1 point was noted in 72.7% of cases and dispersion of 2 points was noted in 26.5%. Substantial score dispersion among observers was noted very rarely, in only 0.8% of the cases (Figure 5).

DISCUSSION

Liver biopsy is a complex diagnostic procedure performed in many medical centers and evaluated in many laboratories. The aim of the biopsy is to obtain objective information about the condition of the liver tissue. However, there are many factors that influence the objectivity of this examination. Rousselet *et al*[8] have shown that the pathologists' experience is one of the most important factor influencing the inter-observer agreement on viral hepatitis liver biopsy assessment. The other factors that can be taken into account are: the place of liver biopsy sampling; the size of liver biopsy; fixation and staining of the liver tissue specimen; patient's age and reason for liver damage[9].

To our best of knowledge, there are no comprehensive studies addressing the inter-observer variability in liver biopsies taken from children with HBV infection. We, therefore, aimed to investigate the inter-observer error among pathologists who receive a series of biopsies taken in every day practice.

When analyzing the inter-observer variability, one must take into account the definition of agreement. Peutz *et al*[10] showed that the tolerance ±1 point markedly increases the reproducibility of staging results on the Ishak scale. The complexity of assessment scale and number of observers can also influence the variability of diagnoses. Gronback *et al*[11], in their report on a series of 46 liver biopsies analyzed independently by 5 observers, showed that the more complex the scale, the bigger the variability of results. The variability in different pathologists' assessment of the liver biopsy may lead to variability of therapeutic decisions based on liver biopsy reports. Thus, in our study, we compared the results obtained from different pathologists for the same biopsies. To provide a practical value of the results for every day life, we have resigned from sophisticated statistics. The questions that we asked were very simple but vital for the clinician who collaborates with different independent pathologists.

In our biopsy series, we demonstrated that inter-observer variability depended on the scale complexity and agreement cut-off. When the same opinion of at least 60% of observers (≥4 out of 6, or ≥3 out of 5 observers) was used as a cut-off point, the inter-observer agreement for representativity reached 97% and for technical accuracy of the biopsy reached 98%. Even with the higher cut-off, the inter-observer agreements for these two parameters were satisfactory. These positive results could be obtained in a simple scale with a two-option (yes/no) selection[12].

The Batts-Ludwig grading and staging scale is more complex. With the same cut-off as specified above (agreement of at least 60% of observers), the inter-observer agreement for grading reached in 51.6% of cases and for staging 75.7% of cases. A large number of observers was probably the reason why the agreement with the higher cut-off level was very low.

Moreover, a large number of observers was also the reason why the dispersion between the maximal and minimal score for single biopsies was high. In a four-step scale, a maximal difference of 1 point should probably be used as acceptable tolerance. This level of dispersion was achieved for grading in 44.5% of cases and for staging in 72.7%. We found a higher inter-observer agreement and a lower score dispersion for staging than for grading, showing that staging assessment is more reliable and probably more reproducible than grading assessment. This has also been confirmed by other studies[1,8].

The analysis of inter-observer grading and staging agreements and score dispersion showed that despite the same assessment scale being used by pathologists, their reports differed. This is because grading and staging assessments are essentially subjective[13] and this is not a unique situation for liver biopsy only. Similar discrepancies have been reported for other diagnostic techniques in hepatology as well[14]. However, despite the inter-observers variability, the numbers of children with extremely low and extremely high grading or staging were small. Our data showed that the vast majority of children with chronic HBV infection had mild to moderate inflammatory changes and mild to moderate fibrosis. The risk of biopsy-proven HBV-related cirrhosis in this age group is minimal. This observation confirms the clinical observations of Bortolotti *et al*[15] who could not find a progression to liver cirrhosis in children with HBV infection. On the other hand, our data indicated that the chance of completely normal liver tissue in children with chronic HBV infection is small. These observations suggest that the approach to liver biopsy should be changed. Liver biopsy should not be a mandatory examination in all children with active HBV infection, but it should be performed only in selected patients: those with persistently abnormal ALT despite HBeAg clearance, or those with initial signs of liver destruction. In everyday practice, the grading and staging scores should not replace the descriptive biopsy report, as scoring systems are not reliable and they reduce the amount of information on liver tissue. Grading and staging scores become important information in a liver biopsy

report when the series of liver biopsies for the same patient are analyzed for research or follow-up reasons, providing, however, that all the patients' biopsies are reviewed by the same pathologist on the same occasion. It seems important that both clinician and pathologist are aware of the liver biopsy limitations and they closely collaborate with each other.

In conclusion, our study shows that pathologists differ in their assessment of grading and staging of liver biopsy, but the inter-observer variability for staging is lower than that for grading. The majority of children with chronic HBV infection have mild to moderate inflammation and mild to moderate fibrosis. Thus, the value of liver biopsy as a guide for current therapeutic decision in children with chronic HBV infection is limited. The liver biopsies should rather be used as a tool for research or long-term disease dynamics assessment.

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BASIC RESEARCH

Effects of betaine on ethanol-stimulated secretion of IGF-I and IGFBP-1 in rat primary hepatocytes: Involvement of p42/44 MAPK activation

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Abstract

AIM: To evaluate the effects of betaine on the ethanol-induced secretion of IGF-I and IGFBP-1 using radioimmunoassay and Western blotting, respectively, in primary cultured rat hepatocytes.

METHODS: Hepatocytes isolated from male Sprague-Dawley rats were incubated with various concentrations of ethanol and PD98059 procedures. The hepatocytes were also treated with different doses of betaine (10^{-5}, 10^{-4}, and 10^{-3} mol/L). We measured IGF-I and IGFBP-1 using radioimmunoassay and Western blotting, respectively.

RESULTS: The ethanol-induced inhibition of IGF-I secretion was attenuated by betaine in a concentration-dependent manner in primary cultured rat hepatocytes. At 10^{-3} mol/L, betaine significantly increased IGF-I secretion but decreased IGFBP-1 secretion. In addition, p42/44 mitogen-activated protein kinase (MAPK) activity was accelerated significantly from 10 min to 5 h after treatment with 10^{-3} mol/L betaine. Furthermore, the changes in IGF-1 and IGFBP-1 secretion resulting from the increased betaine-induced p42/44 MAPK activity in primary cultured rat hepatocytes was blocked by treatment with the MAPK inhibitor PD98059. Betaine treatment blocked the ethanol-induced inhibition of IGF-I secretion and p42/44 MAPK activity, and the ethanol-induced increase in IGFBP-1 secretion.

CONCLUSION: Betaine modulates the secretion of IGF-I and IGFBP-1 via the activation of p42/44 MAPK in primary cultured rat hepatocytes. Betaine also alters the MAPK activations induced by ethanol.

Key words: Betaine; IGF-I; IGFBP-1; p42/44 MAPK; Hepatocytes; Ethanol

Lee MS, Kim MS, Park SY, Kang CW. Effects of betaine on ethanol-stimulated secretion of IGF-I and IGFBP-1 in rat primary hepatocytes: Involvement of p42/44 MAPK activation. *World J Gastroenterol* 2006; 12(11): 1718-1722

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INTRODUCTION

Betaine, an organic osmolyte, is a precursor of S-adenosylmethionine (SAMe) in the liver. It has been shown that betaine administration has the capacity to elevate hepatic levels of SAMe and to prevent ethanol-induced fatty liver[1]. Kanbak *et al*[2] reported that betaine protects the liver from ethanol-mediated hepatotoxicity in rats, and it was reported recently that it also protects the liver from ischemia/reperfusion injury, niacin toxicity, CCl_4-, hyperosmolarity-, and ethanol-induced apoptosis.

Alcohol consumption is associated with the nutritional metabolism system, which reduces hepatic glucose production and insulin secretion, and increases protein catabolism and triacylglycerol levels. The liver is predominantly responsible for ethanol metabolism and it appears to be the primary source of blood-borne insulin-like growth factor (IGF)-I. Impairment of hepatic synthesis could be responsible for at least a portion of the alcohol-induced decrease in serum levels of IGF-I. Alcohol can affect various organ conditions, thus alcohol consumption may regulate IGF, IGF binding protein (IGFBP) and the IGF-I receptor, which may be involved in growth disorders. It has been shown that IGFBPs are associated with alcohol metabolism[3,4], and we found recently that alcohol administration not only reduces the level of IGF-I, but also increases the level of IGFBP-1 in rat liver, kidney, and serum[5]. It may also contribute to the metabolic dysfunction that occurs following chronic

alcohol consumption. Acute alcohol ingestion also reduces the level of IGF-I in serum, but promptly increases serum IGFBP-1 levels in human subjects[3]. In a pilot study, we observed a reduction in the level of IGF-I in alcohol-treated primary rat hepatocytes.

Based on these previous results, we can speculate that betaine will affect IGF systems in the liver. However, the effects of betaine on the ethanol-mediated secretions of IGF-I and IGFBP-1 in primary cultured rat hepatocytes remain poorly understood. In the present study, therefore, we investigated the effects of betaine on the ethanol-mediated secretions of IGF-I and IGFBP-1 in primary rat hepatocytes.

MATERIALS AND METHODS

Materials

IGF-I antigen, and IGF-I and IGFBP-1 antibodies were purchased form Gropep (Thebarton, Australia). The mitogen-activated protein kinase (MAPK) inhibitor PD98059 was purchased from New England Biolabs (Beverly, MA, USA), and antibodies against phosphor-p42/44 MAPK, p42/44 MAPK were obtained from Cell Signaling (Beverly, MA, USA). All routine culture media were obtained from Gibco-BRL (Grand Island, NY, USA). Aqualsol, reflection X-ray film, and I^{125} (isotope) were purchased from Dupont-NEN. Immobilon-P polyvinylidene difluoride (PVDF) membranes were purchased from Millipore. Bovine serum albumin (fraction V), glycine, sodium dodecylsulfate (SDS), acrylamide, glycerol, and Tween 20 were obtained from Sigma Co. (St. Louis, MO, USA).

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats weighing 200-300g by a two-step perfusion procedure with 0.5g/L collagenase that has been described previously (Seglen, 1976: Weng and Shukla, 2000). Cell viability, as assessed by exclusion of trypan blue, was 90% ± 5%. Isolated hepatocytes were plated onto collagen-coated plastic culture dishes (60 mm in diameter) at a density of 5×10^4 cells/cm^2 in William's medium E containing 100ml/L fetal bovine serum (FBS) in an incubator with an atmosphere of 50ml/L CO_2. After 3 h, the medium was replaced with FBS-free-Williams medium E and various concentrations of ethanol and PD98059 were carefully added to the cells. The dishes immediately were sealed tightly with parafilm and the cells were incubated in this way for 10-180 min at 37℃.

Cells were rinsed twice with ice-cold phosphate-buffered saline and lysis buffer was added (20 mmol/L HEPES, pH 8.8, 136 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 g/L Triton X-100, 10 mmol/L KCl, 2 mmol/L MgCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L benzamidine, 10-B-glycerophosphate, 10 mg/L aprotinin, 10 mg/L leupeptin, and 1 mg/L pepstatin A). Cell lysates were collected and sonicated for 5 min in a VibraCell ultrasonic processor. After centrifugation of the sonicated samples at 12000 × *g* for 10 min at 4℃, the supernatant was collected and protein concentrations were estimated using a bicinchoninic acid assay kit.

IGF-I radioimmunoassay

Recombinant human IGF-I was iodinated to a specific radioactivity of 5.55 - 11.10 MBq/g with the isotope I^{125} using a modified version of the chloramin-T method (Kodak, NY, USA). The specific activity of the iodinated IGF-I was approximately 2.22 - 4.07 MBq/g protein. The iodination mixture was purified on a Sephadex G-50 column (150 cm) and pre-equilibrated with phosphate-buffered saline (0.1 mol/L, pH 7.4). Serum and tissue IGFBPs were separated using the method of Lee *et al*[6], and IGF-I immunoreactivity was determined using the method of Lee *et al*[7]. IGF-I data are expressed in terms of nanograms of pure human IGF-I per milliliter assuming equal cross-reactivity of rat and human IGF-I in the RIA. Fifty microliters of rat polyclonal IGF-I antibody diluted to 1:1,500 was added to 100 μL of each sample/standard and then incubated for 1 h at room temperature. [^{125}I]-IGF-I (20000 cpm) was then added, and the sample/standard was incubated for an additional 18 h at 4℃. Fifty microliters of horse serum (Sigma) was added to the incubated sample, which was then centrifuged at 3000*g* for 30 min. The supernatant was discarded, and the radioactivity of the precipitate containing bound [^{125}I]-IGF-I was counted in a gamma scintillation counter (Wallac, Finland). All assays were performed in duplicate. Intra- and interassay coefficients of variation for IGFs were 8% and 10%, respectively.

Western blotting

Supernatants were concentrated for 5 h using a Centricon processor (Millipore) at 4℃. Equal amounts of concentrated protein and cell lysates (20-30 μg) were separated on 100 g/L SDS-polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, proteins were transferred to a PVDF membrane. The membrane was washed with Tris-buffered saline containing Tween 20 (TBS-T; 25 mmol/L Tris, pH 7.4, containing 137 mmol/L NaCl and 10 g/L Tween-20) and then blocked with TBS-T containing 50g/L nonfat dry milk for 2 h at room temperature. Blots were incubated with antibodies against IGFBP-1 overnight at 4℃ and then incubated with anti-rabbit horseradish peroxidase. After washing, the specific protein band was visualized using an enhanced chemiluminescence detection system (Pierce Chemical, IL, USA). For MAPK blotting, cell lysates were separated on 100 g/L SDS-PAGE and blotted with p-p42/44 MAPK antibody.

Statistical analysis

All experiments were repeated at least three times. The data obtained from this investigation were analyzed using ANOVA and Student's *t* test and are expressed as mean ± SD values.

RESULTS

Regulatory effects of betaine on ethanol-mediated IGF-I and IGFBP-1 secretion

Chronic alcohol treatment is associated with liver and kidney damage. IGF is the major growth factor that is affected by alcohol consumption. Recently, we found that

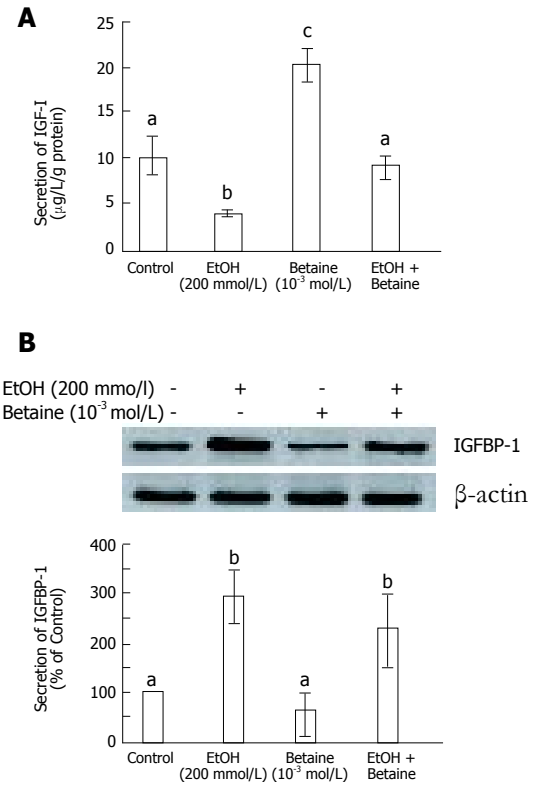

Figure 1 Betaine inhibits the ethanol-stimulated reduction in IGF-I and increases in IGFBP-1 secretion in primary rat hepatocytes (mean ± SD). **A**: IGF-I, **B**: IGFBP-1.

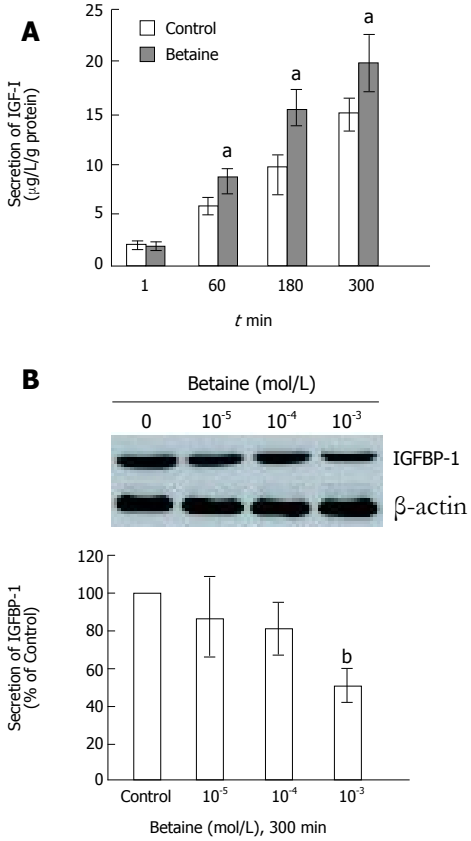

Figure 2 Betaine stimulates IGF-I secretion and decreases IGFBP-1 secretion in primary rat hepatocytes (mean ± SD). **A**: IGF-I, **B**: IGFBP-1. $^{a}P<0.05$; $^{b}P<0.01$ *vs* control.

alcohol reduces the level of IGF-I and increased that of IGFBP-1 in the serum, liver, and kidney of Sprague-Dawley rats[5]. To examine the possible effect of betaine, an osmolyte, on the regulation of alcohol-stimulated IGF-I and IGFBP-1 secretion, we measured the secretion of IGF-1 and IGFBP-I after cotreatment with betaine (for 300 min) and alcohol (for 180 min) in primary cultured rat hepatocytes. Whereas the secretion of IGF-I was significantly reduced by alcohol treatment alone, co-treatment with betaine resulted in IGF-I secretion being maintained at control levels (Figure 1A, $P<0.05$). In addition, betaine reduced alcohol-stimulated IGFBP-1 secretion (Figure 1B). Taken together, these results suggest that betaine ameliorates the alcohol-mediated secretion of IGF-I and IGFBP-1 in primary cultured rat hepatocytes.

Effects of betaine on IGF-I and IGFBP-1 secretion in primary rat hepatocytes

To evaluate the modulatory effects of betaine, we examined its effect on IGF-I and IGFBP-1 secretion in primary rat hepatocytes. Confluent cells were treated with 10^{-3} mol/L betaine for the times indicated in Figure 2. Supernatant was removed and the levels of IGF-I were measured by RIA (Figure 2A). The secretion of IGF-I increased significantly with increasing time after betaine treatment (Figure 2A, $P<0.05$ *vs* Control). To examine the effect of betaine on IGFBP-1 secretion, rat hepatocytes were treated with the concentrations of betaine indicated in Figure 2B for 300 min, and secretion of IGFBP-1 was monitored. Betaine decreased IGFBP-1 secretion in primary rat hepatocytes in a dose-dependent manner (Figure 2B). In summary, IGF-I secretion was significantly increased by betaine treatment, whereas IGFBP-1 secretion was decreased.

Involvement of MAPK in the betaine-induced stimulation of IGF-I and reduction of IGFBP-1 secretion.

To determine the molecular mechanism by which betaine stimulates IGF-I and reduces IGFBP-1 secretion in rat hepatocytes, we examined the involvement of MAPK. The effects of phosphorylation of p42/44 MAPK on the secretions of IGF-I and IGFBP-1 were monitored for 300 min after treatment with betaine alone (10^{-3} mol/L) and with betaine (10^{-3} mol/L) after pretreatment with PD98059 (10 μmol/L). Pretreatment with PD98059 not only significantly decreased betaine-stimulated IGF-I secretion (Figure 3A), but also attenuated the betaine-induced reduction in IGFBP-1 secretion (Figure 3B).

Given the effects of PD98059 on IGF-I and IGFBP-1 secretion, we performed an immunoblot analysis with antibody binding to phosphorylated MAPK 1/2 molecules. Whole lysates of cells treated with betaine were subjected to 100 g/L SDS-PAGE and blotted with phospho-p44/42 MAPK. The phosphorylation of MAPK 1/2 was increased at 60, 180, and 300 min after betaine stimulation, reaching a maximum after 300 min (Figure 4A). Betaine stimulated the phosphorylation of p42/p44 MAPK in a dose-dependent manner (Figure 4B). A significant ($P<0.05$) phosphorylation of p42/p44 MAPK was observed at a betaine concentration of 10^{-3} mol/L compared with the

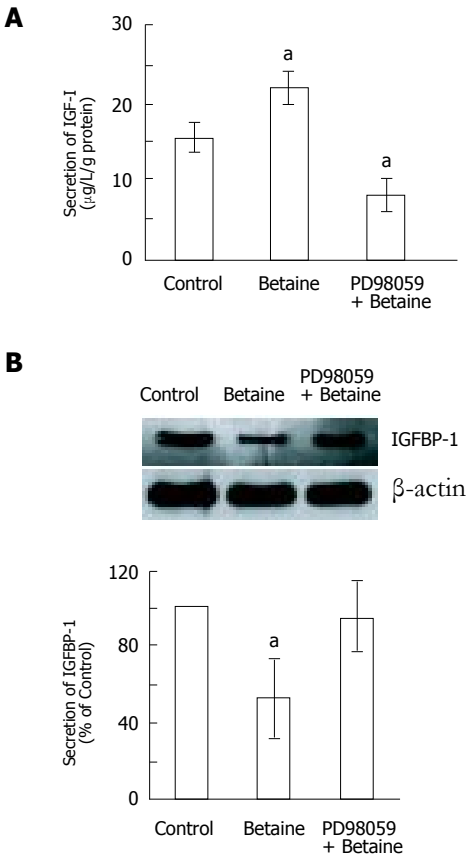

Figure 3 PD98059 attenuates the stimulation of IGF-I and attenuation of IGFBP-1 secretion effected by betaine (mean ± SD). **A**: IGF-I, **B**: IGFBP-1. $^{a}P<0.05$, *vs* control.

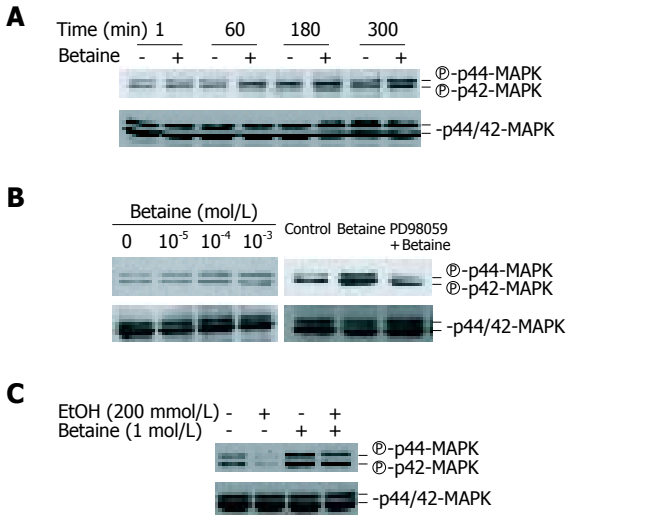

Figure 4 Betaine stimulates the phosphorylation of p42/44 MAPK in primary rat hepatocytes. **A**: Time-dependent activation of p42/44 MAPK by 10^{-3} mol/L betaine, **B**: Dose-dependent activation of p42/44 MAPK by betaine for 300 min (left panel). Treatment with betaine following pretreatment with PD98059 for 10 min (right panel), **C**: Betaine-stimulated recovery of p42/44 MAPK activation in ethanol-treated cells.

control (Figure 4B). Western blot of anti-phospho-MAPK was then reprobed with a specific antibody for MAPK 1/2. The resulting bands coincided with the immunoreactive bands of MAPK 1/2 (Figure 4B). Betaine ameliorated the activation of MAPK, although alcohol treatment inhibited the basal phosphorylation of p42/44 MAPK (Figure 4C). The effect of betaine on MAPK activation was also monitored at 300 min after treatment of the hepatocytes with betaine (10^{-3} mol/L); betaine induced the activation of p42/44 MAPK.

DISCUSSION

Alcohol abuse is associated with deleterious effects on several organs of the body, particularly the liver and brain[8]. Alcohol-induced liver damage is one of the major causes of morbidity and mortality among alcoholics, exposure to ethanol leading to both short- and long-term changes in liver function. Ethanol alters hepatic carbohydrate and lipid metabolism, as well as the synthesis of protein and DNA, which leads to liver dysfunction and cirrhosis[9]. The mechanisms and mediators underlying this liver injury, however, are not clearly understood. Kanbak *et al*[2] reported that betaine protects the liver from ethanol-mediated hepatotoxicity in rats, and it was reported recently that betaine also protects the liver from ischemia/reperfusion injury, niacin toxicity, and CCl_4-, hyperosmolarity-, and ethanol-induced apoptosis.

In the present study we found that the secretion of IGF-I, which is inhibited by ethanol, is increased by treatment with betaine, whereas that of IGFBP-1, which is increased by ethanol is reduced by betaine. It has been reported recently that lipid peroxidase activity is involved in the effects of ethanol on the secretions of IGF-I and IGFBP-1, but the underlying mechanism remains to be elucidated[10]. It has also been reported that acute ingestion of ethanol raises the plasma levels of IGFBP-1 in normal subjects[3,11]. Intake of ethanol influences the bioavailability of IGF-I in normal individuals. Barak *et al*[1] has shown that betaine, which is a naturally occurring substance, may be used as a protective agent against hepatotoxic substances such as ethanol and CCl_4. It may prevent the generation of reactive oxygen species in hepatocytes that results from treatment with ethanol, which is thought to be responsible for attenuation of IGF-I and increase in IGFBP-1 secretion.

This study also aimed to determine whether changes in IGF-I and IGFBP-1 secretion induced by betaine involve signal transduction in hepatocytes. Dose-dependent influences of betaine on the secretion of IGF-I and IGFBP-1, and on MAPK activity were observed. A significant increase in the secretion of IGF-I, a decrease in the secretion of IGFBP-1, and a significant increase in the phosphorylation of p42/p44 MAPK were observed 300 min after treatment with 200 mmol/L ethanol. The activation of p42/44 MAPK was blocked by the MAPK inhibitor PD98059 (10 μmol/L), and this greatly influenced the secretion of IGF-I and IGFBP-1.

In conclusion, the modulation of hepatocyte secretion of IGF-I and IGFBP-1 by betaine involves alteration of p42/44 MAPK activity; betaine may prevent ethanol-induced changes in IGF-I and IGFBP-1 secretion in hepatocytes.

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CLINICAL RESEARCH

Cerebral processing of auditory stimuli in patients with irritable bowel syndrome

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Abstract

AIM: To determine by brain functional magnetic resonance imaging (fMRI) whether cerebral processing of non-visceral stimuli is altered in irritable bowel syndrome (IBS) patients compared with healthy subjects. To circumvent spinal viscerosomatic convergence mechanisms, we used auditory stimulation, and to identify a possible influence of psychological factors the stimuli differed in their emotional quality.

METHODS: In 8 IBS patients and 8 controls, fMRI measurements were performed using a block design of 4 auditory stimuli of different emotional quality (pleasant sounds of chimes, unpleasant peep (2000 Hz), neutral words, and emotional words). A gradient echo T2*-weighted sequence was used for the functional scans. Statistical maps were constructed using the general linear model.

RESULTS: To emotional auditory stimuli, IBS patients relative to controls responded with stronger deactivations in a greater variety of emotional processing regions, while the response patterns, unlike in controls, did not differentiate between distressing or pleasant sounds. To neutral auditory stimuli, by contrast, only IBS patients responded with large significant activations.

CONCLUSION: Altered cerebral response patterns to auditory stimuli in emotional stimulus-processing regions suggest that altered sensory processing in IBS may not be specific for visceral sensation, but might reflect generalized changes in emotional sensitivity and affective reactivity, possibly associated with the psychological comorbidity often found in IBS patients.

Key words: Visceral hypersensitivity; Irritable bowel syndrome; Brain processing; fMRI; Auditory stimulation; Emotion

Andresen V, Poellinger A, Tsrouya C, Bach D, Stroh A, Foerschler A, Georgiewa P, Schmidtmann M, van der Voort IR, Kobelt P, Zimmer C, Wiedenmann B, Klapp BF, Monnikes H. Cerebral processing of auditory stimuli in patients with irritable bowel syndrome. *World J Gastroenterol* 2006; 12 (11): 1723-1729

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INTRODUCTION

Visceral hypersensitivity has been shown to play a pathogenic role in the irritable bowel syndrome (IBS)[1,2]. At least in a subgroup of patients, this might be caused by altered brain processing of visceral sensation as has been suggested on the basis of brain imaging studies. Another important characteristic of IBS is the extraintestinal comorbidity. There is a high prevalence of non-gastrointestinal functional diseases, such as fibromyalgia or chronic fatigue syndrome, and also psychological disorders, such as depression and anxiety[3,4]. The frequent co-occurrence of these different disorders on the one hand, and the impact of psychological factors on all of them on the other hand[5], leads to the question whether these disorders might share a common pathogenesis such as a generalized increase in emotional and sensory sensitivity that could be involved in the alterations of sensory brain processing observed in these disorders[6-9].

Indeed, there is some evidence that altered cerebral

response patterns in IBS may not be specific for gastr-ointestinal stimuli. In patients with IBS and concomitant fibromyalgia, alterations in the central processing of painful somatic stimuli have been observed[7]. Moreover, even IBS patients without a manifest fibromyalgia may exhibit a somatic hypersensitivity with altered cerebral processing of somatic sensation[10]. These forms of somatic hypersensitivity, which are in contrast to earlier descriptions of somatic hyposensitivity states in IBS, are caused by viscerosomatic convergence mechanisms. The latter are thought to occur, because visceral and somatic sensations are both passed through the dorsal root ganglia and the dorsal columns of the spinal cord, where neural interactions have been described[11]. Therefore,any peripherally generated visceral hypersensitivity could subsequently induce a somatic hypersensitivity in the corresponding somatic dermatoma, and altered cerebral response pattern to somatic stimuli would actually reflect increased sensory input from the periphery. However, further support for cerebrally located processing alterations in IBS is provided by two studies demonstrating an increased reactivity of event-related potentials to auditory stimuli in IBS patients[12,13]. Since auditory stimuli are transmitted directly to the brain via the eighth cranial nerve, there is no peripheral connection to visceral sensory input and changes in the central reactivity could be allocated directly to the sensory processing circuits of the brain.

With this study, we aimed to test the hypothesis that IBS patients differ from healthy controls in the cerebral processing of non-visceral stimuli, detected by functional magnetic resonance imaging (fMRI). To be able to focus on the cerebral processing level, we chose auditory cues as non-visceral stimuli. The specific aims were 1) to analyze by fMRI the cerebral response patterns to non-visceral, auditory stimuli in IBS patients compared with healthy controls, and 2) to evaluate whether different brain responses are influenced by the emotional impact of the stimulus.

MATERIALS AND METHODS

Subjects

Eight right-handed patients with IBS [five females, three males; mean age, 41.3 (27-64) years; diarrhea-predominant, $n=5$, alternating stools, $n=3$] established by Rome II criteria [14] were recruited at our institution's outpatient clinic for gastrointestinal functional disorders. To exclude other causes for bowel symptoms, all patients had undergone a thorough work-up including laboratory tests, stool analysis for bacterial, fungal, or parasite infection, abdominal ultrasound, colonoscopy, lactose- and fructose hydrogen breath test. Moreover, a rectal sensitivity testing by computerized barostat was performed. The mean individual perception threshold of first sensation of the rectal barostat distension was 16.4 mmHg (SD 6.2). None of the patients had previous abdominal surgery and all patients had symptoms for more than 1 year. None of the patients used centrally acting agents to treat bowel symptoms, and peripherally acting IBS treatments were stopped 7 d prior to the study. As controls, 8 right-handed healthy volunteers [5 males, 3 females; mean age, 39.4 (24-54) years] were selected after exclusion of individuals with concomitant or previous GI-disorders by history and the IBS symptom score-questionnaire [15]. In all subjects, written informed consent as approved by the institutional ethical committee was obtained, and concomitant psychiatric disorders were excluded by using the standardized German Diagnostic Interview for Psychiatric Disorders (DIPS[16]), a structured clinical interview based on the Anxiety Disorders Interview Schedule[17].

Personality-questionnaires

For the assessment of personality characteristics, all study participants were asked to fill in the following questionnaires: The German form of the State-Trait-Anxiety-Inventory (STAI)[18] for the assessment of depression, neuroticism and complaints, the German form of the Beck's Depression Index (BDI)[19] for the assessment of depression, and the German form of the NEO-Five-Factor-Inventory (NEO-FFI)[20] for the assessment of neuroticism, openness to experience, extroversion, agreeableness, and conscientiousness.

Stimulation protocol for MRI-scanning

We used a block design of four auditory stimuli of different emotional impact: an unpleasant peep (a sound of 2000 Hz), pleasant sounds of chimes, words with emotional impact and neutral words. The part of the protocol using neutral words had served as a neutral, non-visceral control stimulus, in a previous study investigating visceral stimulation[21]. All words were chosen from a list of words that had been evaluated as neutral or emotional by a group of 20 healthy volunteers. The stimulation paradigm had been evaluated in event-related potential studies for patients with psychosomatic disorders. The stimulation phases of 48 s each were separated by resting phases and applied in the following order: peep, chimes, neutral words, chimes, emotional words, neutral words, peep.

MRI acquisition

Magnetic resonance images were collected on a 1.5 T whole body scanner (Siemens Magnetom Vision, Erlangen, Germany) with a standard head coil. A vacuum pad was used to minimize head movements. First, a T1-weighted localizer scan was recorded. Next, T2-weighted oblique scans were obtained (TR/TE 4500/128 ms, field of view 230 mm), primarily to aid Talairach transformation for data analysis. For the functional scans, an echo-planar sequence (TR/TE 4000/66 ms; flip angle 90 degrees; field of view 230 mm; matrix 128 × 128; slice thickness 6 mm, interslice gap 0.6 mm; in-plane resolution 1.8 mm × 1.8 mm) was used. One hundred and twenty images per slice were acquired. Sixteen slices adjusted at a transverse-to-coronal angle of approximately 20° covering the whole brain with the exception of the most superior frontal and superior parietal lobe, inferior temporal pole, and cerebellum (most superior z about 60 and most inferior z about -25 according to Talairach and Tournoux[22]) were obtained for all studies. Structural 3D data sets were acquired using a T1-weighted sagittal sequence with isotropic voxels (TR/TE 11.4/4.4 ms; flip angle 15 degrees; number of slices

Table 1 Personality characteristics (mean ± SD)

Personality test	Control	IBS	IBS *vs* control	Reference-population [18-20]
STAI X2	34.8 ± 6.1	41.3 ± 11.0	$P = 0.17$	35.7 ± 9.4
BDI	5.4 ± 5.3	8.7 ± 4.4	$P = 0.19$	6.5 ± 5.2
NEO: Neuroticism	1.7 ± 0.5	2.2 ± 0.6	$P = 0.11$	1.8 ± 0.7
NEO: Extroversion	2.8 ± 0.4	2.6 ± 0.3	$P = 0.24$	2.4 ± 0.6
NEO: Openness to experience	2.5 ± 0.5	2.4 ± 0.3	$P = 0.73$	2.7 ± 0.5
NEO: Agreeableness	2.4 ± 0.6	2.4 ± 0.3	$P = 0.81$	2.4 ± 0.5
NEO: Consciousness	3.0 ± 0.6	3.2 ± 0.5	$P = 0.46$	2.6 ± 0.6

STAI=State-Trait-Anxiety-Inventory, with X2=Trait-Anxiety Subtest, BDI= Beck's Depression Index, NEO= NEO-Five-Factor-Inventory

160, matrix 256 × 256, field of view 256 mm, voxel size 1 mm3).

Data analysis

For data analysis we used the Brainvoyager® software (Brain Innovation B.V, Postbus, Maastricht, Netherlands). The 2D functional data were reconstructed within the 3D structural data set. The 3D-data were then transformed into the standardized Talairach spaced brain[22]. Finally, the reconstructed data set underwent subsequent procedures of motion correction, intensity scaling and detrending.

Statistical maps were constructed using the general linear model module of the Brainvoyager software. The stimulation conditions were used as predictors, and the contrasts of each stimulation condition versus rest were analyzed. For the between group comparison, predictors were defined for each stimulus type as the interaction of the group with this stimulus type. Therefore, each predictor represented a larger signal increase of one group at the referred stimulus type.

Activated clusters were only accepted if they showed highly significant ($P < 0.001$) activation increase. In order to correct for multiple comparisons, a minimal cluster size of 6 voxels was defined.

Definition of regions of interest (RoI)

As regions of interest (RoI) for the data analysis (Table 2), we selected brain areas known to be involved in emotional processing[23]. All regions were neuroanatomically predefined by an expert neuroradiologist according to the coordinates of Talairach *et al*[22], and according to neuroanatomical visualization.

RESULTS

Subject characteristics

The personality traits were not significantly different between IBS patients and healthy controls. Results are summarized in Table 1. Descriptively, IBS patients had higher values for anxiety ($P = 0.17$), depression ($P = 0.19$) and neuroticism ($P = 0.11$) relative to healthy controls (Table 1).

Group analysis-auditory stimulation

Both groups responded to all stimuli with a significant activation ($P < 0.001$) in the auditory cortex, with a higher signal response to the word processing stimulus conditions and in the speech processing areas for the word stimulations (data not shown). With regard to the emotional processing regions we observed the following:

Response to unpleasant peep

To unpleasant peep, both groups responded with significant deactivations ($P < 0.001$) in the anterior cingulate cortex (ACC) and prefrontal cortex (PFC) with a relatively stronger ACC deactivation in controls, as indicated by the number of deactivated voxels. By contrast, only IBS patients responded with significant deactivations ($P < 0.001$) in the amygdala and the hippocampus (HC) (Table 2).

Response to pleasant sounds of chimes

To pleasant sounds of chimes, both groups responded with significant deactivations ($P < 0.001$) in the PFC and the posterior cingulated cortex (PCC) with a relatively stronger PCC deactivation in controls, as indicated by the number of deactivated voxels. Only controls responded with deactivations ($P < 0.001$) in the Insula and HC, and with significant activations ($P < 0.001$) in the ACC and a different region of the PFC. In IBS patients, on the contrary, the ACC was significantly deactivated ($P < 0.001$) (Table 2).

Response to emotional words

To emotional words, both groups responded with significant deactivations ($P < 0.001$) in the parietal sensory association cortex (PSAC), the ACC, the PFC, and the HC. However, the overall deactivation response in the ACC, PFC and HC, as indicated by number and size of deactivated clusters, was much stronger in IBS patients relative to controls (Table 2, Figure 1). By contrast, only controls responded with a significant deactivation ($P < 0.001$) in the PCC, while only in IBS patients, emotional words induced deactivations ($P < 0.001$) in the insula and moreover a significant activation ($P < 0.001$) in a different region of the PFC (Table 2).

Response to neutral words

To neutral words, both groups responded with significant activations ($P < 0.001$) in the PFC and PSAC. However, the overall activation response, as indicated by number and size of activated clusters, was much stronger in IBS patients relative to controls. Only in controls, neutral words induced a deactivation ($P < 0.001$) in the ACC. By contrast, only IBS patients responded with significant activations ($P < 0.001$) in the ACC, PCC, HC, and insula (Table 2, Figure 2).

DISCUSSION

Altered cerebral processing of visceral sensation has been proposed to play a pathogenic role in IBS. Whether these alterations are specific for visceral stimuli is currently not

Table 2 Significant activations (signal increase) and deactivations (signal decrease) induced by different acoustic stimuli

			[1]Largest cluster				
I) PEEP							
A: Control deactivation							
RoI	Side	No. of Clusters	No. of Voxels[1]	x[1]	y[1]	z[1]	% signal change*
ACC	L	1	-793	-8	37	-10	-0.53
PFC	B	7	-1506	37	35	-7	-0.52
B: IBS deactivation							
ACC	L	1	-271	-6	31	7	-0.28
PFC	B	7	-1724	23	57	8	-0.52
Amyg-dala	R	1	-452	25	1	-20	-0.33
HC	B	2	-436	33	-22	-24	-0.38
II) CHIMES							
A: Control activation							
ACC	B	1	699	-1	29	2	0.40
PFC	B	7	1957	-4	37	-6	0.39
Deactivation							
PFC	B	4	-1781	31	47	23	-0.48
PCC	L	1	-707	-5	-4	51	-0.24
Insula	R	1	-850	32	1	7	-0.25
HC	R	1	-113	26	-14	-15	-0.14
B: IBS deactivation							
ACC	B	2	-1212	2	18	44	-0.38
PCC	B	1	-159	0	-17	32	-0.36
PFC	R	3	-2095	54	13	13	-0.42
III) EMOTIONAL WORDS			[1]Largest cluster				
A: Control deactivation							
ACC	L	1	-724	-5	36	13	-0.33
PFC	B	4	-275	-4	47	-2	-0.36
PCC	L	1	-134	-1	-33	44	-0.35
PSAC	B	1	-2048	0	-62	33	-0.36
HC	R	1	-31	25	-24	-15	-0.21
B: IBS activation							
PFC	L	1	609	-46	29	-1	0.25
Deactivation							
ACC	R	1	-1207	2	40	5	-0.41
PFC	B	4	-2076	9	43	26	-0.32
PSAC	B	1	-2048	0	-46	42	-0.35
HC	B	2	-1010	24	-35	-12	-0.29
Insula	R	1	-331	34	-10	1	-0.19
IV) NEUTRAL WORDS							
A : Controls activation							
PFC	R	1	45	26	55	6	0.40
PSAC	R	1	45	26	-42	53	0.05
Deactivation							
ACC	L	1	-118	-3	31	-6	-0.44
B : IBS activation							
ACC	R	2	223	1	6	46	0.19
PFC	B	8	1408	0	46	-11	0.18
PSAC	B	4	257	26	-65	41	0.08
HC	B	2	652	27	-27	19	0.14
PCC	L	2	102	-2	-39	37	0.12

RoI= region of interest, R=right, L=left, B= bilateral, ACC= anterior cingulate cortex, PCC= Posterior Cingulate Cortex, PFC=prefrontal cortex, PSAC=parietal sensory association cortex, HC=hippocampus, x+y+z=coordinates of the Talairach space.

clear. In this study, we analyzed cerebral processing of non-visceral, auditory stimuli using fMRI. In a separate protocol, all IBS patients included in this study also underwent rectal stimulation by balloon distension adapted to the individual rectal perception thresholds, which were lower in IBS patients than in controls[21]. In IBS patients, decreased ACC and PFC activation with subliminal and supraliminal rectal stimuli and increased HC activation with supraliminal stimuli suggested disturbances of the associative and emotional processing of visceral sensation[21]. Our current findings demonstrate that IBS patients exhibit differences in the central processing of auditory stimuli of different emotional impact in brain regions involved in emotional sensory processing. Our main observations were: 1) To distressing auditory stimuli (peep and emotional words) both controls and IBS patients responded with significant deactivations in emotional processing regions demonstrating stronger responses in a greater variety of brain areas in IBS patients. 2) To pleasant auditory stimuli (chimes) IBS patients also responded with significant deactivations only, while in controls significant activations in the ACC and PFC were observed. 3) To neutral auditory stimuli (neutral words) IBS patients, unlike controls, responded with large significant activations in a variety of emotional processing regions. Taken together, IBS patients reacted more intensively with deactivations following emotional auditory stimuli, not differentiating, unlike controls, between distressing or pleasant sounds, and more intensively reacted with activations to neutral auditory stimuli.

Thus, this fMRI study indicates alterations of auditory sensory processing in IBS patients in brain regions participating in emotional stimulus processing. The cerebral processing differences involve both activation and deactivation responses. However, the specific cerebral function of deactivations observed in fMRI is still not completely understood and has been widely discussed[24]. Within the context of BOLD (Blood Oxygen Level Dependent) responses, on which the fMRI technique is based on, an activation reflects an increase in regional oxyhemoglobin that is believed to result from an increased blood flow coupled to neural activation ("neurovasular coupling"). In contrast, a "deactivation" reflects a stimulation-induced regional decrease of oxyhemoglobin of which the functional meaning is unclear. Vascular steal mechanisms or "neurovascular decoupling" resulting in reduced oxyhemoglobin have been suggested as possible cause of signal decrease[24]. In this case, the signal decrease would also reflect a neural *activation*. On the other hand, there is some evidence that the signal decrease could actually reflect a reduction of neural activity[25-27]. In fact, it has been proposed, that deactivations might contribute to cortical protection from incoming stimuli, for example in the context of chronic repetitive subconscious somatic stimuli[28] or in the context of sleep preservation[26,27]. A study demonstrating that the same auditory stimulus can induce either activation or deactivation responses of the amygdala just depending on the psychosocial background of the subjects[29],also supports a distinct neuro-functional meaning of deactivations. However, the uncertain functional meaning of deactivations to date makes the detailed interpretation of our findings difficult and we therefore restrict the following discussion to a rather descriptive evaluation of the dem-

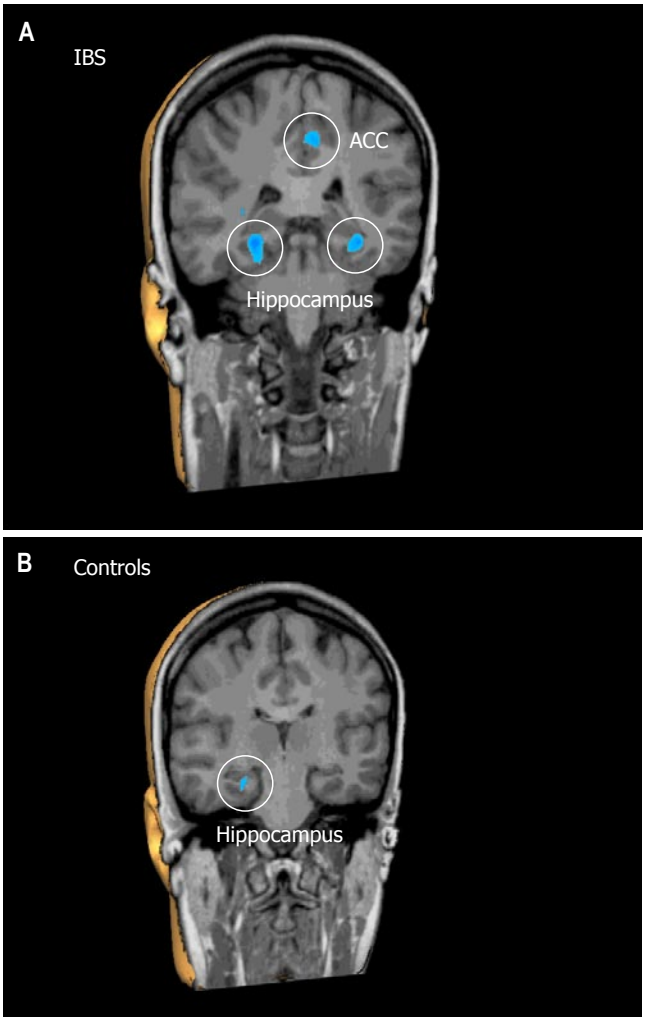

Figure 1 Auditory stimulation with emotional words induced significant deactivations of the hippocampus bilateral, and of the anterior cingulated cortex (ACC) in IBS patients, but only a very small deactivation of the right hippocampus in controls ($P < 0.001$).

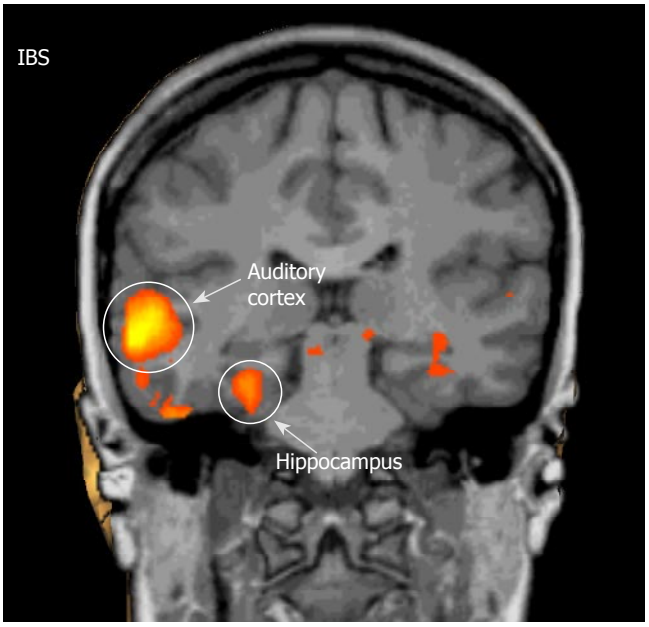

Figure 2 In IBS patients, auditory stimulation with neutral words induced significant activation of the hippocampus ($P < 0.001$).

onstrated brain processing differences.

Though both IBS patients and controls responded to distressing emotional stimuli mainly with deactivations, the deactivated regions in IBS patients were larger and involved a greater variety of emotional processing regions, among them parts of the limbic system like the amygdala or the hippocampus. In general, these observations could suggest a greater emotional reactivity to distressing stimuli in IBS patients as compared with healthy controls. This is in line with previous literature that demonstrate a central hyperreactivity of IBS patients to rectal stimuli in the presence of distressing auditory or visual stimuli[30-32].

Interestingly, IBS patients responded to pleasant sounds with similar deactivation patterns as to distressing stimuli whereas controls also reacted with activations. It is conceivable that an underlying alexithymia, a disorder characterized by the difficulty to recognize or express emotions and found to be significantly increased in IBS patients[33], could possibly accord for this finding. However, this has to remain speculative, because alexithymia was not specifically assessed in this study.

Neutral words, as expected, did hardly induce any reaction in emotional processing centers in healthy controls. By contrast, IBS patients responded with significant activations in these brain regions. These findings could indicate that IBS patients have a generalized increase in the sensitivity and reactivity of emotional processing brain regions even in response to neutral stimuli. Interestingly, the reaction to neutral stimuli is limited to activation-responses, while the emotional stimuli induced deactivation patterns in the same brain regions. This provides further evidence that activations and deactivations represent different processing functions depending on the emotional impact of the stimulus[29].

Overall, our findings indicate that IBS patients relative to controls show a greater cerebral reactivity to auditory stimuli with a larger involvement of limbic structures suggesting a higher sensitivity of emotional processing brain regions. Thereby, our results underline earlier observations by Blomhoff *et al* and Berman *et al*[12,13,34] who demonstrated a cerebral hyperreactivity to both emotional and neutral auditory stimuli in IBS patients. While altered cerebral processing in IBS has previously been demonstrated in the context of visceral stimulation[6,35,36], the current findings suggest that differences in cerebral stimulus processing in IBS might not be restricted to visceral sensation. Rather it is conceivable that differences in the emotional state might be an underlying factor. In fact, Blomhoff could demonstrate that the hyperreactivity to auditory stimuli is especially found in IBS patients with concomitant phobic anxiety disorders[34]. Accordingly, in our study, higher levels of anxiety, depression, and neuroticism in the IBS patient group (though differences were not reaching the level if significance possibly due to small sample sizes) could account for some of the observed differences in brain activation responses.

We acknowledge that this study has several limitations: The sample size of $n = 8$ for each group is rather small, and the bowel dysfunctions are heterogeneous in the IBS group. There is some evidence that diarrhea- versus

constipation-predominant IBS patients have different cerebral response patterns to rectal stimulation[37]. Whether this could also affect the cerebral processing of auditory stimuli is unknown. Furthermore, it is conceivable that the increased scores for anxiety, neuroticism and depression can alone account for the different stimulus processing of patients versus controls independent of IBS status. Future studies should include more patients, compare also to other psychologically distressed patients free of IBS, assess further psychometric variables such as alexithymia and allow for different subgroup analyses to further elucidate the nature of altered sensory processing in IBS.

Overall, the current observations of altered cerebral response patterns to neutral and emotional auditory stimuli in IBS patients indicate that altered emotional stimulus processing in IBS may not be specific for visceral sensation, but might reflect a generalized increase in emotional sensitivity and affective reactivity. This could account for the frequent association of IBS with psychological or extra-intestinal functional disorders[3].

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CLINICAL RESEARCH

Effect of IBD sera on expression of inducible and endothelial nitric oxide synthase in human umbilical vein endothelial cells

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Abstract

AIM: To study the expression of endothelial and inducible nitric oxide synthases (eNOS and iNOS) and their role in inflammatory bowel disease (IBD).

METHODS: We examined the effect of sera obtained from patients with active Crohn's disease (CD) and ulcerative colitis (UC) on the function and viability of human umbilical vein endothelial cells (HUVEC). HUVECs were cultured for 0-48 h in the presence of a medium containing pooled serum of healthy controls, or serum from patients with active CD or UC. Expression of eNOS and iNOS was visualized by immunofluorescence, and quantified by the densitometry of Western blots. Proliferation activity was assessed by computerized image analyses of Ki-67 immunoreactive cells, and also tested in the presence of the NOS inhibitor, 10^{-4} mol/L L-NAME. Apoptosis and necrosis was examined by the annexin-V-biotin method and by propidium iodide staining, respectively.

RESULTS: In HUVEC immediately after exposure to UC, serum eNOS was markedly induced, reaching a peak at 12 h. In contrast, a decrease in eNOS was observed after incubation with CD sera and the eNOS level was minimal at 20 h compared to control (18% ± 16% *vs* 23% ± 15% $P<0.01$). UC or CD serum caused a significant increase in iNOS compared to control (UC: 300% ± 21%; CD: 275% ± 27% *vs* 108% ± 14%, $P<0.01$). Apoptosis/necrosis characteristics did not differ significantly in either experiment. Increased proliferation activity was detected in the presence of CD serum or after treatment with L-NAME. Cultures showed tube-like formations after 24 h treatment with CD serum.

CONCLUSION: IBD sera evoked changes in the ratio of eNOS/iNOS, whereas did not influence the viability of HUVEC. These involved down-regulation of eNOS and up-regulation of iNOS simultaneously, leading to increased proliferation activity and possibly a reduced anti-inflammatory protection of endothelial cells.

Key Words: Crohn's disease; Human umbilical vein endothelial cells; Inflammatory bowel disease; Nitric oxide synthase; Ulcerative colitis

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INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are characterized by chronic inflammatory ulceration of the intestine. In an early[1], and in some recent studies[2, 3], the contribution of mucosal microvascular dysfunction is implicated in the development of CD. Histological analy-

Table 1 Data of patients with ulcerative colitis and Crohn's disease

Group	*n*	Age (yr)(range)	M/F	Endoscopic activity score	Clinical activity score	CRP (mg/L)	Treatment
UC	21	27 (18-33)	3/8	G_1: 4 G_2: 9 G_3: 8	4≤UC-DAI	>10	5-ASA
CD	18	31 (23-45)	13/5	8≤CDEIS≤14	150≤CDAI≤450	>10	5-ASA
Control	16	29 (25-39)	8/8				

G_{0-3}: Endoscopic grading of UC (Br Med J 1964; 1:89); CDEIS: Crohn's disease endoscopic index of severity (Gut 1989; 30:983-89); UC-DAI: Ulcerative colitis disease activity index (Br Med J 1955; 2:1041); CDAI: Crohn's disease activity index (*Gastroenterology* 1979; 77:843)

sis of IBD colonic mucosa indicated structural changes of the vascular system. In CD, arterial thickening and the appearance of an increasing number of capillaries were observed[1, 3]. Dysfunction in the vasodilatory capacity of microvessels from areas of chronic inflammatory damage was also shown in IBD[2]. Inflammatory cytokines modify and regulate the level of NO, and they are also involved in vascular endothelial response to inflammatory injury[3, 4]. Apoptosis of endothelial cells induced by proinflammatory cytokines and reactive oxygen species is counteracted by the endothelial nitric oxide (NO). The suppression of apoptosis may contribute to the anti-inflammatory and pro-angiogenic effect of endothelial NO[5].

The endothelial form of nitric oxide synthase (eNOS) plays a significant protective role against experimental colitis[6]. Decrease of eNOS immunoreactivity in the endothelium is suggested as a putative cause of arterial thickening and the appearance of an increasing number of capillaries in the mucosa of Crohn's colitis[3]. Moreover, weak eNOS immunoreactivity in the endothelium together with structural alteration of the vascular wall, both found in biopsies of Crohn's patients suggest that malfunction of local blood supply may also contribute to the development of the disease[3]. Human umbilical vein endothelial cells (HUVEC) are frequently used as a specific model for studying endothelial function. HUVECs are also sensitive to pro-inflammatory stimuli induced by interleukins. NO is produced in HUVEC constitutively by eNOS and by inducible nitric oxide synthase (iNOS) in the case of different stimuli[7, 8]. NO also contributes to the regulation of apoptosis, cell differentiation and proliferation (angiogenesis) in this cell type[9-11].

The changes in the activity of eNOS and iNOS as well as their role in the endothelium of IBD patients have not been examined in detail. In the present study, we examined the effect of sera from patients diagnosed with UC and CD on cultured HUVEC. The expression and localization of eNOS and iNOS were determined in highly confluent and in semi-confluent HUVEC cultures, and the apoptotic/necrotic characteristics and proliferation activity upon distinct treatments were also assessed.

MATERIALS AND METHODS

Collection of blood sera

Blood was sampled for examination from patients who had relapsing UC or CD. Among 21 UC patients, 16 were on their 1st relapse (mean time from diagnosis [*mtd*] was 1 yr), and 5 on 2nd relapse (*mtd* = 3 yr), whereas all the 18 CD patients were on their 1st relapse (*mtd* = 4 yr). Control sera were obtained from 16 healthy volunteers. They received no systemic or locally applied corticosteroids, and their medication was stable for the last four weeks before sampling. All the patients were on 5ASA oral therapy. Patients diagnosed as having UC had left side colitis (Table 1). Crohn's patients had colonic localization and a non-stricturating, non-penetrating form of the disease (Table 1) according to the Vienna classification (1998), and an elevated C-reactive protein level (CRP>10 mg/L), without abscess or sign of infection (all of the patients had negative abdominal ultrasound). In cases which raised the suspicion of abdominal or pelvic abscess, CT or MRI (to exclude the presence of perianal / perirectal abscesses) was performed. Five milliliters of blood were taken from patients of UC, CD and control subjects respectively, placed into collecting tubes, and centrifuged at 2000 r/min for 10 min. The sera were removed and mixed, and kept frozen at -80℃ until use.

Preparation and culturing of HUVEC

Freshly isolated umbilical cords were obtained from the Department of Obstetrics and Gynecology (University of Debrecen, Medical and Health Science Center). Umbilical cords were immersed into a sterile flask containing culture medium (components are described below) and carried to the cell culture room. Both ends of the cords were bound and the ends of the umbilical vein were cannulated. The vein vessel was washed through the cannulas by sterile PBS, followed by Hanks' Balanced Salt Solution (HBSS), and M199 media (all purchased from Sigma, Budapest, Hungary). Then, the vein vessel was filled with 1 g/L collagenase in M199, and placed in a CO_2 incubator for 12 min at 37 ℃. Endothelial cells were isolated in a sterile centrifuge tube from the vessel by washing twice with 20 mL M199 medium. Cell suspension was centrifuged at 1000 r/min for 15 min, and the supernatant was discarded and the cells were resuspended in the culture medium.

The cells (2×10^9 cells/L) were inoculated into a plastic culture dish. The culture medium contained the following components: 500 mL/L normal human serum, 20 g/L HEPES, 10 g/L L-glutamine, 10 g/L amphotericin, 10 g/L penicillin, 450 mL/L sterile filtered M199. One day after inoculation, dishes were washed with 37 ℃ HBSS, and put into the culture media again. After 4-5 d of culturing, dur-

ing which time cells grew extensively over the entire space of the dish, cells were washed with HBSS, then digested with 1 mL tripsin-EDTA (tripsin : EDTA = 1 : 25). The cells were suspended in 1 mL culture medium and collected into a sterile flask, then mixed well. The cells were inoculated into the culture dish and the cell propagation and collection procedure were repeated again as described above. Then, cells were passed onto culturing plates at about 2 $\times 10^9$ cell/L concentration. Cell monolayers that reached 20%-30% or 70%-80% confluency were used for the experiments. The cells were kept in 50% normal, 50% UC or 50% CD sera dissolved in culturing medium for 2, 4, 8, 12, 20, 24, 36, and 48 h, then processed for visualization of eNOS or iNOS.

Western blot

After removal of the culture media, cells were washed with ice-cold PBS, then harvested by a cell scraper into 50 μL RIPA lysis buffer (0.01 mol/L NaH_2PO_4, 10 g/L Nonident P-40, 10 g/L Na-deoxycholate, 1 g/L Na-dodecyl sulfate (SDS), 0.15 mol/L NaCl, 2 mmol/L EDTA), containing 100 kU/L protease inhibitor mix (Sigma-Aldrich, Budapest, Hungary). Cell lysis was accelerated by continuous shaking. Five microliters of this mixture were added to 45 μL RIPA buffer for protein measurement. All protein solutions were frozen and kept at -80 ℃ until use.

The protein concentration of the lysates was determined by the BCA method using BSA as standard in an ELISA reader at 540 nm. Data were analyzed by GraphPad Prism software using linear regression to determine the protein concentration (g/L). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the conditions of Laemmli (1970) on 100 g/L acrylamide gels in a Bio-Rad MiniProtean apparatus[3]. Samples for immunoblot analysis were diluted in the RIPA buffer, dissolved in an equal volume of 2 ×SDS sample buffer (100 mmol/L Tris pH 6.8, 2 g/L bromophenol blue, 200 mL/L glycerol, 200 mL/L β-mercaptoethanol) and boiled for 5 min. From each solution, aliquots (30 μg protein) were applied to SDS-PAGE and electrophoresed at 120 V for 1 h. Separated proteins were blotted onto a nitrocellulose membrane (Pharmacia, Vienna, Austria) at 100 V for 90 min during the continuous mixing and cooling of the transfer buffer (192 mmol/L glycine, 25 mmol/L Tris-base, 200 mL/L methanol). Membranes were rinsed twice in 0.1 mol/L PBS (pH 7.6) containing 1 g/L Tween 20 (PBS-Tween), then blocked for 1 h with 50 mL/L non-fat dried milk in PBS-Tween. After 3 × 5 min of washing with PBS-Tween, the membranes were incubated overnight at 4 ℃ with the anti-eNOS (diluted into 1 : 5000, Cat. No.: 482726, Merck-Calbiochem, Darmstadt, Germany) or anti-iNOS (1 : 2000, Cat. No.: AB5384, Chemicon, Temecula, CA) polyclonal antibodies raised in rabbits. After washing with PBS-Tween for 3 × 5 min, membranes were incubated with horseradish peroxidase conjugate of anti-rabbit IgG (diluted into 1 : 5000 in PBS-Tween, Vector, Bulingame, CA) for 30 min. After washing the membranes subsequently with PBS-Tween for 2 ×5 min, and once with PBS for 5 min. The blots were developed with the enhanced chemiluminescent (ECL) reagent kit (Pierce, Rockford, IL) and the immunoreactions were visualized on X-ray films.

Densitometry of the blots was performed and the scans were analyzed by the Volume Analyse feature of the Molecular Analyst Software (Bio-Rad, Hercules, CA). The densities for the NOS bands were normalized in each blot to the NOS-IR band obtained from the sample of 2 h treatment with normal serum and expressed as percentage of this control. Means of the percentages obtained from three independent experiments were presented in figures as mean ± SEM. The significance of the data between groups (normal, UC, CD) was tested by analysis of variance (ANOVA). Differences were considered significant for values of $P < 0.01$.

Immunofluorescence

Cells were cultured on coverglasses coated with sterile filtered 100 g/L gelatine in PBS. Cultures showing 20%-30% confluence were used for detection of proliferation, cell distribution and apoptosis/necrosis characteristics, whereas those with 70%-80% confluency were used for visualization of NOS isoforms. After treatments, cells were fixed in an ascendant ethanol gradient (500 ml/L, 700 mL/L, 960 mL/L), then rehydrated with PBS. For specific detection of eNOS and iNOS proteins, non-specific binding sites of primary antibodies were blocked by incubation of the fixed cells for 1 h with 50 mL/L normal goat serum in PBS also containing 1 g/L bovine serum albumin (BSA), 1 g/L Triton-X 100 and 0.1 g/L Na-azide (referred to as solution-A below). NOS primary antibodies used for immunocytochemistry were the same as those used for immunoblotting. Both eNOS and iNOS antibodies were applied at 1 : 200 dilution in solution-A overnight at 4 ℃. After extensive washing with PBS (3×5 min), cells were incubated with FITC conjugated anti-rabbit IgG diluted to 1 : 40 in solution-A for 30 min at room temperature. Cells were washed for 3 ×5 min with PBS and cover glasses were mounted on microscope slides (with one drop of PBS-glycerol (1 : 1) solution on them).

For evaluation of the proliferation activity, HUVECs were incubated in a medium containing 500 mL/L normal, 500 mL/L UC or 500 mL/L CD sera, or in a medium containing 500 mL/L normal sera plus 10^{-4} mol/L nitro-L-arginine methyl-ester (L-NAME, Sigma-Aldrich), an NOS inhibitor. Cells were exposed to a monoclonal antibody (at 1 : 100 dilution) specific for the proliferation marker Ki-67 antigen (clone No. MIB-1, Dako, Denmark) for 2 h at room temperature. In this case, FITC-conjugated anti-mouse IgG (1 : 40 in solution-A, Dako) was used as a secondary antibody. For testing of the effect of normal and IBD sera on the viability of HUVEC, apoptotic/necrotic cells were assessed by the annexin-V-biotin kit (Cat. No. 1828690, Boehringer-Mannheim, Germany). Briefly, after removal of the incubation media and washing with PBS, cells were labeled with the annexin-V-biotin/propidium iodide solution (1 : 50 annexin-V-biotin stock solution diluted in a 0.01 mol/L HEPES buffer, pH 7.4, containing 0.14 mol/L NaCl, 0.005 mol/L $CaCl_2$, and 1 mg/L propidium iodide) for 15 min. After subsequent washing with HEPES, cells were fixed with methanol-ethanol (1 : 1) solution for 1 min, and labeled with 5 mg/L avidin-fluorescein for 15 min. After consecutive washing with

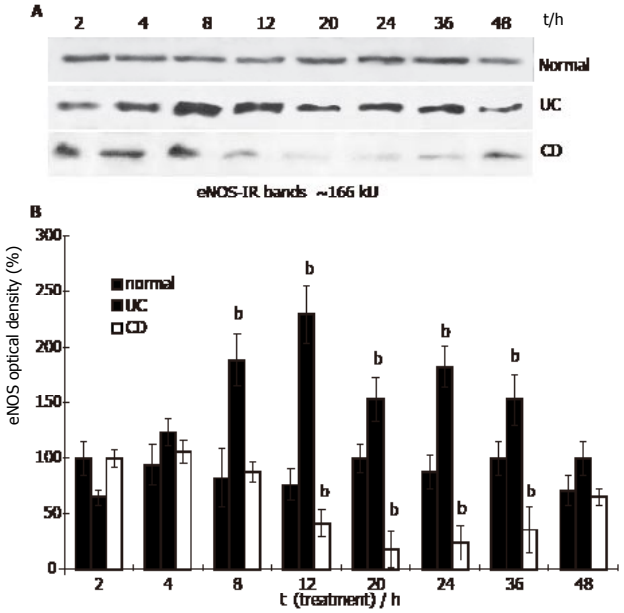

Figure 1 eNOS content in HUVEC treated with IBD sera (Western blot. Mean ± SE, $n = 3$; $^bP<0.01$ *vs* normal).

HEPES and PBS, cells were put on microscope slides as described above. Preparations used for the assay of proliferation activity and assessment of apoptosis/necrosis were stained prior to covering with 10 g/L toluidin blue in PBS. The latter labeled the cell nuclei and helped to determine total cell numbers for calculation of apoptotic/necrotic indices. The integrity of the cytoskeleton was examined by visualization of actin filaments by incubation with 0.2 mg/L phalloidin-FITC (Sigma-Aldrich) for 1 h.

Examination was carried out and digital images were captured with an Olympus AX-70 fluorescence microscope. In each case, the brightness-contrast of the images was set in the same manner by the Jasc software Paintshop Pro 7.0. Images were taken from Ki-67 immunoreactive (IR), annexin-labeled, and propidium iodide reactive cell nuclei, respectively. The same sites of the preparations were also captured in bright visual illumination to count the toluidin blue stained nuclei (total cell number). The images were quantitatively analyzed by the computer image analysis software Olympus analysis 2.11 (SiSoft), developed and validated by Olympus (Tokyo, Japan). At least 10^3 cells were counted for each data point and the ratios of proliferative, apoptotic and necrotic cells were given as the percentage of total cell numbers.

Statistical analyses

Statistical analyses were performed to compare the data obtained from the different treatments groups of IBD and normal sera using the ANOVA test. $P<0.05$ was considered as significant.

RESULTS

eNOS

Western blots from HUVEC lysates detected eNOS immunoreactive bands at approximately 160 kU, corresponding to the molecular mass of human eNOS (Figure 1A).

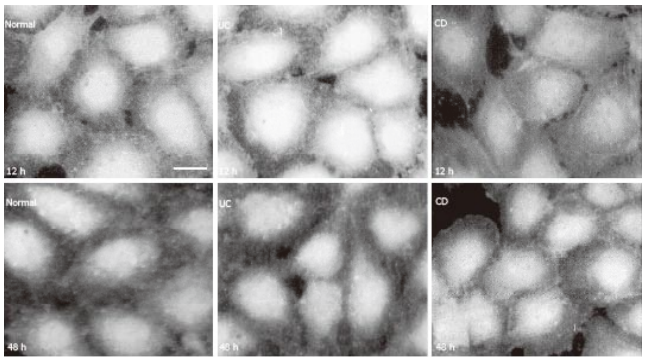

Figure 2 eNOS in HUVEC (immunoreaction was visualized by FITC. bar: 10 μm).

HUVEC was cultured in a medium containing 500 mL/L normal human serum. The amount of eNOS did not change significantly during the experimental period (0-48 h). However, in the presence of 500 mL/L UC serum, eNOS level increased after 8 h, and peaked at 12 h (relative optical density value was 188% ± 23% and 229 %± 26%, $P < 0.01$, respectively). At 12 h, eNOS level appeared to have doubled compared to the normal level at 2 h (100% ± 15%). Over the 12 h period in the presence of 500 mL/L UC serum, eNOS level began to decline (36 h, 153% ± 23%, $P < 0.01$), and reached control level at 48 h (100%± 8%). In the presence of 500 ml/L CD serum, the level of eNOS was close to normal until 8 h, and decreased after 12 h (41% ± 12%, $P < 0.01$), reaching the lowest level at 20-24 h (18% ± 16% and 23% ± 15%, $P < 0.01$, respectively), and then slowly increased rising close to normal at 48 h (65% ± 8%, Figure 1A, B).

Localization of eNOS in HUVEC by immunofluorescence revealed that the enzyme mostly accumulated in the nuclear area of the cytoplasm and was also detected at the periphery of the cells (Figure 2). Normal human serum had no effect on the distribution and intensity of eNOS immunoreactivity in HUVEC. Prolonged treatment (12 h) with UC serum extended eNOS immunoreactive area and simultaneously enhanced slightly the intensity of eNOS immunofluorescence in HUVEC. In contrast, in the presence of CD serum (after more than 4 h), eNOS immunoreactivity seemed to contract toward the centre of the cells and its immunofluorescence intensity decreased (Figure 2). At the end of the experimental period (48 h treatment), no visible difference could be detected in eNOS immunofluorescence of HUVEC in the presence of normal, UC or CD sera.

iNOS

Western blots in HUVEC lysates detected faint iNOS immunoreactive bands at around 130 kU, corresponding to the molecular mass of human iNOS (Figure 3A). HUVEC was cultured in the presence of 500 mL/L normal human serum, the relatively low level of iNOS expression did not change significantly during the 48 h incubation period. In the presence of 500 mL/L UC serum, iNOS level increased at 4 h (relative optical density value was 225% ± 20%, $P < 0.01$), peaked at 8 h (300% ± 21%, $P < 0.01$), and then declined to about normal level at 48 h (117% ± 15%). In the presence of 500 mL/L CD serum

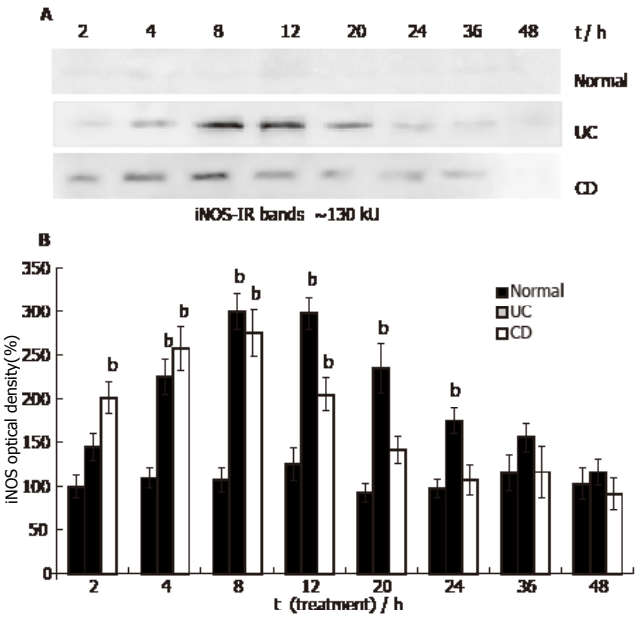

Figure 3 iNOS content in HUVEC (Western blot. Mean ± SE, n=3; ^bP<0.01 *vs* normal).

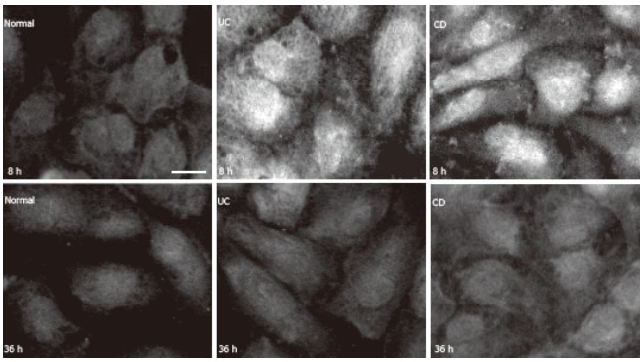

Figure 4 iNOS in HUVEC (immunoreaction was visualized by FITC. bar: 10 μm).

the iNOS level in HUVEC increased after 2 h (201% ± 19%, $P < 0.01$) and peaked at 8 h (275% ± 27%, $P < 0.01$). From 12 h iNOS level declined and was not distinguishable from the control during the 24-48 h period (at 48 h: 92% ± 18%, Figure 3A, B).

In the presence of 500 mL/L normal serum, HUVEC exhibited faint iNOS immunofluorescence (Figure 4). Expression of iNOS in HUVEC by immunofluorescence could obviously be detected in the presence of IBD sera during the 4-12 h period. Figure 4 shows that iNOS was dispersed in the cytoplasm, accumulated in certain areas showing a high intensity of fluorescent signal. Both UC and CD sera significantly increased iNOS immunofluorescence at the 4-12 h period. Before and after this time interval, iNOS could not be detected unequivocally in HUVEC by immunofluorescence (Figure 4).

HUVEC proliferation activity

Ten to fifteen percent of HUVEC was observed to be in the state of proliferation in the cultures with 20%-30% confluency at the beginning of the experiments as revealed by Ki-67 immunostaining (Figure 5). During the 2 to 48 h period no difference was detected in the ratio of Ki-67-IR/toluidin blue stained cell nuclei in cultures in

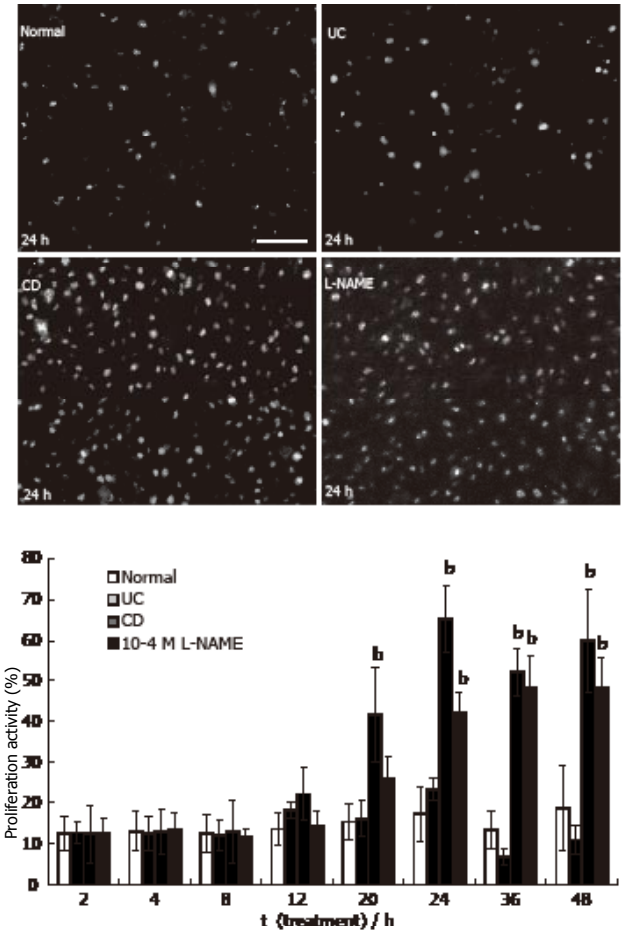

Figure 5 Proliferation activity of HUVEC (Ki-67 immunoreactivity was visualized by FITC. bar: 50 μm. mean ± SE, n = 3; ^bP<0.01 *vs* normal).

the presence of 500 mL/L normal (at 48 h: 19% ± 10%) or 500 mL/L UC sera (at 48 h: 10% ± 3%). In the presence of 500 mL/L CD serum a significant increase in the number of proliferating cells was apparent at 20 h (41% ± 12%, $P < 0.01$). About 50%-65% of HUVEC nuclei exhibited Ki-67 immunofluorescence in the presence of CD serum for 24-48 h (Figure 5). The presence of 10^{-4} mol/L L-NAME in the medium containing 500 ml/L normal serum, increased the proliferation activity significantly from 24 h (42% ± 5%, $P < 0.01$) to 48 h (48% ± 7%, $P < 0.01$).

HUVEC viability

By the annexin-V-biotin method, early apoptotic cells revealed fluorescent signals only on the cell membrane (Figure 6A), whereas necrotic cells showed propidium iodide binding to DNA (nuclear fluorescent signal, Figure 6B). Both signals could be detected in late apoptotic cells (Figure 6C). During the experimental period (2-48 h), the number of apoptotic cells was low (1%-3%) in the presence of 500 mL/L normal serum, but a continuous increase of apoptotic cell numbers was observed in the presence of 500 mL/L CD serum (2%-5%, with a maximum level at 36 h), which proved not to be significant (Figure 7A). In the presence of UC serum the apoptotic index did not differ from that of the control. Incubation of HUVEC with normal or CD sera slightly increased the number of necrotic cells during the examination period (2-48

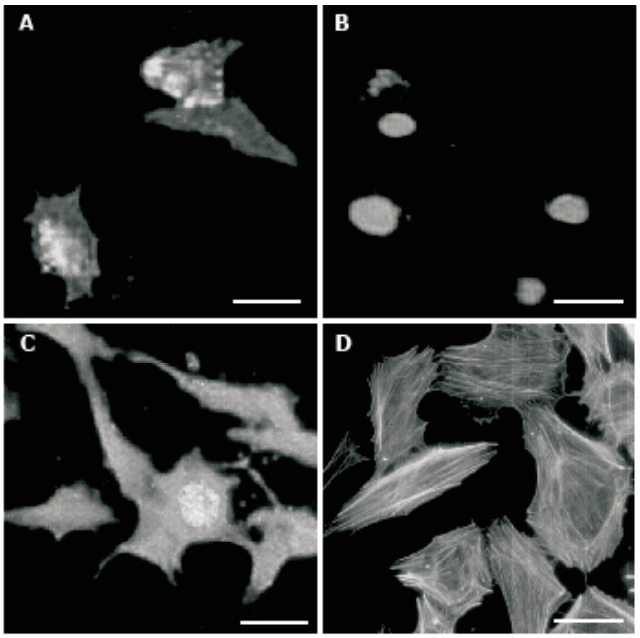

Figure 6 HUVEC viability. **A**: Early apoptotic cells; **B**: necrotic cells; **C**: Late apoptotic cell; **D**: Phalloidin conjugated with TRITC labeled actin filaments. bars: 10 μm.

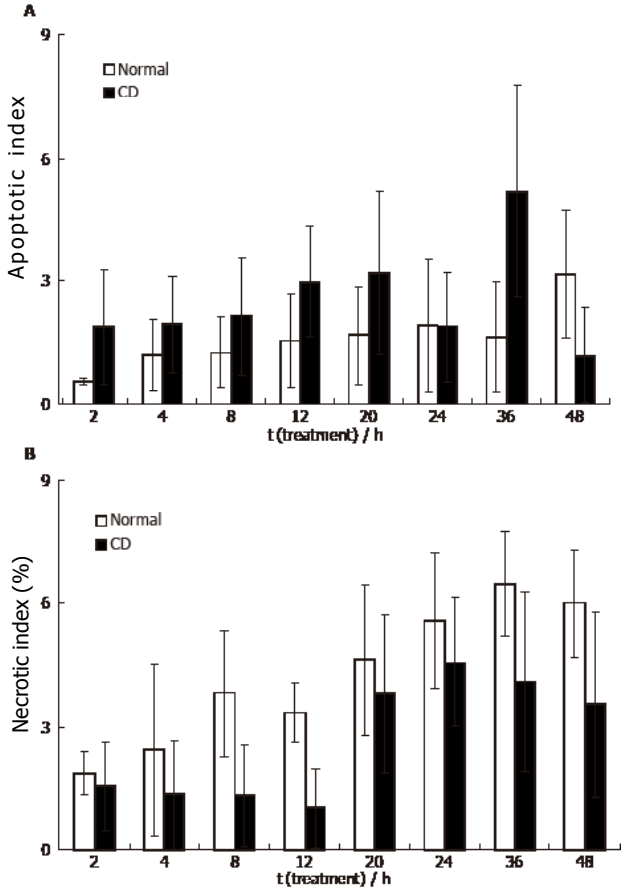

Figure 7 HUVEC 48 h incubation with CD sera (mean ± SE, *n* = 3).

h), but no significant changes were observed between the necrotic indices (Figure 7B). Actin filaments visualized by phalloidin-FITC seemed to be intact in cells independent of the media used and the time of incubation (Figure 6D).

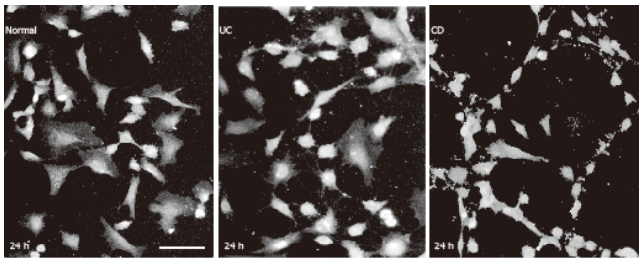

Figure 8 HUVEC visualized by eNOS immunofluorescence in culture. bar: 50 μm.

Shape and structure of HUVEC

HUVEC cultures of 20%-30% confluency were used also to investigate the effect of IBD sera on the distribution and shape of the cells. Cells visualized by eNOS immunofluorescence were partly found to be accumulated in islands exhibiting polygonal shape and attached to each other. In addition, single cells exhibiting long processes were also present. In the presence of 500 ml/L normal or 500 ml/L UC sera cells did not change in distribution and shape over the 48 h experimental period. However, in the presence of 500 mL/L CD serum, after 20 h cells were distributed in a two dimensional reticular network, which changed the shape of the culture reminiscent of the appearance of early vascular vessels in tissues (Figure 8).

DISCUSSION

In this study, we investigated the effect of sera from patients with active UC or CD on the expression and localization of eNOS and iNOS in HUVEC cultures. Induction of both iNOS and eNOS was observed in the presence of UC serum. In contrast, upon addition of CD serum to HUVEC media a transient increase in the expression of iNOS was observed in parallel with a marked decrease in the expression of eNOS. The number of apoptotic and necrotic cells was not altered significantly, and the structural integrity of the cytoskeleton (revealed by staining of the actin filaments) remained intact during the exposure of the cells to either CD or UC serum.

It has been shown previously that NO might enhance or reduce the cascade reaction of chronic inflammation in humans and animal models[12-14]. We found opposite changes in the expression of eNOS in HUVEC exposed to UC and to CD sera. The generation of eNOS is modified by elevated levels of cytokines (Th1 in CD) and the cytokine-induced expression and presentation of endothelial cell adhesion molecules[15]. UC and CD sera are known to contain a distinct cytokine and pro-inflammatory molecule profile. It was reported that TNF-α reduced eNOS protein expression in a time dependent manner in human endothelial cells[16, 17]. Moreover, TNF-α downregulated eNOS mRNA by decreasing its stability[18]. In our experiment, the low eNOS level in the presence of CD serum, could be due to TNF-α, which is a key cytokine in Th1 mediated inflammation in CD. Inflammation could result in functional and antigenic changes of the endothelium, including eNOS down-regulation and consequently increased expression of leukocyte adhesion molecules (ICAM, VCAM, E-selectin)[19-23]. At the same time, a lag in the fall of enzyme

activity was observed which may reflect a greater stability of eNOS protein compared to its mRNA, or may involve posttranslational modification(s) in the increase and stabilization of the enzyme activity[9]. Leukocyte-endothelial binding is exacerbated in an eNOS deficient state and injury during ischemia-reperfusion is more severe[24, 25].

A high amount of iNOS is generated during inflammation which can be harmful, worsening the course of disease in the animal colitis model[26]. In Crohn's colitis, human intestinal microvascular endothelial cells may lack the capacity to express iNOS, which makes them susceptible to leukocyte mediated injury[27, 28]. In UC, it has been shown that there is an elevation in iNOS level which is well correlated with disease severity[29]. Elevated iNOS activity was demonstrated in the mesenteric microvascular endothelial cells in active UC, indicating a close relationship between vascular activation and the pathogenesis of UC[30]. Stimulation of HUVEC with pro-inflammatory cytokines (Il-1β, TNF-α) or hormones (eg. relaxin, estrogen) induces the expression of iNOS and the NO produced increases the biosynthesis of prostaglandins and other mediators[31-33]. The maximal level of iNOS detected in primary cell cultures was not related to lipopolysaccharide contamination. It did not affect cell proliferation and was decreased when cells became confluent[34]. Dual modulation of NOS expression was detected upon estradiol treatment in a concentration dependent manner; estradiol in nanomolar range could induce eNOS, whereas in micromolar range it could evoke iNOS expression[35]. In previous studies, INF-γ in conjunction with TNF-α, or together with IL-1β, increased NOS activity, whereas these cytokines alone had different effects on NOS expression[9, 36, 37]. Up-regulation of iNOS by IBD sera may be an adaptive response of HUVEC to this culture environment.

The effect of UC and CD sera on eNOS expression could be of special interest and may be implicated in the distinct pathophysiological and morphological features of the two diseases. Inhibition of NO production *in vivo* results in reduced VEGF induced angiogenesis and vascular permeability[38]. Recent studies have provided evidence that basic fibroblast growth factor (bFGF), which is an important component of CD serum, induced angiogenesis by different mechanisms[39]. An impaired VEGF response was suggested in CD with down-regulation of the VEGF receptor (VEGF-Ets-1) and the angiogenic cascade supporting the vascular hypothesis of the disease[40-43]. The deficiency in eNOS expression and concomitant alteration of VEGF-induced angiogenesis and vascular permeability could be an important step in the pathomechanism of CD. However, bFGF, the most potent angiogenic molecule in CD has an NO independent activity[39, 44]. In addition, the pathomechanism of UC appears to be VEGF independent[39, 44]. The reciprocal reaction of cytokines and angiogenic molecules results in changes in the expression of eNOS and iNOS and causes an imbalance between the two isoforms which may play a role in the dysfunction of angiogenesis in IBD.

Angiogenesis is an important pathophysiological process in chronic inflammation and it includes proliferation, migration, and tube formation of endothelial cells. This process is tightly regulated by different angiogenic factors, and one of them is NO which has a pro-angiogenic effect[45, 46]. HUVEC, in the presence of CD serum, but not in the presence of UC serum, showed morphological changes reminiscent of vascular like structures, forming loop like rearrangement of the cells. It was shown recently that reactive oxygen species (ROS) have a putative pro-angiogenic activity *in vivo* possibly via iNOS up-regulation[47]. ROS also play a significant role in proliferation through regulation of eNOS activity, whereas antioxidants stimulate NO production and proliferation of HUVEC[48]. However, it was suggested also that antioxidants may enhance the activity of NOS[49, 50]. This dual effect could have a role in the pro-angiogenic and proliferation activity of HUVEC.

Recent data revealed that NO, by regulating mitochondrial respiration and cytochrome c release, triggers a defensive mechanism against cell death induced by pro-apoptotic stimuli[51-53]. In the present study, HUVEC incubated in the presence of CD serum exhibited a low expression level of eNOS, which could explain the slightly elevated apoptotic index. It also suggests a defective anti-apoptotic mechanism. The TNF-α level is known to be high also in CD serum and this could alter cell viability by inducing apoptosis in an NO independent pathway[54]. In contrast, in control and UC sera treated cells, the higher eNOS level may result in protection against apoptosis.

Our present results correlate well with our previous immunohistochemical findings through biopsy studies, and suggest the presence of a cytokine composition, which is able to evoke changes in the eNOS/iNOS ratio in endothelial cells in the blood of IBD patients. Simultaneous down-regulation of eNOS and up-regulation of iNOS, together with the increase of cell proliferation activity may reduce the anti-inflammatory capacity of endothelial cells. The latter may contribute to the initiation of pro-inflammatory cascade reactions leading to endothelial barrier dysfunction and causing structural changes in the microvessels in Crohn's mucosa.

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RAPID COMMUNICATION

Role of bile acids, prostaglandins and COX inhibitors in chronic esophagitis in a mouse model

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Abstract

AIM: To develop a new experimental model of esophagitis that serves a complementary tool to clinical investigation in an insight into the mechanism of the damage to the esophagus mucosa by aggressive factors, and role of COX inhibitors in this process.

METHODS: The study was conducted in 56 male mice. Animals were divided into seven groups: (1) perfused with HCl, (2) perfused with HCl and physiologic concentration of pepsin (HCl/P), (3) perfused with similar HCl/P solution enriched with conjugated bile acids (glycho- and tauro-sodium salts) designated esophageal infusion catheter under the general anesthesia, (4) perfused as in group 2 treated with indometacin, (5) perfused as in group 2 treated with NS-398, (6) perfused as in group 3 treated with indometacin, and (7) perfused as in group 3 treated with NS-398. The esophagus was divided into 3 parts: upper, middle and lower. The PGE2 concentration was measured in all parts of esophagus using RIA method. Esophagus of sacrificed animals was macroscopically evaluated using a low power dissecting microscope (20×). Specimens, representing the most frequently seen changes were fixed, stained with H&E and assessed microscopically using the damage score, and inflammatory score.

RESULTS: The macroscopic changes were significantly severer in HCl/P than those in HCl animals (77%) and in HCl/P/BA group (43%). In HCl/P NS-398 group we noticed significantly less changes than those in not treated group (42%) and in analogical group treated with indomethacine (45%). In HCl/P/BA INDO group we observed significantly severer changes than that in not treated group (52%). We noticed less changes in HCl/P NS-398 than that in group with indomethacine (46%). In HCl/P/BA NS-398 group we had less changes than that in indometacin group (34%). The microscopic changes observed in HCl/P/BA INDO group were severer than that in not treated group (48%). Esophagitis index in HCl group was significantly lower than in HCl/P and also HCl/P/BA group (32% and 33 %). In HCl/P/BA/INDO group the esophagitis surface was larger than that in not treated group (33%). In HCL/P group the surface of esophagus with ulceration was significantly larger (10-fold) than that in HCl/P/BA group. The PGE2 concentration was significantly higher in HCl/P group than in HCl/P/BA group. The PGE2 concentration in lower part of esophagus was also significantly higher in middle than those in HCl and HCl/P/BA groups. In upper part of esophagus the PGE2 concentration was significantly higher in HCl/P/BA group than that in group treated with indomethacine (46%). We also observed higher PGE2 concentration in middle part of esophagus in HCl/P/BA group than those in group treated with indomethacine and in group treated with indometacin and NS-398 (by 52% and 43% respectively).

CONCLUSION: Pepsin is the pivotal factor in the development of chronic esophageal injury. Bile acids diminish chronic esophageal injury induced by HCl/P, indicating its potential negative impact on pepsin proteolytic potential, pivotal for mucosal injury in low pH. The role of selective COX inhibitors is still unclear, and needs more investigations. This novel chronic experimental esophagitis is an excellent model for further study on the role of cytokines in genetically modified animals.

Key words: Bile acids; ProstaglIndins; Oesophagitis mouse model

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INTRODUCTION

Primary gastroesophageal reflux disease (GERD) is an ac-

id-related disease in majority of patients. However, there is evidence that in some patients with GERD reflux of duodenal contents into the stomach and esophagus may be involved in the disease[1, 2].

Chronic GERD may induced Barrett`s metaplasia[3]. This clinical situation has increased risk for the development of esophageal adenocarcinoma and is considerate to be a premalignant condition[4]. The complications in Barrett's esophagus was accompanied with presents of duodenal juice in gastroesophageal refluxate (GER)[3]. In the patients with esophagitis, Barrett`s esophagus strictures compared to patients with minimal injury the concentration of bile acids in refluxate was significantly higher [5]. The concentration of bile was significantly higher in patients with early adenocarcinoma arising in Barrett`s esophagus, compared to GERD patients, esophagitis group and asymptomatic volunteers[6]. Clinical trials have begun in order to assess the efficacy of selective COX-2 inhibitors to prevent the progression of Barrett's esophagus to adenocarcinoma. Bile salts and acid are likely to early induce COX-2 in this sequence, although other factors, such as proinflammatory cytokines, inducible nitric oxide synthase and growth factors such as TGF-beta, are potential COX-2 inducers in the esophagus[7]. In animal studies it has been shown that reflux of gastric contents with addition of duodenal juice into the esophagus may lead to esophageal adenocarcinoma[2]. The carcinogenetic effect of duodenal contents on gastric mucosa was clearly demonstrated[8].

Bile acid may induce mucosal injury by two mechanisms. The detergent properties of bile salts may destabilize membranes and increase permeability, disrupt cellular homeostasis and potentially result in cell death[9]. Bile acids may also create cytotoxic effect through cellular absorption of bile salts, witch is dependent upon the salt`s ionization[10].

COX-1 activity is constitutive in the rabbit esophageal mucosa, but both COX-2 and COX-1 activity are increased under the impact of acidified pepsin. Treatment with the COX-2 inhibitor DFU is associated with improvement of mucosal damage, which may have therapeutic implications[11].

PGE2 plays the important role in development of Barrett`s esophagus and adenocarcinoma of the esophagus. The concentration of PGE2 was significantlly higher in high grade displasia cells and also in adenocarcinoma cels of esophagus [12].

Our new experimental model of chronic esophagitis seems to be very useful tool to determinate the role of HCl/P/BA, major components of duodenogastroesophageal reflux, and the role of COX inhibitors on pathological changes in mucosa of the esophagus during refluxate episodes.

MATERIALS AND METHODS

The study was conducted in 56 male mice (CD1 strain from Charles River) according to study protocol approved by Animal Research Committee at KUMC. Animals were divided into seven groups: (1) Animal perfused with HCl (100 mmol/l, pH1.1), (2) Animals perfused with HCl (100 mmol/l, pH1.1) and physiologic concentration of pepsin (0.5 mg/l of HCl) (HCl/P), (3) Animals perfused with similar HCl/P solution enriched with conjugated bile acids (glycho- and tauro-sodium salts) designated esophageal infusion catheter under the general anesthesia, (4) Animals perfused as in group 2 treated with indometacin (5 mg/kg b.w. s.c.), (5) Animals perfused as in group 2 treated with NS-398 (10 mg/kg b.w. p.o), (6) Animals perfused as in group 3 treated with indometacin (5 mg/kg b.w. s.c.), and (7) Animals perfused as in group 3 treated with NS-398 (10 mg/kg b.w. p.o). The total perfusion time per day for each mouse was 1.5 h. At the end of experimental procedure the animals were sacrificed under prolonged metoxyflurane anesthesia, esophagus was removed, opened and evaluated microscopically after stained with Alcian blue (0.1%, pH 5.8), using a low power dissecting microscope (20×) with stage micrometer for measurement of the area of macroscopic damage. The macroscopic changes was evaluated based on macroscopic score: 0 - no changes; 1 - erosion - max 3, size - 3-6 mm; 2 - erosion - 6 and up, size - 6-9 mm; 3 - ulcer without perforation with small hemorrhagic areas; 4 - ulcer with perforation and large hemorrhagic areas.

Specimens, representing the most frequently seen changes were fixed, stained with hematoxylin and eosin, and assessed microscopically using the damage score [15]: 1-normal esophagus, 2-submucosal edema or separation of epithelial layer, 3-focal areas of intramural hemorrhage or partial epithelial loss, 4 - large areas of hemorrhage or complete epithelial desquamation; and inflammatory score[16]: 0 - no infiltration, 1-very mild infiltration, 2-mild infiltration, 3 - moderate infiltration, 4 - marked infiltration.

The concentration of PGE2 was measured in 1/3 upper, 1/3 middle and 1/3 lower parts of esophagus using RIA kit (*Amersham, Arlington Heights, Illinois*).

Statistical analysis was performed with S-Stat (Jandel Sci. Co).

RESULTS

The macroscopic score was significantly higher in animals perfused with HCl/P than those in groups with HCl/P/BA and with HCl (3.69 ± 0.23 *vs* 2.58 ± 0.25 and 3.69 ±0.23 *vs* 2.08 ± 0.11, $P < 0.05$). The macroscopic score was significantly lower in group HCl/P/NS-398 than that in not treated group (2.13 ± 0.21 *vs* 3.69 ± 0.23, $P < 0.05$) and also lower than that in analogical group treated with indometacin. In HCl groups, the microscopic changes were less evident in groups with HCl and HCl/P/BA than that in HCl/P (2.63 ± 0.38 *vs* 3.90 ± 0.10 and 2.64 ± 0.27 *vs* 3.90 ± 0.10, $P < 0.05$, respectively). The microscopic score was the same in HCl group and in HCl/P/BA group. The microscopic changes were significantly severer in group HCl/P/BA/INDO than that in not treated group (3.90 ± 0.10 *vs* 2.64 ± 0.27, $P < 0.05$) and also than that in analogical group treated with NS-398 (3.90 ± 0.10 *vs* 2.58 ± 0.14, $P < 0.05$). We noticed significantly higher score of microscopic changes in group HCl/P/INDO than that in group treated with NS-398 (3.92 ± 0.07 *vs* 2.12 ± 0.13, $P < 0.05$). Inflammation of esophagus in HCl group was significantly lower than that in HCL/P group (2.63 ± 0.24 *vs* 3.90 ± 0.10, $P < 0.05$). The inflammation score in HCl/P/BA group was also lower than that in HCl/P (2.23 ± 0.26 *vs* 3.90 ± 0.10, $P < 0.05$). Inflammation was less evident in HCl/P/NS-398 than those in not treated group and in group treated with indometacin (2.21 ± 0.11 *vs* 3.90 ± 0.10 and

Table 1 Macroscopic and microscopic changes in mice esophageal mucosa (mean ± SE)

Model	Grades of acroscopic changes	Grades of microscopic changes	Grades of inflammation	Surface of esophagitis (% of all esophagus)	Ulcers of esophagus (mm^2)
HCl	2.08 ± 0.11	2.63 ± 0.38	2.63 ± 0.24	15.50 ± 2.02	0 ± 0.0
HCl/P	3.69 ± 0.23[a]	3.90 ± 0.10[a]	3.90 ± 0.10[a]	23.00 ± 2.31[a]	7.09 ± 2.17[a]
HCl/P/BA	2.58 ± 0.25[c]	2.64 ± 0.27[c]	2.23 ± 0.26[c]	23.46 ± 3.85[a]	0.71 ± 0.49[ac]
HCl/P/INDO	3.90 ± 0.08	3.92 ± 0.07	3.86 ± 0.11	25.12 ± 2.14	8.11 ± 2.31
HCl/P/NS-398	2.13 ± 0.21[cg]	2.12 ± 0.13[g]	2.21 ± 0.11[cg]	19.12 ± 1.34	5.12 ± 2.13
HCl/P/BA/INDO	3.92 ± 0.06[e]	3.90 ± 0.10[e]	3.93 ± 0.03[e]	31.17 ± 2.45[e]	1.35 ± 0.11[e]
HCl/P/BA/NS-398	2.32 ± 0.11[g]	2.58 ± 0.14[g]	2.42 ± 0.13[g]	21.23 ± 1.21[g]	0.98 ± 0.27

[a]P<0.05 *vs* HCl; [e]P<0.05 *vs* HCl/P/BA; [g]P<0.05 INDO; [c]P<0.05 *vs* HCl/P.

2.21 ± 0.11 *vs* 3.86 ± 0.11 respectively, $P < 0.05$). Inflammation score was higher in HCl/P/BA/INDO than those in not treated group and in group treated with NS-398 (3.93 ± 0.03 *vs* 2.23 ± 0.26 and 3.93 ± 0.03 *vs* 2.42 ± 0.13 respectively, $P < 0.05$). Esophagitis index in HCl group was significantly lower than those in HCl/P and HCl/P/BA groups (15.50 ± 2.02 *vs* 23.00 ± 2.31 and 15.50 ± 2.02 *vs* 23.46 ± 3.85 % of all esophagus surface, $P < 0.05$). Surface area of esophagitis was significantly larger in HCl/P/BA/INDO group than that in not treated one (31.17 ± 2.45 *vs* 23.46 ± 3.85, $P < 0.05$) and also larger than that in group treated with NS-398 (31.17 ± 2.45 *vs* 21.23 ± 1.21, $P < 0.05$). In the HCl group of animals we did not observed any ulceration of the esophagus. In HCL/P group the surface of esophagus with ulceration was significantly larger than that in HCl/P/BA group (7.09 ± 2.17 *vs* 0.71 ± 0.49 mm^2, $P < 0.05$). The surface of ulceration in esophagus was significantly larger in HCl/P/BA/INDO group than that in not treated group (1.35 ± 0.11 *vs* 0.71 ± 0.49, $P < 0.05$). All data are showed in Table 1.

In the HCl group the concentration of PGE2 in middle part of esophagus was significantly higher than in lower part (1 027 ± 166 pg/mg of protein *vs* 378 ±69 pg/mg of protein, $P < 0.05$). We also observed the higher concentration of PGE2 in the middle part of esophagus than that in lower one in animals from HCL/P/BA groups (1 264 ± 134 pg/mg of protein *vs* 332 ± 59 pg/mg of protein, $P < 0.05$). In the HCL/P/BA group the concentration of PGE2 was significantly higher in the middle part of esophagus than that observed in the HCl/P (1 264 ± 134 pg/mg of protein *vs* 766 ± 95 pg/mg of protein, $P < 0.05$). The concentration of PGE2 in upper part of esophagus in HCl/P/BA/INDO group was significantly lower than that in not treated group (553 ± 50 *vs* 807 ± 111 pg/mg of protein, $P < 0.05$). We noticed lower PGE2 concentration in middle part of esophagus in HCl/P/BA treated with indometacin and NS-398 than that in not treated analogical group (614 ± 64 *vs* 1264 ± 134 and 733 ± 67 *vs* 1 264 ± 134 pg/mg of protein respectively, $P < 0.05$). All data are shown in Table 2.

Table 2 Concentration of pge2 in mouse esophagus

Model	1/3 upper part	1/3 middle part	1/3 lower part
HCl	801 ± 103	1027 ± 166[a]	378 ± 69[a]
HCl/P	674 ± 107	766 ± 95[c]	405 ± 39
HCl/P/BA	807 ± 111[e]	1264 ± 134[aceg]	332 ± 59[a]
HCl/P/INDO	500 ± 59	569 ± 60	388 ± 35
HCl/P/NS-398	576 ± 34	663 ± 59	324 ± 32
HCl/P/BA/INDO	553 ± 50	614 ± 64	324 ± 26
HCl/P/BA/NS-398	607± 50	733 ± 67	368 ± 43

Mean ± SE pg/mg of protein; [a]P<0.05 middle *vs* lower; [e] P<0.05 *vs* INDO; [g]P<0.05 *vs* NS-398; [c]P<0.05 HCl/P *vs* HCl/P/BA.

DISCUSSION

In our current study we demonstrated the significantly increase of macroscopic damage score in esophageal mucosa in animals perfused with HCl/P when compared with HCl and HCl/P/BA group. In addition in group perfused with HCl/P the microscopic changes were significantly remarkable compared to that in HCl and HCl/P/BA perfused animals. Inflammation of esophagus in HCl/P group was evidently severer than that in HCl only perfused animals. Inflammation of the esophageal mucosa in group with perfusion mimicking duodenogastroesophageal reflux was significantly higher than in HCl and HCl/P/BA perfused groups. The total surface of esophagitis in HCl/P perfused animals was significantly larger than that in HCl perfused group of animals. We observed wider surface of esophagitis in group perfused with HCl/P/BA – mimicking duodenogastroesophageal reflux, than in HCl/P perfused animals but the differences was not significant. However, the surface of esophagus with esophagitis in group mimicking the duodenogastroesophageal reflux was significantly larger than that observed in HCl perfused group. In group of mice with perfusion of esophagus with HCl we did not find any ulceration. We demonstrated in group with HCl/P significantly larger surface of esophagus with ulcer (10-fold) than in HCl/P/BA group. In our study we also found the significant decrease of macroscopic damage in esophageal mucosa in group of animals perfused with HCl/P and treated with NS-398. Macroscopic score in this group was lower than that in analogical group treated

with indomethacine. We also observed this phenomenon in microscopic and inflammatory scores. We noticed that animals perfused with HCl/P/BA and treated indomethacine had higher values of all macro- and microscopical scores than in not treated group. In addition, HCl/P/BA group of mice treated with NS-398 showed lower values of all macro-and microscopical scores than that observed in animals treated with indomethacine.

It was recently documented that in patients with reflux esophagitis the concentration of bile acids in refluxate is significantly higher than that in asymptomatic volunteers[15,16].

Chronic GERD may induced Barrett`s metaplasia[3]. This clinical situation has increased risk for the development of esophageal adenocarcinoma and is considered to be a premalignant condition[4]. The concentration of bile was significantly higher in patients with early adenocarcinoma arising in Barrett`s esophagus, compared to GERD patients, esophagitis group and asymptomatic volunteers[6].

In our previous clinical study we demonstrated that perfusion with acid, pepsin and bile acids, mimicking the duodenogastroesophageal reflux episodes increased the esophageal protective components secretion in asymptomatic volunteers, and less evidently in GERD patients.

There are some surgical experimental model of esophagitis, Barrett`s esophagus and also adenocarcinoma of esophagus[17,18]. In animal studies it has been shown that reflux of gastric contents with addition of duodenal juice into the esophagus may lead to esophageal adenocarcinoma[19]. The carcinogenetic effect of duodenal contents on gastric mucosa was clearly demonstrated [8]. The higher concentration of PGE2 in esophagus may be connected with deeper impact of bile acids on the esophagus wall, and induction of COX-2 in the esophagus muscle cells [5]. The role of COX-2 inhibitors in that phenomenon is still unclear and need more experiments.

Our new experimental model of esophagitis in mice mimicking the clinical scenario of gastroesophageal or duodenogastroesophageal reflux seems to be a useful tool to investigate some pathological problems in esophageal pathophysiology.

In conclusion, pepsin is the pivotal factor in the development of chronic esophageal injury. Bile acids diminish chronic esophageal injury induced by HCl/P, indicating its potential negative impact on pepsin proteolytic potential, pivotal for mucosal injury in low pH. The COX-2 inhibitors are much more active than not selective inhibitors in patients with esophageal mucosa injury, especially during duodenogastroesophageal reflux scenario. This novel chronic experimental esophagitis is an excellent model for further study on the role of cytokines in health and disease of the esophageal mucosa in genetically modified animals.

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RAPID COMMUNICATION

Expression of Ets-1 proto-oncoprotein in gastrointestinal stromal tumors, leiomyomas and schwannomas

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Abstract

AIM: Gastrointestinal stromal tumors (GISTs) are rare. GISTs differ from other mesenchymal tumors of the gastrointestinal tract (e.g. leiomyomas and schwannomas). The purpose of this study was to investigate the role of Ets-1 in the growth and differentiation of GISTs.

METHODS: Twenty-eight GISTs, nine leiomyomas and six schwannomas were examined by immunohistochemical staining method for Ets-1 in this study. Specimens were selected from surgical pathology archival tissues at Nagasaki University Hospital.

RESULTS: Ets-1 protein was expressed in the cytoplasm of cells in all of these tumors. Immunohistochemical staining revealed that 27 GISTs (96.4%), six leiomyomas (66.7%), and five schwannomas (83.3%) were positive for Ets-1. Ets-1 expression was statistically different between GISTs and leiomyomas ($P < 0.005$). However, there was no correlation between Ets-1 expression and clinical risk categories.

CONCLUSION: Ets-1 plays an important role in the growth and differentiation of GISTs, leiomyomas and schwannomas.

Key words: Ets-1; GISTs; Leiomyoma; Schwannoma; Immunohistochemistry; Risk category

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that may occur from the oesophagus to the anus, including the omentum. These tumors have a wide clinical spectrum from benign, incidentally detected nodules to frankly malignant tumors[1]. Small GISTs are often detected incidentally during surgery for other conditions, during gastroscopy, or on routine X-ray[1,2]. GISTs may present with bleeding, perforation, pain, obstruction or a combination of these symptoms[3-6]. The mechanisms of tumorigenesis, progression and differentiation of GISTs are unknown. Traditionally, all primary mesnchymal spindle cell tumors of the gastrointestinal (GI) tract were uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas or leiomyosarcomas). Tumors with epithelioid cytologic features were designated leiomyoblastomas or epithelioid leiomyosarcomas[7]. Recently, Sircar *et al* postulated that GISTs origin-ate from Cajal cells in the gastrointestinal tract and differ from leiomyomas and schwannomas, which are of mesenchymal cell origin[8]. Cajal cells are thought to be gastrointestinal pacemaker cells that regulate intestinal motility[9]. GISTs are characterized by frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[10] and the c-kit proto-oncogene[8,11,12]. Ets-1 was characterized originally as the v-ets retroviral gene, one of two oncogenes (v-myb and v-ets) of the avian leukemia retrovirus, E26[13]. The ets family of genes encodes transcription factors for mesodermal cell development during embryogenesis[14, 15].

Ets-1 plays a role in the regulation of physiological processes such as cell proliferation and differentiation[16]. Ets-1 also is expressed in astrocytes and vascular smooth muscle cells[17,18] but its expression has not been reported in Cajal cells. Increased Ets-1 expression was observed in several tumors in our previous studies[19-22]. We reported

Table 1 Ets-1 immunohistochemistry in intestinal stromal tumors. *n* (%)

	n	-	+	++
GISTs	28	1 (3.6)	4 (14.3)	23 (82.1)[a]
Leiomyomas	9	3 (33.3)	3 (33.3)	3 (33.3)
Schwannomas	6	1 (16.7)	1 (16.7)	4 (66.7)

[a]$P<0.005$ *vs* leiomyomas.

that Ets-1 is correlated with the progression of carcinoma cells of the stomach, pancreas, and thyroid and cells of astrocytic tumors[19-22]. These studies suggest that Ets-1 is involved in tumor growth and differentiation. However, there are no data concerning Ets-1 expression in GISTs, leiomyomas or schwannomas or the role of Ets-1 in the etiology of these tumors. The purpose of this study was to investigate the expression of Ets-1 in GISTs.

MATERIALS AND METHODS

Samples

A total of twenty-eight GISTs included 24 cases from the stomach and four from the small intestine. Nine leiomyomas included four from the oesophagus, two from the stomach and three from the large intestine, and five schwannomas included four from the stomach and one from the large intestine. Specimens were selected from surgical pathology archival tissues at Nagasaki University Hospital between 1999 and 2004. The GISTs were 0.8 - 12.0 cm in diameter, the leiomyomas were 0.1 - 4.5 cm, and the schwannomas were 0.6 - 5.0 cm. In this study, GISTs were defined as expressing both c-kit and CD34 surface antigens. GISTs were classified by risk categories, mitosis counts and tumor size[23]. The number of mitoses was determined by counting 50 high-power fields (HPF, x400) in Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined as expressing α-smooth muscle actin (SMA) but not c-kit, CD34 and S100-protein. Schwannomas were defined as expressing S100-protein but not c-kit, CD34 and SMA. Tumor identification/classification was determined by two independent pathologists (T. Nakayama and I. Sekine).

Immunohistochemical staining

The subcellular localization of Ets-1 was determined in GISTs using a monoclonal antibody directed against the unique middle sequence of Ets-1 and this antibody was devoid of any cross-reaction with other proteins in the Ets family. Formalin-fixed and paraffin-embedded specimens were cut into 4 μm thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4°C with the primary monoclonal antibody to human Ets-1 (1 g/L; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then with a horseraddish peroxidase conjugated goat anti-mouse IgG antibody (0.4 g/L; Santa Cruz Biotechnology, Santa Cruz, CA). The reaction products were resolved using diaminobenzidine (DAB; DAKO Ltd., Glostrap, Denmark). Negative controls involved replacing the primary antibody with non-immunized mouse serum and human gastric cancer tissue served as the positive control[19]. Ets-1 expression was classified into three categories depending upon the percentage of cells stained and/or the intensity of staining: -, 0% to 10% tumor cells positive; +, 10% to 50% tumor cells positive; and ++, >50% tumor cells positive.

Table 2 Ets-1 immunohistochemistry and risk categories in GIST. *n* (%)

	n	-	+	++
Total	28	1 (3.6)	4 (14.3)	23 (82.1)
Risk categories				
High	4	0 (0.0)	0 (0.0)	4 (100.0)
Intermediate	5	0 (0.0)	0 (0.0)	5 (100.0)
Low	14	1 (7.1)	3 (21.4)	10 (71.4)
Very low	5	0 (0.0)	1 (20.0)	4 (80.0)
Mitosis counts (per 50 HPF)				
<2	14	1 (7.1)	3 (21.4)	10 (71.4)
2-5	6	0 (0.0)	1 (1.7)	5 (83.3)
6-10	3	0 (0.0)	0 (0.0)	3 (100.0)
10<	5	0 (0.0)	0 (0.0)	5 (100.0)
Tumour size (cm)				
<2	5	0 (0.0)	1 (20.0)	4 (80.0)
2-<5	18	1 (5.6)	3 (16.7)	14 (77.8)
5-<10	4	0 (0.0)	0 (0.0)	4 (100.0)
10<	1	0 (0.0)	0 (0.0)	1 (100.0)

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyses. Analyses comparing the degree of Ets-1 expression in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

RESULTS

The results of immunohistochemical staining for Ets-1 are summarized in the Table 1. Ets-1 expression was heterogeneous in GISTs and localized to the cytoplasm (Figure 1). Twenty-three of the GISTs (82.1%) were strongly positive, four (14.3%) were positive and one (3.6%) was negative for Ets-1. Similarly, four of the schwannomas (66.7%) were strongly positive, one (16.7%) was positive and one (16.7%) was negative. However, only three of the leiomyomas (33.3%) were strongly positive, three (33.3%) were positive and three (33.3%) were negative. There is a statistical difference in Ets-1 expression between the GISTs and the leiomyomas ($P<0.005$). Positively stained cells, i.e. those classified as ++ or +, were found in 96.4% of the GISTs, 66.7% of the leiomyomas and 83.3% of the schwannomas. Ets-1 was expressed in the cytoplasm of cells in all three tumors. However, normal stromal cells and smooth muscle cells showed faint or focal positivity of expression. GISTs were classified by risk categories, mitosis counts and tumor size in Table 2. In risk categories, all nine cases of high and intermediate groups were strongly expressed Ets-1 protein. In mitosis counts, all eight cases with over 6 mitoses per 50 HPF strongly expressed Ets-1. In tumor size, all five cases with over 5 cm strongly expressed Ets-1. However, there was no correlation between

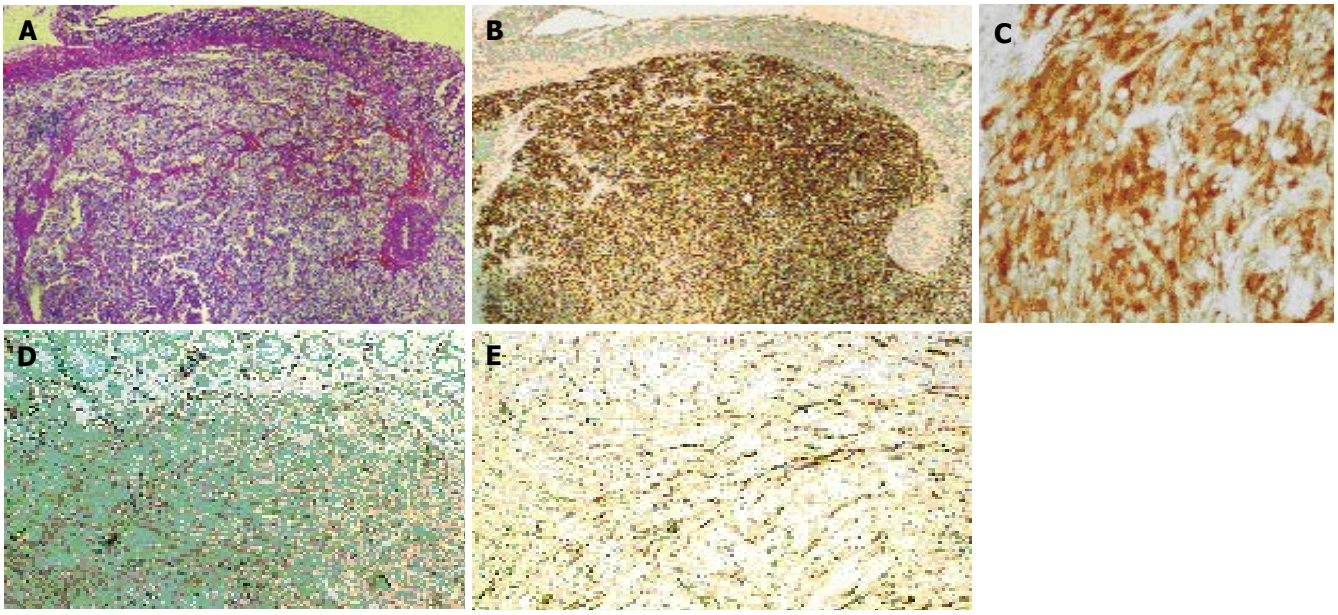

Figure 1 Ets-1 expression in GISTs (**A**-**C**), Leiomyomas (**D**) and Schwannomas (**E**). (magnification; **A**, **B**:x20, **C**: x 200, **D**, **E**: x100).

Ets-1 expression and each classification.

DISCUSSION

GISTs are known to originate from the Cajal cells of the neural crest[8] and schwannomas are thought to originate from the peripheral nerve sheath cell[24]. In this study, Ets-1 expression was higher in GISTs and schwannomas than in leiomyomas. Ets-1 expression has been reported in neural cells and astrocytes[22], but not yet in Cajal cells, cells that are all of neurons origin. Vascular smooth muscle cells also express Ets-1[18]. These findings suggest that Ets-1 may play a role in neural differentiation of intestinal stromal tumors. Previous studies have demonstrated Ets-1 expression in several tumors and normal stromal cells[19-22,25]. Furthermore, Ets-1 has been shown to play a role in the proliferation and/or differentiation of stromal cells[25]. We have shown already that Ets-1 may function as a growth factor in several tumors[19-22]. However, there have been no studies of Ets-1 expression in GISTs, leiomyomas and schwannomas, or of the potential role of Ets-1 in the growth of these tumors. Our results demonstrate substantial levels of Ets-1 expression in the cytoplasm of GIST, leiomyoma and schwannoma cells. These results suggest that Ets-1 may play a role in the growth and/or differentiation of intestinal tumors.

Ets-1 regulates the expression of many proteins, such as matrix metalloproteinases, urokinase type-plasminogen activator and parathyroid hormone-related peptide (PTHrP), which promote tumor growth and/or progression[26,27]. In our previous study, PTHrP and its receptor were found to be highly expressed in GISTs, leiomyomas and schwannomas[28]. Ets-1 may promote tumor growth and/or progression through regulating the expression of these proteins.

In recent studies, mutations affecting c-kit that cause constitutive tyrosine kinase activation have been shown to be important for the pathogenesis of GIST[29,30]. Joensuu *et al* reported a patient in whom STI-571 (imatinib, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST[31]. And STI-571 has proven to be remarkably efficacious in heavily pretreated GISTs patients with advanced disease in phase II clinical trials[32]. The expression of the Ets family protein is upregulated by the activation of tyrosine kinase through the mitogen-activated protein kinase pathway[33]. Ets-1 expression may be upregulated by the c-kit/tyrosine kinase pathway.

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RAPID COMMUNICATION

Roles of adipocyte derived hormone adiponectin and resistin in insulin resistance of type 2 diabetes

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Abstract

AIM: To detect plasma levels of new adipocyte derived hormone adiponectin and resistin in type 2 diabetes patients and to explore their potential roles in insulin resistance in type 2 diabetes.

METHODS: According to the body mass index (BMI), 60 type 2 diabetes patients were divided into two groups, one group was non-obese diabetes patients with BMI < 25Kg/M^2 (30 cases) and the other group was obese diabetes patients with BMI > 25Kg/M^2 (30 cases). There were 28 healthy persons in the control group. ELISA technique was employed to determine the plasma adiponectin and resistin concentrations. The fasting blood glucose, insulin and blood lipid were detected respectively by electrocheminescence immunoassay and immunoturbidimetric assay. Insulin resistance index and insulin sensitive index were calculated by the homeostasis model assessment (HOMO).

RESULTS: The levels of plasma adiponectin were decreased significantly in diabetes group compared to that in control group (non-obese: 8.58 ± 0.86, obese: 6.22 ± 1.34 *vs* 10.53 ± 1.47 $P < 0.05$); moreover, adiponectin concentration in obese diabetes group was significantly decreased compared to that in non-obese diabetes group (6.22 ± 1.34 *vs* 8.58 ± 0.86, $P < 0.05$). The levels of plasma resistin were increased significantly in diabetes group compared to that in control group (obese: 18.64 ± 4.65, non-obese: 24.05 ± 9.07 *vs* 14.16 ± 5.25, $P < 0.05$, $P < 0.05$); furthermore, the levels of resistin in obese diabetes group were increased significantly compared to that in non-obese diabetes group ($P < 0.05$). Plasma adiponectin was correlated negatively with BMI, blood glucose, insulin resistance index and triglyceride (respectively, $r = -0.55$, $P < 0.01$; $r = -0.51$, $P < 0.05$; $r = -0.52$, $P < 0.05$; $r = -0.39$, $P < 0.05$), while it was positively correlated with insulin sensitive index (r=0.45, $P < 0.05$). Conversely, plasma resistin correlated positively with BMI, blood glucose, triglyceride and insulin resistance index (respectively, $r = 0.40$, $P < 0.05$; $r = 0.52$, $P < 0.05$; $r = 0.46$, $P < 0.01$; $r = 0.27$, $P < 0.05$), and negatively correlated with insulin sensitive index ($r = -0.32$, $P < 0.05$).

CONCLUSION: Plasma adiponectin and resistin are associated with the disorder of metabolism of glucose and lipid in diabetes. The relationship between these hormone and insulin sensitivity suggests that they may take part in the development of insulin resistance of type 2 diabetes.

Key words: Adiponectin; Resistin; Adipocyte; Type 2 diabetes

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INTRODUCTION

The prevalence of obesity is rapidly increasing with the changes of life style and structure of meals. The high prevalence of obesity has led to an increase in medical conditions that accompany obesity, especially type 2 diabetes (also known as non-insulin dependent diabetes mellitus, NIDDM), hypertension, cardiovascular disease (CVD), and certain cancers. Perhaps most importantly, obesity generally confers a significantly increased rate of mortality when compared with individuals of normal body weight[1].

Adipose tissue was once considered to be an inert storing depot for energy, in the form of triglyceride. However, in recent years, a line of evidence has

Table 1 Subject characteristics and plasma glucose, insulin, and lipid levels

Parameter	Control group	Non-obese diabetes group	Obese diabetes group
n	28	30	30
Age (yr)	47±11	48 ± 8	52 ± 9
Gender (M/F)	14/14	14/16	16/14
BMI (kg/m^2)	24.03±4.05	23.13±1.62	27.25±1.71^{ac}
FPG (mmol/L)	4.97±0.56	9.9±2.03^a	10.03±2.08^a
FINS (mU/L)	6.25±4.22	9.52±7.81	8.72±4.64
IR	0.50±0.35	0.98±0.59^a	1.22±0.53^a
ISI	-3.61±0.35	-4.09±0.59^a	-4.33±0.54^a
TCH (mmol/L)	4.79±1.07	5.79±1.42^a	5.61±1.79^a
TG (mmol/L)	1.48±1.74	2.45±1.46^a	3.05±1.92^a
NEFA (mmol/L)	0.71±0.18	0.95±0.21^a	1.13±0.24^a

BMI: body mass index; FPG: fasting plasma glucose; FINS: fasting insulin; IR: insulin resistance; ISI: insulin sensitive index; TCH: total cholesterol; TG: plasma triglyceride; NEFA: non-esterified fatty acids. $^aP<0.05$ *vs* control group; $^cP<0.05$ *vs* non-obese diabetes group.

demonstrated a much more complex function of adipose tissue, as an endocrine organ that releases hormones into the blood stream to take part in their potential implication in insulin resistance, obesity and diabetes[2]. The recent boom of interest in adipocyte derived factors has resulted in identification of a large group of adipocyte specific proteins, such as adiponectin, acylation stimulating protein, resistin, leptin, etc[3]. These adipocyte derived hormones are presently subject to intensive research concerning their involvement in the regulation of adipose tissue physiology, and in particular, their potential implication in insulin resistance, obesity and diabetes[4].

Both adiponectin and resistin are new hormones secreted exclusively from adipose tissue. They have important biological activity on glucose and lipid metabolism; moreover, they can affect insulin resistance. The present study was carried out based on the hypothesis that plasma adiponectin and resistin would play some roles in type 2 diabetes.

MATERIALS AND METHODS

Subjects and fasting blood samples

A total of 88 subjects were recruited from outpatient clinics at the Tongji Medical Centre, TongJi Hospital, Wuhan, Hubei province, China. All participants gave informed consent and the study was approved by Tongji Hospital Ethics Committee. Type 2 diabetic patients ($n=60$) were recruited at the time of their initial screening at the Endocrinology Clinic. Non-diabetic control subjects ($n=28$) were normal healthy adults recruited at their yearly checkups. Diabetes was defined in conformity with 1999 World Health Organization. According to the obesity criteria established in 2000 of Asia area, the diabetes patients were divided into two groups: (1) Non-obese diabetes group: BMI <25 ($n=30$); (2)Obese diabetes group: BMI $\geqslant 25$ ($n=30$). The type 2 diabetic participants had not taken any medication (including antidiabetic medication or herbal preparations) before we collected the blood samples. Subjects in diabetic and control groups were confirmed to have no known disease (including cardiovascular disease, thyroid disease, hypertension or any other acute and chronic disease condition) or any current infectious condition.

Table 2 Plasma adiponectin and resistin levels in diabetes patients divided by body weight

	Control group	Non-obese diabetes group	Obese diabetes group
Adiponectin (mg/L)	10.53±1.47	8.58±0.86^a	6.22±1.34^{ac}
Resistin (mg/L)	14.16±5.25	18.64±4.65^a	24.05±9.07^{ac}

$^aP<0.05$ *vs* control group; $^cP<0.05$ *vs* non-obese diabetes group.

Blood samples were drawn after an overnight fast from an antecubital vein. Fasting plasma samples were used for measurement of all parameters. The samples were centrifuged, aliquoted and immediately frozen at -80℃ for analyses of lipoproteins and proteins

Analytical procedures

Plasma samples were analyzed for concentrations of adiponectin, resistin, insulin, glucose, non-esterified fatty acids (NEFA), triglycerides (TG), total cholesterol (TCH). Plasma triglycerides (TG) were measured by GPO-PAP method and total cholesterol was measured by COD-PAP method. The plasma NEFA concentration was determined by colorimetric enzymatic assay (WAKO Chemicals, Tokyo, Japan).

Plasma glucose was determined by glucose-oxidase method (AVE-852 half-auto biochemical analyzer). Plasma insulin was measured by electrocheminescence immunoassay (Elecsys 1010, Roche Instrument Center AG).

Plasma adiponectin was measured by ELISA (B-Bridge International, Phoenix, AZ, USA) and plasma resistin also measured by ELISA (BioVendor Laboraory Medicine, USA).

Calculation

Body mass index (BMI) was calculated as weight (kg) per height (m^2). Insulin resistance index (IRI) was calculated by homeostasis model assessment, HOMA-IR as (fasting insulin IU/L) × (fasting glucose mmol/L) / 22.5 as previously reported by Matthews[5]. Insulin sensitivity index (ISI) was calculated as 1/[(fasting insulin IU/L) × (fasting glucose mmol/L)].

Statistical analysis

All results are displayed as mean ± SE (standard error of mean) unless stated otherwise. ANOVA analyses were used to compare means among the groups and correlations were calculated using Pearson correlation coefficient or multiple regression analysis. $P<0.05$ was considered statistically significant for all analyses.

RESULTS

The age and gender had no significant differences between diabetes group and control group. Subject characteristics are presented in Table 1.

The results of adiponectin and resistin are shown in

Table 3 Correlation analysis of fasting plasma adiponectin and resistin with the other parameters

	Adiponectin		Resistin	
Parameter	*r*	*P* value	*r*	*P* value
BMI	-0.55	0	0.4	0
FPG	-0.51	0	0.52	0
FINS	0.15	0.16	0.1	0.39
IR	-0.52	0	0.46	0
ISI	0.45	0	-0.32	0
TCH	-0.15	0	0.07	0.53
TG	-0.39	0.02	0.27	0.01
NEFA	-0.45	0	0.29	0

BMI: body mass index; FPG: fasting plasma glucose; FINS: fasting insulin; IR: insulin resistance; ISI: insulin sensitive index; TCH: total cholesterol; TG: plasma triglyceride; NEFA: non-esterified fatty acids. $^{a}P<0.05$ *vs* control group; $^{c}P<0.05$ *vs* non-obese diabetes group.

Table 2. There was a significant decrease in adiponectin in diabetes group as compared to that in control group ($P<0.05$). Furthermore, adiponectin in obese diabetes group was lower than that in non-obese diabetes group ($P<0.05$), while plasma resistin levels of non-obese diabetes group and obese diabetes group were apparently increased as compared to that in control group ($P<0.05$). Moreover, resistin level of obese diabetes group was also apparently higher than that of non-obese diabetes group ($P<0.05$).

There were significant differences in blood lipid parameters, including TG, TCH and NEFA, as given in Table 1. Cholesterol and triglyceride in non-obese diabetes group and obese diabetes group were apparently higher than that in control group ($P<0.05$), but there was no significant difference between non-obese diabetes group and obese diabetes group. The level of fasting insulin was not significantly different among the three groups ($P>0.05$). Fasting plasma glucose in diabetes group was apparently higher than that in control group ($P<0.05$), and there was no significant difference between non-obese and obese diabetes groups.

Pearson correlation analysis was used to identify the factors that most closely related to the hormones adiponectin and resistin, as shown in Table 3. Plasma adiponectin correlated negatively with BMI, blood glucose, insulin resistance index and triglyceride (respectively, $r=-0.55$, $P<0.05$; $r=-0.51$, $P<0.05$; $r=-0.52$, $P<0.05$; $r=-0.39$, $P<0.05$); whereas it was positively correlated with insulin sensitive index ($r=0.45$, $P<0.05$). Plasma resistin was correlated positively with BMI ($r=0.39$, $P<0.05$), fasting plasma glucose ($r=0.37$, $P<0.05$) and triglyceride ($r=0.41$, $P<0.05$); and it was correlated positively with insulin resistance index ($r=0.49$, $P<0.05$), but negatively with insulin sensitive index ($r=-0.34$, $P<0.05$). There was no significant correlation between plasma resistin and insulin and cholesterol, but there was significant correlation between plasma adiponectin and cholesterol ($r=-0.15$, $P<0.05$).

DISCUSSION

Recent clinical and experimental data have radically modified the concept of adipose tissue as one solely devoted to energy storage and release. Adipose tissue is also an important endocrine organ. It can release hormones into the blood stream in response to specific extra cellular stimuli or changes in metabolic status. The factors secreted by adipose tissue are actively involved in energy homeostasis in that they signal to the brain, the pancreatic β-cells, the liver and skeletal muscle in order to adapt to changes in energy stores by modulating feeding, insulin secretion and insulin sensitivity [6]. Secreted factors may include leptin; steroids and glucocorticoids; peptide hormone precursors, e.g. angiotensinogen; complement factors, e.g. adipsin, acylation-stimulating protein (ASP) adiponectin; pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNF-α), interleukin 6 (IL-6); resistin; etc. Most of these products probably act through paracrine or autocrine mechanisms[7].

Adiponectin, also known as adipose most abundant gene transcript 1 (apM1), adipoQ and adipocyte complement related protein of 30 Kdaltons (ACRP30) was originally identified as the product of a highly induced gene after 3T3-L1 differentiation. It is a relatively abundant plasma protein (around 0.01% of total plasma proteins) that is exclusively synthesized and secreted by white adipose tissue (WAT)[8]. Adiponectin has many metabolic actions involving peripheral tissue and the regulation of energy homeostasis, particularly energy expenditure. The new adipocykin can decrease plasma glucose, increase clearance of a glucose load and ameliorate insulin resistance in mouse models with normal (C57Bl/6J), reduced (ob/ob, db/db) or absent (lipoatrophic mice) adiponectin pathways[9]. Acute administration in mice reduces elevated postprandial NEFA resulting from ingestion of a high-fat test meal or lipid intravenous injection[10]. Daily administration of adiponectin in mice on a high-fat/ high-sucrose diet induces marked and sustainable weight loss without affecting food intake[11]. Adiponectin decreases muscle and liver TG content, increases gene expression related to muscle NEFA uptake and utilization, increases muscle NEFA oxidation, and enhances hepatic insulin-mediated suppression of glucose production[12]. Adiponectin knockout mice demonstrate insulin resistance after a glucose load or after a high-fat/ high-sucrose diet (2 wk), delayed NEFA clearance and decreased expression of NEFA transporters in muscle[13]. It is apparent that adiponectin is very important for the metabolism of lipid and glucose, as well as insulin sensitivity.

Adiponectin is the only WAT derived hormone whose levels are downregulated in obesity. Human, primate and mouse models of obesity and/or insulin resistance (Type II DM and ob/ob mice) have reduced circulating adiponectin concentrations and expression in WAT[14]. In a recent study in adolescents, plasma adiponectin was shown to correlate with insulin sensitivity but was inversely proportional to plasma triglyceride and intra-myocellular lipid, suggesting its functional link to insulin resistance[15]. During weight-reduction or prolonged negative energy balance, circulating adiponectin is elevated in human[16]. We found that plasma adiponectin was decreased significantly in diabetes group, especially in obese diabetes group. Moreover, plasma adiponectin correlated negatively with insulin resistance

index, triglyceride and correlated positively with insulin sensitive index. It is well known that adiponectin improves insulin sensitivity of the whole body, so we speculated the decreased adiponectin in type 2 diabetes may be one of the reasons to cause insulin resistance. The change of plasma adiponectin was correlated with the development of insulin resistance in diabetes. It is unclear why adiponectin levels were decreased in obese diabetes with more fat tissue which can produce adiponectin. Some researchers speculated that the suppression of adiponectin production in obesity and diabetes may be mediated by an autocrine negative feedback inhibition in WAT, for instance, lower expression of adiponectin mRNA was found in cultured human visceral WAT and microarray study demonstrated that adiponectin expression is suppressed with the development of obesity and diabetes in mice[17].

Resistin is another protein identified recently as a hormone secreted by adipocytes which leads to insulin resistance *in vivo* and *in vitro* and might therefore be an important link between obesity and diabetes[18]. 3T3-L1 cells can secrete abundant resistin into the medium and its mRNA is induced (reduced?) markedly during adipocyte differentiation of 3T3-L1 cells[19]. When these adipocytes were exposed to specific resistin antibody, the insulin-stimulated glucose uptake was increased significantly. Consistent with the results, treatment of 3T3-L1 adipocytes with purified resistin reduced insulin-stimulated glucose uptake[20]. These studies suggest that resistin functions as a signal to decrease insulin-stimulated glucose uptake. Administration of anti-resistin antibody also can improve blood glucose and insulin action in mice with diet-induced obesity[21]. Resistin gene and protein expression were increased in human abdominal adipose tissue. In this study we found that plasma resistin levels of non-obese diabetes group and obese diabetes group were apparently higher than that in control group. Furthermore, resistin levels in obese diabetes group were increased as compared to non-obese diabetes group. Meantime plasma resistin was correlated positively with insulin resistance index. Al-Harithy *et al*[22] had similar result in their study on type 2 diabetes. It was well known that resistin levels were increased in differentiation of adipocytes, obesity and people on high fat diet?, and it is increased in abdominal vs. thigh adipose tissue. There is a potential link between central obesity and type 2 diabetes and/or cardiovascular disease[23]. Functionally, resistin impairs glucose tolerance, insulin action, and increases hepatic glucose production, while a neutralizing antibody normalizes glucose levels and tissue glucose uptake[24]. It suggests that increased plasma resistin may be correlated with the development of insulin resistance in type 2 diabetes.

Type 2 diabetes mellitus is characterized by target-tissue resistance to insulin. It is strongly linked to obesity as over 80% of sufferers are obese[25]. While the molecular basis for this link has remained a mystery. Insulin resistance is the core pathogenic factor for diabetes. In addition, it is also strongly associated with obesity, hypertension and cardiovascular disease, etc. The new adipocytes derived hormones adiponectin and resistin may be an important link between increased fat mass and insulin resistance and disorder of metabolism of lipid and glucose in diabetes. Adipocytes have become the center for the study of the association of insulin resistance and type 2 diabetes[26]. Studies of the roles of adiponectin and resistin will shed new light on prevention and treatment of type 2 diabetes, and open a new field for the development of new drugs to improve insulin resistance.

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RAPID COMMUNICATION

Chronic hepatitis B serum promotes apoptotic damage in human renal tubular cells

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Abstract

AIM: To investigate the effect of the serum of patients with chronic hepatitis B (CHB) on apoptosis of renal tubular epithelial cells in vitro and to study the role of hepatitis B virus (HBV) and transforming growth factor-β1 (TGF-β1) in the pathogenesis of hepatitis B virus associated glomerulonephritis (HBV-GN).

METHODS: The levels of serum TGF-β_1 were measured by specific enzyme linked immunosorbent assay (ELISA) and HBV DNA was tested by polymerase chain reaction (PCR) in 44 patients with CHB ,and 20 healthy persons as the control. The normal human kidney proximal tubular cell (HK-2) was cultured together with the sera of healthy persons, CHB patients with HBV-DNA negative(20 cases) and HBV-DNA positive (24 cases) for up to 72 h. Apoptosis and Fas expression of the HK-2 were detected by flow cytometer.

RESULTS: The apoptosis rate and Fas expression of HK-2 cells were significantly higher in HBV DNA positive serum group 19.01±5.85% and 17.58±8.35%, HBV DNA negative serum group 8.12±2.80% and 6.96 ± 2.76% than those in control group 4.25±0.65% and 2.33 ± 1.09%, respectively ($P < 0.01$). The apoptosis rate and Fas expression of HK-2 in HBV DNA positive serum group was significantly higher than those in HBV DNA negative serum ($P < 0.01$). Apoptosis rate of HK-2 cells in HBV DNA positive serum group was positively correlated with the level of HBV-DNA ($r = 0.657$). The level of serum TGF-β_1 in CHB group was 163.05 ± 91.35 μg/L, significantly higher as compared with 81.40 ± 40.75 μg/L in the control group ($P < 0.01$).

CONCLUSION: The serum of patients with chronic hepatitis B promotes apoptotic damage in human renal tubular cells by triggering a pathway of Fas up-regulation. HBV and TGF-β_1 may play important roles in the mechanism of hepatitis B virus associated glomerulonephritis.

Key Words: Renal tubular epithelial cells; HBV; TGF-β_1; Apoptosis

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INTRODUCTION

Hepatitis B (HBV) viruses are well-recognized causes for chronic hepatitis, cirrhosis, and even for hepatocellular carcinoma. Apart from liver disease, these viral infections are known to be associated with a spectrum of extrahepatic manifestations [1]. Hepatitis B is prevalent in China and hepatitis B virus associated glomerulonephritis has been one of the common renal damages secondary to HBV infection in Chinese children [2]. The cause of HBV-GN is generally believed to be immune mediated [3]. Recent studies showing expression of HBV viral antigens in kidney tissue and HBV DNA distributed generally in the nucleus and cytoplasm of epithelial cells of renal tubules suggest direct viral-induced pathological alterations and chronic immunologic injury [4,5]. Apoptosis is an active mode of cell death that promotes cell loss during both acute and chronic renal damage [6,7,8]. Here we investigated cell apoptosis in human renal tubular epithelial cells induced by sera of patients with chronic HBV infection and explored its mechanism.

MATERLALS AND METHODS

Patients and serum samples

Forty-four patients with chronic HBV infection (mild degree) came from Department of Infectious Diseases of the Affiliated Hospital of Luzhou Medical College, China

during 2003-2004. All the patients conformed to the revised standard of the Sixth National Meeting of Infectious Disease and Parasitosis in 2000. There were 38 men and 6 women. The median age was 34 years (range: 17-62 years). According to the HBV DNA levels in serum assayed by polymerase chain reaction (Positive criteria: $>1\times10^6$ copies/L), the patients were divided into HBV DNA positive patients group (24 cases) and HBV DNA negative patients group (20 cases). Twenty healthy persons whose serum markers of HBV were negative were used as the control. The blood collected from these persons was centrifuged at 4℃ and the serum samples were stored at -80 ℃ until examination. The serum samples were in the water bath at 56 ℃ for 30 min to inactivate complement. The level of HBV DNA in the serum samples was estimated by polymerase chain reaction (Shanghai Fortune Medical Science Co. Ltd, China) and TGF-β1 protein concentration was determined by specific-ELISA (Jingmei Biotech Co. Ltd, China).

Cell culture

All experiments were performed using the human kidney proximal tubular epithelial cell line (HK-2)[9] (American Type Culture Collection, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. Fresh growth medium was added to cells every three to four days until confluent.

Flow cytometry analysis

HK-2 was cultured in the presence of the serum (50 μL) of healthy persons, CHB patients with HBV-DNA negative and HBV-DNA positive for up to 72 h, respectively. The survival rate and apoptotic rate of HK-2 cells were determined by Annexin V and propidium iodide (PI)(Bio Vision, USA) staining. HK-2 cells were digested by 0.25% trypsin. Cells (5×10^5) were suspended in Binding Buffer. Annexin V (5 μL) and PI (5 μL) were then added and incubated with cells at room temperature for 5 min in the dark until detected by flow cytometry. HK-2 cells (5×10^5) suspended in binding buffer were stained at room temperature with CD95-FITC (20 μL) (Bio Vision, USA) for 20 min in the dark and evaluated by flow cytometry to determine Fas expression.

Statistical analysis

The data are expressed as means ± SD. Statistical analysis of data was performed using *t* test or *F* test, with a value of $P < 0.05$ considered to represent a significant difference.

RESULTS

Detection of HBV DNA

Serum samples of forty-four patients with chronic HBV infection were assayed by polymerase chain reaction. There are 24 cases with HBV DNA positive (9.36×10^6-1.57×10^{12} copies/L) and 20 cases with HBV DNA negative.

Detection of TGF-β1

The level of serum TGF-β1 in CHB group was 163.05 ± 91.35 μg/L, significantly higher as compared with 81.40 ± 40.75 μg/L in the control group (Table 1, $P<0.01$).

Table 1 Comparison between levels of TGF-β 1 in two groups

Group	Cases	TGF-β1 (μg/L)
Control	20	81.40 ± 40.75
CHB serum	44	163.05 ± 91.35[b]

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

Table 2 Apoptosis of HK-2 in all groups

Group	Cases	Apoptosis (%)
Control	20	4.25 ± 0.65
HBV DNA negative serum	20	8.12 ± 2.80[b]
HBV DNA positive serum	24	19.01 ± 5.85[bd]

[b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* HBV DNA negative serum.

Table 3 Expression of Fas of HK-2 cells in all groups

Group	Cases	Fas (%)
Control	20	2.33 ± 1.09
HBV DNA negative serum	20	6.96 ± 2.76[b]
HBV DNA positive serum	24	17.58 ± 8.35[bd]

[b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* HBV DNA negative serum.

Detection of apoptosis by flow cytometry

The apoptosis rate of HK-2 cells was significantly higher in HBV DNA positive serum group 19.01 ± 5.85% and HBV DNA negative serum group 8.12±2.80% than that in control group 4.25 ± 0.65%, respectively ($P < 0.01$) (Table 2, Figure 1). The apoptosis rate of HK-2 in HBV DNA positive serum group was significantly higher than that in HBV DNA negative serum group($P < 0.01$) (Table 2). Apoptosis rate of HK-2 cells in HBV DNA positive serum group was positively correlated with the level of HBV-DNA ($r = 0.657$) (Figure 2).

Detection of expression of Fas by flow cytometry

The expression of Fas of HK-2 cells was significantly higher in HBV DNA positive serum group 17.58 ± 8.35% and HBV DNA negative serum group 6.96 ± 2.76% than that in control group 2.33 ± 1.09%, respectively ($P<0.01$) (Table 3, Figure 3). The expression of Fas of HK-2 cells in HBV DNA positive serum group was significantly higher than that in HBV DNA negative serum ($P < 0.01$) (Table 3).

DISCUSSION

Apoptosis plays an essential role in the regulation of renal cell number in both healthy and diseased kidneys [10]. Efficient deletion by apoptosis of excessively damaged, or nonfunctioning renal cells and infiltrating inflammatory cells may be beneficial. However, the loss of resident renal cells by uncontrolled apoptosis is detrimental as it may in-

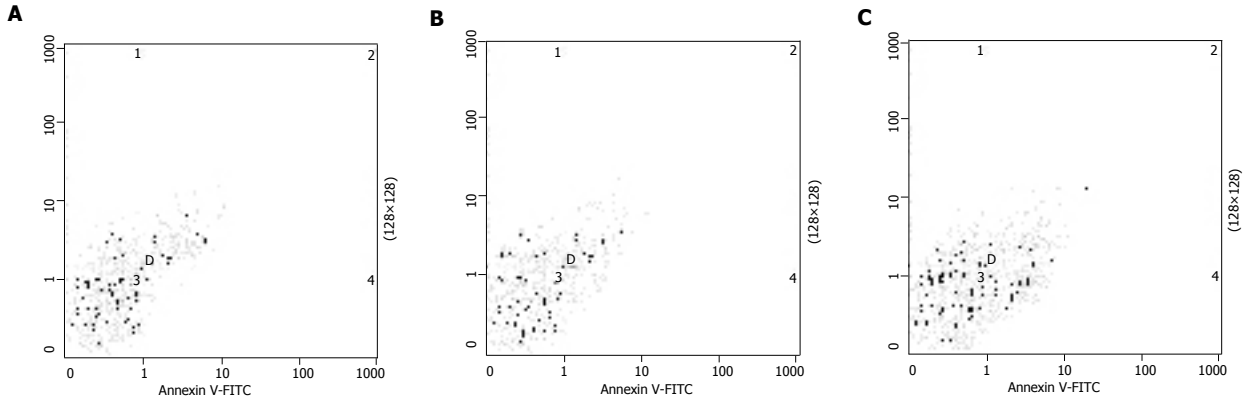

Figure 1 Flow cytometric analysis of HK-2 cells stained with Annexin V and PI. **A**: Treated with serum of healthy persons. **B**: Treated with HBV DNA negative serum. **C**: Treated with HBV DNA positive serum.

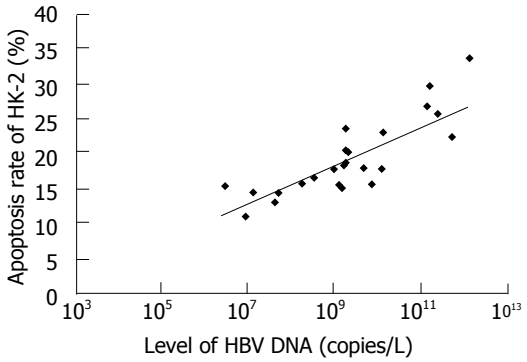

Figure 2 Relationship between apoptosis of HK-2 and levels of HBV DNA in HBV DNA positive serum.

duce a reduction of functional renal mass and lead to renal insufficiency [11-13]. Several external and internal signals can induce apoptosis, which is then effected via a number of pathways. These pathways are either the Fas/FasL pathway and downstream MAPK (mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinase) signal transduction, or the RANK/RANK-L (receptor activator of NFkB) pathway via activation of the caspase cascade [14,15]. Finally, irreversible branch of these pathways is the release of cytochrome c from the mitochondria, leading to nuclear fragmentation [16,17]. Previous studies indicated that pathological factors had a toxic effect on HK-2 cells by triggering an apoptotic pathway involving caspase activation, Fas up-regulation, Fas ligand (FasL) expression, the increase of Bax, the decrease of Bcl-2 protein expression and JNK signaling transduction[18,19], thus potentially interacting with mechanisms of cell loss which have been already shown to be activated in chronic renal diseases. In this study, we showed that CHB serum induced a significant increase in apoptosis in HK-2 cells detected by flow cytometry. It suggested that CHB serum might play a crucial role in the apoptosis of HK-2 cells. Excessive apoptosis of renal proximal tubular cells might be associated with renal injury in patients with chronic HBV infection.

Hepatitis B virus associated glomerulonephritis is occurring at a high prevalence in most Asian endemic areas [20]. The detailed mechanism of renal tubular epithelial cells (RTC) injury in HBV-GN remained unclear. The cause of HBV-GN is generally believed to be immune mediated [3]. Recent studies suggest hepatitis B viral direct infection in RTC may be correlated with renal pathological alterations. HBV DNA and HBV antigen were distributed generally in epithelial cells of renal tubules and the positive results from HBV DNA in situ hybridization correlated well with HBV antigen assays[4,5,20]. The analyses implied that the more extensive the existence of HBV DNA in the nephron unit, the more severe the clinical manifestation. The duration of proteinuria in cases with HBV DNA in renal tubules was much longer than in those with no HBV DNA in renal tubules [5]. Our previous study has observed that the rate of early impairment of renal cells in HBV DNA positive patients with HBV chronic infection was higher than that in HBV DNA negative patients with HBV chronic infection[21]. In this study, we demonstrated that apoptosis of HK-2 cells stimulated by HBV DNA positive serum was significantly higher than that of HBV DNA negative serum, and the apoptosis rate of HK-2 cells was correlated with serum level of HBV-DNA positively. These data suggested that the level of HBV DNA in CHB serum may have a significant effect on the apoptosis of HK-2 cells, and supported the viewpoint that hepatitis B viral direct infection in RTC could lead to cellular apoptosis and might cause renal pathological alterations.

Epithelial cell loss characterized as tubular atrophy is considered to be a hallmark in the development of chronic renal interstitial fibrosis[22]. Large amounts of research demonstrated that the proximal tubular cell may contribute to the pathogenesis of renal interstitial fibrosis[23,24]. Although the mechanism of epithelial cell loss remains uncertain, it is conceivable to assume that tubular atrophy primarily results from apoptotic cell death under pathologic conditions[25,26]. Previous studies demonstrated that TGF-β1 markedly potentiated cell apoptosis initiated by accelerating the activation of pro-caspase-9 and -3[27]. We observed that the level of TGF-β1 significantly increased in CHB serum compared with healthy person serum. Apoptosis of HK-2 cells stimulated by HBV DNA negative serum was notablely higher than that of healthy person serum. We speculate that TGF-β1 may contribute to progressive renal dysfunction after severe tubular injury.

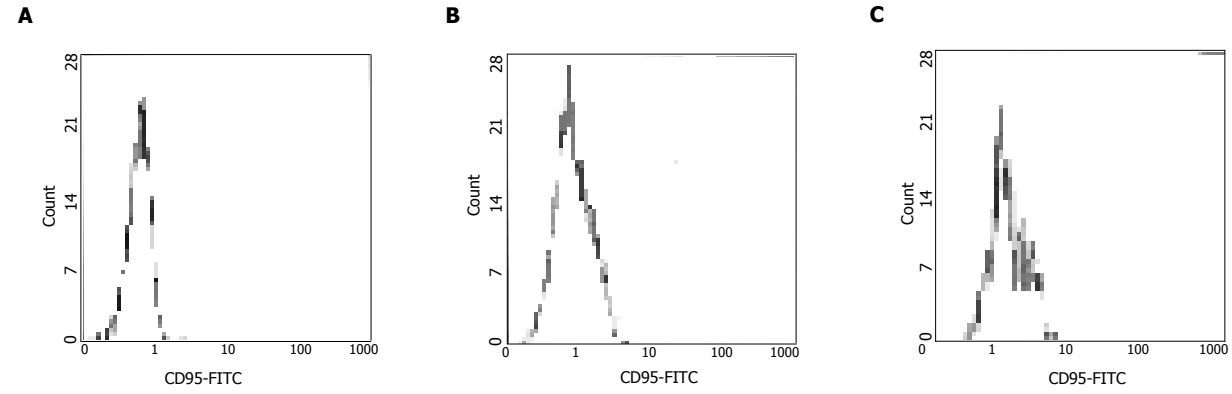

Figure 3 Flow cytometric analysis of HK-2 cells stained with CD95-FITC. **A**: Treated with serum of healthy person. **B**: Treated with HBV DNA negative serum. **C**: Treated with HBV DNA positive serum.

Fas (CD95, APO-1) is a ubiquitously expressed member of the tumor necrosis factor receptor superfamily, which mediates diverse cellular responses, including proliferation, inflammation, angiogenesis, and apoptosis[28]. Fas and FasL are exclusively expressed in RTC undergoing apoptosis. Fas and FasL expression were related to the apoptosis of RTC[29,30]. The Fas-mediated cell death of a tubular cell may be true cell suicide or fratricide in which the FasL is derived from the same or neighboring tubular cell [31]. Binding of FasL to Fas leads to receptor oligomerization and recruitment of the cytoplasmic adapter protein FADD (i.e., Fas-associated death domain protein)[8]. FADD binds caspase 8, which subsequently becomes autoactivated. The catalytically active caspase 8 then initiates the caspase cascade, which leads to the rapid demise of the cell [32]. Our study showed that Fas was clearly up-regulated in HK-2 cells stimulated by CHB serum. These results indicate that apoptosis of proximal tubule kidney cells effects by triggering a pathway of Fas up-regulation. The extracellular microenvironment of injured kidneys may sensitize parenchymal renal cells to Fas induced death, leading to cell loss in the course of acute or chronic renal damage.

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RAPID COMMUNICATION

Morphological observation of tumor infiltrating immunocytes in human rectal cancer

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Abstract

AIM: To investigate the morphological characterization of tumor infiltrating dendritic cells (TIDCs) and tumor infiltrating lymphocytes (TILs) in human rectal cancer.

METHODS: Light and electron microscopy as well as immunohistochemistry were used to observe the distributive and morphological changes of TIDCs and TILs.

RESULTS: TIDCs were mainly located in tumor-surrounding tissue. The number of TIDCs in the earlier stage was higher than that in the later stage ($P < 0.01$). TILs were mainly seen in adjacent tissue of cancers and tumor-surrounding tissue. There were more TILs in the earlier stage than that in the later stage ($P < 0.01$). Under electron microscope, TIDCs were irregular in shape and exhibited many dendritic protrusions. It isn't obvious that cancer cells perforated the basement membrane and TILs were arranged along the basement membrane in the earlier stage. In the later stage, it is explicit that cancer cells perforated the basement membrane and surrounded by TILs. There were contacts among TIDCs, TILs and tumor cell. One TIDCs contacted one or several TILs which clustered around TIDCs. Glycogen granules were seen between TIDCs and TILs.

CONCLUSION: The number of TIDCs and TILs is related with tumor progression There exist close relationships among TIDCs, TILs and tumor cell.

Key words: Rectal cancer; Tumor infiltrating dendritic cells; Tumor infiltrating lymphocytes

Xie ZJ, Jia LM, He YC, Gao JT. Morphological observation of tumor infiltrating immunocytes in human rectal cancer. *World J Gastroenterol* 2006; 12(11): 1757-1760

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INTRODUCTION

Antitumor effects of human body are mainly achieved by tumor infiltrating immunocytes, including TIDCs, TILs, natural killer(NK) cells and lymphokine-activated killer(LAK) cells. Because of their prominent role in antitumor effects, TIDCs and TILs have drawn more and more attention. DCs are the most powerful professional antigen-presenting cells (APCs) so far. Immunophenotype and function of DCs in biological therapy of tumor are the focus of immunological study [1,2]. Although studies on tumor immunotherapy involving injection of DCs cultured *in vitro* are reported [3-5], infiltrating DCs in tumor microenvironment have not been fully investigated and studies usually concentrate on the correlation between infiltrating DCs and tumor progression[6,7]. Morphological studies on the relation among TIDCs, TILs and tumor cells, are few. TILs are heterogenous lymphocytes, most of them are T lymphocytes. TILs are a new kind of antitumor effectors after LAK cells. In this study, the morphology of TIDCs and TILs in earlier and later stage of human rectal cancer was investigated, which may provide morphological evidence for antitumor effect of tumor infiltrating immunocytes.

MATERIALS AND METHODS

Patients

Eighteen rectal cancer patients surgically treated in earlier or later stage in the Second and Third Hospitals of Harbin Medical University were studied. According to their clinical manifestations and pathological features, the patients were divided into earlier stage group ($n = 8$) and later stage group ($n = 10$).

Methods

Fresh rectal cancer samples were obtained during surgery. Immediately after surgical resection, tissues were rinsed in redistilled water and then in 0.2 mol/L phosphate-buffered saline (PBS). Tumor-surrounding tissues were obtained and fixed in 3% glutaraldehyde for transmission electron microscopy or in 10% neutrally buffered formalin solution. The cut into slices, embedded in paraffin and sectioned at 4 μm for HE and immunohistochemical staining. The

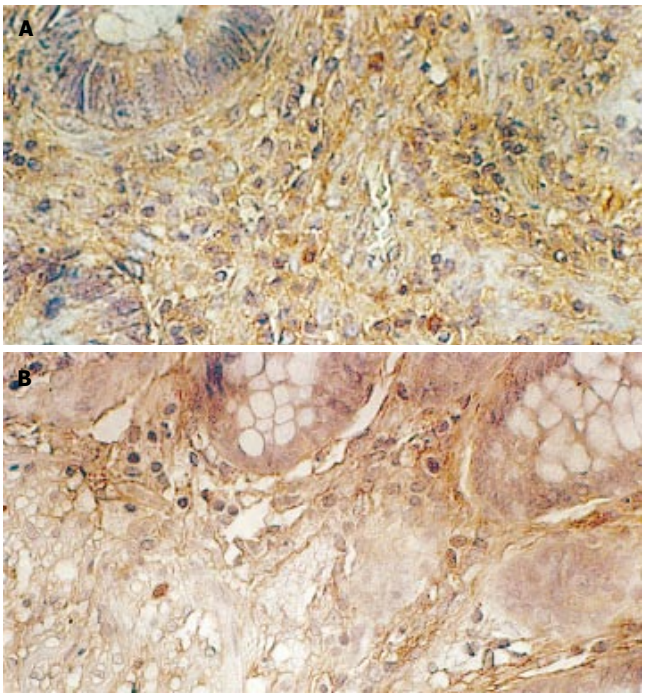

Figure 1 TIDCs in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A, B x 400)

Table 1 Number of immunocytes between earlier and later stages (mean±SD)

	Earlier stage	Later stage	*P*-value
Number of cases	8	10	
Number of TIDCs	49.32 ± 5.48	17.02 ± 3.14	<0.01
Number of TILs	107.38 ± 13.61	48.21 ± 5.13	<0.01

deparaffinized sections were washed with PBS. Primary antibodies used were rabbit-anti human S-100 protein and CD3 (Boster Biological Technology Ltd). Secondary antibody used was mouse-anti rabbit IgG. The procedure was performed with SABC kit following the manufacturer's instructions.

Statistical analysis

Student *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Cell counting

Under the light microscope, the number of infiltrating S-100 (+) and CD3 (+) cells in each section was calculated under 5 high power fields (×400) and statistically analyzed.

RESULTS

Observation of HE and immunohistochemical staining under light microscope

TIDCs were mainly seen in tumor-surrounding area, only a small number of TIDCs were located in cancer tissue scat-

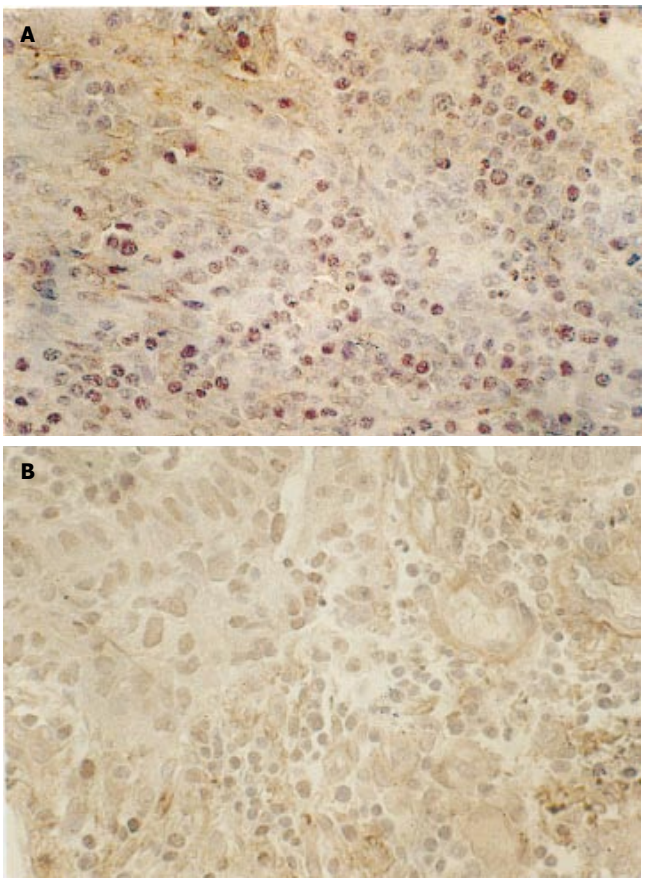

Figure 2 TILs in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A, B x 400)

teredly by HE staining. Using anti-S-100 antibody with the SABC method, Brown S-100 (+) DCs could be observed in each section. Comparatively large DCs exhibited many dendritic protrusions, mainly located in tumor-surrounding tissue. The number of TIDCs was large in the earlier stage (Figure 1A) and comparatively small in the later stage (Figure 1B). The number of TIDCs in earlier stage was significantly higher than that in later stage and the difference was statistically significant ($P < 0.01$, Table 1).

Brown CD3 (+) TILs could be seen in each section. Circular CD3 (+) lymphocytes, little cytoplasm and large nuclei were distributed uniformly and densely. In the earlier stage, the number of TILs was large and TILs were primarily located centrally (Figure 2A). In the later stage, the number of TILs was small and TILs were scattered, only a small number of TILs were distributed centrally (Figure 2B). The number of TILs in earlier stage was significantly higher than that in later stage and the difference was statistically significant ($P < 0.01$, Table 1).

Transmission electron microscopy

Under electron microscope, TIDCs were seen in tumor-surrounding tissue. TIDCs were irregular in shape and displayed many dendritic cytoplasmic protrusions with varying width. The nuclei were irregular in shape and the nucleoli were small. Heterochromatin was highly marginated along the nuclear membrane. Mitochondria and endoplasmic reticulum were prominent in these cells. A fraction

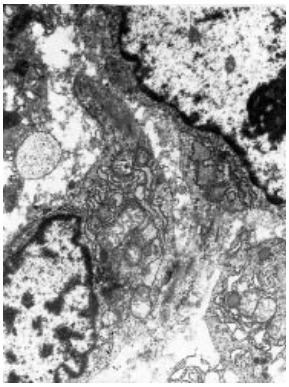

Figure 3 Morphology of TIDCs . D represents TIDCs, T represents tumor cells. (magnification x 10000)

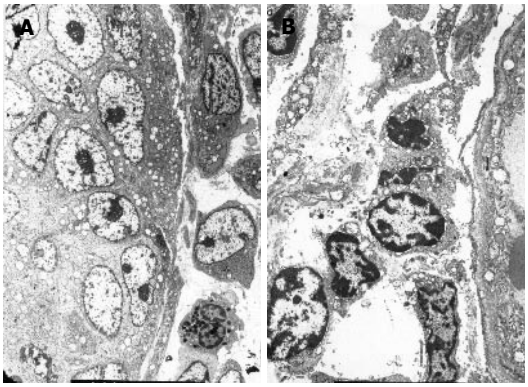

Figure 4 Lymphocytes in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A x4000, B x2000)

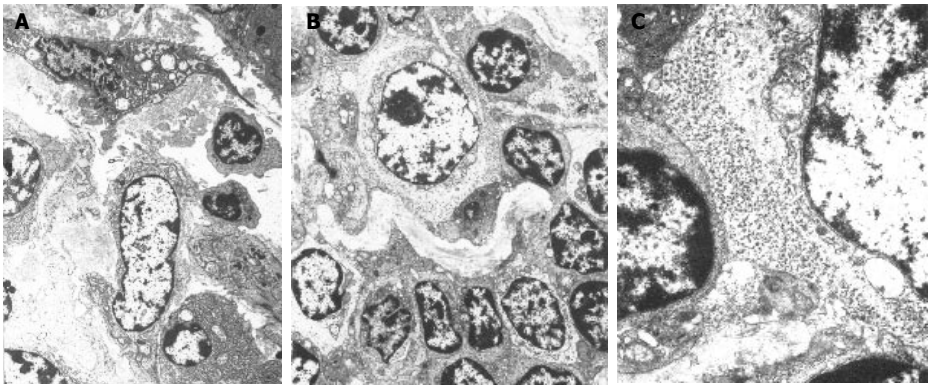

Figure 5 Relations between TILs and tumors cells (**A**), TIDCs and TILs (B) and glycogen granules (**C**). (magnification: A, x 4000, B x3500, C x15000)

of endoplasmic reticulum and some mitochondria were expanded (Figure 3).

It isn't obvious that cancer cells perforated the basement membrane. Lymphocytes were arranged serially along the basement membrane in earlier stage (Figure 4A). However, in the later stage, it is explicit that cancer cells perforated the basement membrane and were surrounded by TILs (Figure 4B). Mitochondria in cancer cells perforating the basement membrane degenerated and the cancer cells were surrounded by DCs, lymphocytes and plasma cells (Figure 5).

TIDCs were contacted with TILs in tumor-surrounding tissue, forming DC-lymphocyte clusters (Figure 5A). Clusters of glycogen granules were seen between TIDCs and TILs (Figure 5B). In addition to contacting TILs, TIDCs also contacted cancer cells at the same time. TILs could be seen surrounding the cancer nests, basement membrane of cancer was fragmentary, and TIDCs contacted cancer cells directly (Figure 5C).

DISCUSSION

DCs are the most powerful professional antigen-presenting cells (APCs)[1]. Studies on immunophenotype and function of DCs are now available[2-5]. Morphological studies on DCs and TIDCs are few. In this study, the morphology of TIDCs and correlation between TIDCs, TILs and cancer cells in human rectal cancer were investigated.

Antitumor immune responses of human body are mainly centralized in local cell-mediated immunity. The infiltrating degree of lymphocytes surrounding tumor tissue reflects the status of antitumor immune responses of human body. Activation of T lymphocytes needs the assistance of APCs. DCs are the most important APCs in human body and are closely related with the function of T lymphocytes. TILs are heterogenous lymphocytes, most of which are T lymphocytes. TILs play an important role in immunity and anti-tumor effects[8,9]. In our study, TILs were closely related with tumor immunity and mainly distributed in adjacent tissue of cancer, suggesting that localization of TILs in tumor-surrounding tissue limits the growth of cancer and confines cancer to a local site, which is the first barrier of anti-tumor effects of human body and contributes to immune response and prevents tumor spreading, which is the second barrier of anti-tumor effects of human body. Infiltration of TILs represents anti-tumor immune responses of human body. The infiltrating degree of TILs plays a decisive role in invasion and metastasis of tumor as well as prognosis of patients. In our study, the number of TILs in earlier stage of rectal cancer was significantly higher than that in later stage ($P < 0.01$), demonstrating that the number of TILs is related with the progression of rectal cancer.

It was reported that metastasis and prognosis of tumor are related with the infiltrating degree of DCs in tumor tissue [6]. Wright-Browne *et al* [7] showed that the number of TIDCs in human gastric cancer, colon cancer and carcinoma of esophagus is negatively correlated with lymph node metastasis, size of tumor and survival time, that is, the more the number of TIDCs, the better the patient's prognosis. In this study, the number of TIDCs in earlier stage was significantly higher than that in later stage ($P < 0.01$), suggesting that the number of TIDC is related with the progression of rectal cancer.

DCs play an important role in tumor immunity. The powerful antigen-presenting function of DCs can induce

efficient and specific anti-tumor immunity activated CTLs can kill tumor cells[10-12]. In this study, TIDCs were closely contacted with TILs. TILs were clustered around TIDCs and cluster of DCs-lymphocytes cluster was formed. Glycogen granules could be seen between TIDCs and TILs. All these suggest that there exists some relation between TIDCs and TILs. It has been reported that DCs mainly present antigen to lymphocytes, stimulate naïve T lymphocyte proliferation and activation to kill tumor cells [13]. The contact of TIDCs and TILs observed in our study may provide morphological evidence and suggests that TIDC can present antigen to TILs and activate TILs. Moreover, fraction of endoplasmic reticulum and some mitochondria in DCs were expanded. These data suggest that these active cells can capture and identify antigens by their cytoplasm protrusions, and subsequently transmit antigens to lymphocytes and prime anti-tumor immunity to kill tumor cells through cytotoxicity.

DCs participate in local anti-tumor immune response and have corresponding changes during oncogenesis and tumor progression, suggesting that DCs are closely related with anti-tumor immunity. DCs are not only professional APCs, but also immunological adjuvant. DCs play an important role in immunoregulation. It has been shown that antigen-sensitized DCs can induce specific CTL *in vivo* and directly inhibit or kill tumor cells [13-16]. But whether DCs can directly inhibit the growth of tumor *in vivo* or directly kill tumor cells remains unclear and should be further studied.

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RAPID COMMUNICATION

Effect of electro-acupuncture at Foot-Yangming Meridian on somatostatin and expression of somatostatin receptor genes in rabbits with gastric ulcer

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Abstract

AIM: To discuss the protective effect of electroacupuncture at the Foot-Yangming Meridian on gastric mucosal lesion, somatostatin (SS) and the expression of SS receptor genes (SSR$_1$mRNA) in rabbits with gastric ulcer and to further explore the relative specificity of meridians and viscera at gene expression level.

METHODS: Forty rabbits were randomly divided into control group (A), gastric ulcer model group (B), Foot-Yangming Meridian group (C), Foot-Shaoyang Meridian group (D) and Foot-Taiyang Meridian group (E). The gastric ulcer model was prepared by infusing alcohol into stomach. Groups C－E were treated with electro-acupuncture at points along the above meridians using meridian stimulating instruments for 7 days respectively. By the end of treatment, the index of gastric ulcer was determined, the amount of epidermal growth factor(EGF) and somatostatin was measured by radioimmunoassay (RIA). SS-R$_1$mRNA expression in gastric mucosa was determined by RT-PCR.

RESULTS: The value of EGF in model group was obviously lower(73.6 ± 14.8 *vs* 91.3 ± 14.9 pg/mL, $P < 0.01$) than that in control group**.** The index of gastric ulcer, content of SS and expression of SSR$_1$mRNA in gastric mucosa were significantly higher than those in control group (24.88 ± 6.29 *vs* 8.50 ± 2.98 scores, $P < 0.01$; 2978.6 ± 587.6 *vs* 1852.4 ± 361.7 mIU/mL, $P < 0.01$; 2.56 ± 0.25 *vs* 1.04 ± 0.36, $P < 0.01$) . The value of EGF in Foot-Yangming Meridian group was higher than that in model group(92.2 ± 6.7 *vs* 73.6 ± 14.8 pg/mL, $P < 0.01$). The index of gastric ulcer, content of SS and expression of SS-R$_1$mRNA in gastric mucosa were significantly lower than those in control group(10.88 ± 3.23 *vs* 24.88 ± 6.29 scores, $P < 0.01$; 1800.2 ± 488 *vs* 2978.6 ± 587.6 mIU/mL, $P < 0.01$; 1.07 ± 0.08 *vs* 2.56 ± 0.25mIU/mL, $P < 0.01$). Compared to the model group, the content of SS and expression of SSR$_1$mRNA in gastric mucosa in Foot-Shaoyang Meridian group decreased (2441.0 ± 488. *vs* 2978.6 ± 587.6 mIU/mL, $P < 0.05$;1.73 ± 0.16 *vs* 2.56 ± 0.25 mIU/mL, $P < 0.01$). But the above parameters in Foot-Taiyang Meridian group did not improve and were significantly different from those in Foot-Yangming Meridian group ($P < 0.05$).

CONCLUSION: Electro-acupuncture at Foot-Yangming Meridian can protect gastric mocusa against injury. The mechanism may be releted to the regulation of brain-gut peptides and the expression of SSR$_1$mRNA.

Key words: Foot-Yangming Meridian; Electro-acupuncture; Somatostatin; SSR$_1$mRNA

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INTRODUCTION

Studies have shown that needling the Foot-Yangming (stomach) Meridian can protect gastric mucosa against injury in rabbits[1-3]. This study was to further explore its mechanism and specificity in the experimental gastric ulcer model of rabbits and to compare its effect on the index of gastric mucosal lesion, brain-gut peptides and expression of SSR$_1$mRNA by investigating the correlation between Foot-Yangming Meridian and stomach.

MATERIALS AND METHODS

Main reagents and instruments

TRIZOL reagent and RT reagent kit as well as TagDNA polymerase were obtained from GZBOD BRL and Pro-

gmego Company respectively. RIA kits of EGF and SS were provided by Beijing SINO-UK Institute of Biological Technology. Hypothermia Eppedorflow-temperature centrifuge, Strategene Eagle Eye II figure recognition analytical system(made in USA), Xuanshou Meridian Unblocking Instrument (China Peace Economic Technic Counseling Corporation) were used in the study.

Preparation of experimental gastric ulcer

Gastric ulcer model was induced by ethanol[4]. In brief, the animals were perfused with 100% ethanol (2.35 mL/kg) into the stomach after fasted for 48 h. Twenty-four hours after the perfusion, the animals were allowed to have normal foods. The model group received normal saline (2.35 mL/kg) through an esophageal cannula.

Grouping and disposing

Forty New Zealand white rabbits of either sex, weighing 1 500-2 500 g were supplied by the Medical Animal Center of Hunan College of Traditional Chinese Medicine. The animals were randomly divided into 5 groups: control group (A), gastric ulcer model group (B), Foot-Yangming (stomach) Meridian group (C), Foot-Shaoyang (gallbladder) Meridian group (D) and Foot-Taiyang (bladder) Meridian group (E). NS was perfused into stomach of the control group. Dehydrated alcohol was administered intragastrically (i.g.) using a metal tube in B-E groups. C-E groups were treated with electro-acupuncture at points along the meridians of stomach, gallbladder and bladder for 7 d respectively. All the animals were killed at the end of the treatment. Gastric mucous membrane was removed for the detection of the indexes.

Location of acupoints and method of electro-acupuncture

Acupoints were selected on the basis of *Experiment Acupuncture Science*[5] and the method was chosen according to the human body[6]. For Foot Yangming (stomach) Meridian, the acupoints of "Neiting", "Jiexi", "Zusanli", "Liangqiu", "Tianshu" and "Liangmen" were chosen as stimulating points. According to the above stimulating points, acupoints in other meridians were selected at the same level of Foot Yangming Meridian in corresponding regions. For example , the acupoints of "Jiaxi","qiuxu", "Yanglingquan","Qiyangguan","Daimo","Jingmen" were selected for Shaoyang Meridian while the acupoints of "Zutonggu","Shenmo","Chengjin","Fuxi","Beishangdian","Beixiadian" were selected for Taiyang Meridian.

The method of stepwise stimulation along the meridians was used as previously described[7]. Then the instrument entered the state of making program and the single way of running was turned at the running speed of 0.5 second. The stimulation parameters included two-direction narrow pulse and continuous wave with a frequency of 50Hz and a width wave of 0.5ms. The output claps of stimulating instrument were clapped with 6 inserted needles at acupoints. Stimulating excitation was turned from low limber to body. The output intensity of instrument was controlled between "2-3" sections. Acupuncture was performed for 30 min a day for 7 d.

Index of gastric ulcer measurement

The abdomen was opened with the stomach removed 7 d after acupuncture. The stomach was then cut from pylorus to cardia. The index of gastric ulcer was calculated as previously described[8].

Determination of EGF and SS

Samples of gastric mucosa were homogenized and disposed with the test kit according to the manufacturer's instructions. EGF and SS were determined by radioimmunoassay.

Determination of SS-R$_1$mRNA

The expression of SS-R$_1$mRNA was analyzed RT-PCR. A small piece of gastric mucosa tissue was cut with a sterilized operating knife and mounted on a mortar stuffed with liquid nitrogen. After the tissue was ground into powders, the total RNA of was extracted by one step method with Trizol reagent kit. The rRNA bands of 28S, 18S and 5S were observed by electrophoresis. The proportion of A260/A280 of the total RNA in all samples was between 1.7 and 1.95. mRNA from 2 μg total RNA in samples was reversed to cDNA at 65℃ using oligo (dT)$_{18}$ as primer for 5 min. Then, 20 μL RT reaction system comprised of 20 uRNA enzyme inhibitor (offered by Promega corporation), 0.5 mmol/L dNTP, 10u AMV RT enzyme as well as 5xRT buffer. PCR was performed with the follow primers. The sense primer of SS-R$_1$ was 5' -CAAGACGACGCCACCGTGAGCCA-3', antisense primer was 5' -GGGGTTGGCACAGCTGTTG-3'. The sense primer of cyclophilin(cyc) was 5'-CCATCGTGTCATCATCAAGGACTTCAT-3' and antisense primer was 5'-TTGCCATCCAGCCAGGAGGTCT-3'. The 50 μL PCR reaction system comprised of 5 μL 10×PCR reaction buffer, 1.5 mmol/L $MgCL_2$, 200 umol/L dNTP, 5 μL cDNA template, 0.1 mmol/L specific primers, and 3u Taq DNA polymerase covered by paraffin oil. The PCR conditions for SS-R$_1$ and cyc cDNA were as follows: predenaturation at 94℃ for 2 min; denaturation at 94℃ for 30s, annealing at 60℃ for 30s, and an extention at 72℃ for 30s for 26 cycles; then a final extention at 72℃ for 5 min. Ten μL product of PCR was put in 1.5% sepharose to go on electrophoresis in ranks in different groups. The products of SS-R$_1$ and cyc were 66 bp and 216 bp, respectively. The products of electrophoresis were stained with ethidium bromide and photographed under ultraviolet lamp. The electrophoresis band was scanned by figure recognition analytical system. The proportion of SS-R$_1$/cyc was calculated as the relative expression level of SS-R$_1$mRNA.

Statistical analysis

All data were presented as mean ± SD and analyzed by the software of SPSS10.0. Statistically significant differences were calculated by analysis of variance (ANOVA). If the mean square deviation was regular, it was analyzed by LSD test. Otherwise it was analyzed by Dunnett T3 test.

RESULTS

Effect of electro-acupuncture on index of gastric ulcer

The highest gastric ulcer index was observed in the model group (24.88 ± 6.29, $P < 0.01$). After electro-acupuncture, the gastric ulcer index in stomach Meridian group was significantly lower than that in the other groups ($P < 0.01$),

Table 1 Index of gastric ulcer in different groups (mean ± SD)

Group	*n*	Index of gastric ulcer
Group A	8	8.50±2.98[b]
Group B	8	24.88±6.29[d]
Group C	8	10.88±3.23[b]
Group D	8	19.38±3.66[d]
Group E	8	24.13±1.64[d]

[b]$P<0.01$ *vs* group B; [d]$P<0.01$ *vs* group C.

Table 2 EGF and SS in gastric mucosa in different groups (mean ± SD)

Group	*n*	EGF(pg/mL)	SS(mIU/mL)
Group A	8	91.3±14.9[b]	1852.4±361.7[b]
Group B1	8	73.6±14.8[d]	2978.6±587.6[d]
Group C	8	92.2±6.7[b]	1800.2±488.1[b]
Group D	8	74.9±9.0[d]	2441.0±488.1[a c]
Group E	8	65.4±12.8[d]	2592.7±426.8[d]

[a]$P<0.05$, [b]$P<0.01$ *vs* group B; [c]$P<0.05$, [d]$P<0.01$ *vs* group C.

Table 3 SS-R1mRNA expression in different groups of rabbits with gastric ulcer (mean ± SD)

Group	*n*	SSR1/cyc
Group A	8	1.04±0.36[b]
Group B	8	2.56±0.25[d]
Group C	8	1.07±0.08[b]
Group D	8	1.73±0.16[b c]
Group E	8	2.39±0.39[d]

[a]$P<0.05$, [b]$P<0.01$ *vs* group B; [c]$P<0.05$, [d]$P<0.01$ *vs* group C.

indicating that electro-acupuncture at Stomach Meridian could significantly decrease the gastric ulcer index in rabbits with experimental gastric ulcer (Table 1).

Effect of electro-acupuncture on content of EGF and SS

The content of EGF in the control group was significantly higher than that in the other groups ($P<0.01$) except for the control group. However, the content of SS in the Stomach Meridian group was significantly lower than that in the other groups except for the control group ($P<0.05$), indicating that electro-acupuncture at Foot-Yangming Meridian could increase the content of EGF in ulcer rabbits and decrease the content of SS (Table 2).

Effect of electro-acupuncture on SS-R$_1$mRNA expression in gastric mucosa

The expression of SS-R$_1$mRNA in model group was obviously lower than that in control group and there was a significant difference ($P<0.01$). The expression of SS-R1mRNA in gastric mucosa after acupuncture treatment was Foot-Yangming Meridian group> Foot-Shaoyang Meridian group> Foot-Taiyang Meridian group. There was a significance difference ($P<0.05$), suggesting that acupuncture at Stomach Meridian had a distinct effect on inhibiting expression of SS-R1mRNA of experimental gastric ulcer (Table 3).

DISCUSSION

Cytoprotection is referred to the fact that some substances can protect epithelial cells of the digestive tract against injury. Studies showed that acupuncture confers quite good protective effect on gastric mucosal lesion[1,2]. The protective mechanism may be as follows. Acupuncture can increase the level of PGE_2 in gastric mucusa[3], inhibit release of GAS, influence metabolism of oxygen free radicles[9], increase gastric mucosal NO and NOS[10], regulate expression of Bcl-2 and Fas and depress gastric mucosal epithelial cell apoptosis[11]. Acupuncture at Stomach Meridian can decrease gastric mucosal ulcer index, thus protecting gastric mucosa against injury.

Epidermal growth factor is secreted by salivary gland, duodenum Brunner gland and pancreas[12], which can resist the destructive effect of gastric and pancreatic protease and chymotrypsin, inhibit secretion of gastric acid and gastric protease, promote proliferation of gastric epithelial cells and synthesis of DNA in gastric mucosa, prevent formation of ulcer[13-15]. Studies showed that epidermal growth factor can accelerate healing process[16-18]. Zhao *et al*[19] showed that electro-acupuncture at "Zusanli" can increase EGF in upper alimentary cannel and depress gastric acid secretion. Jin *et al*[20] reported that EGF remains normal in stress rats and electro-acupuncture can relieve the gastric ulcer index, suggesting that EGF plays an important role in protecting gastric mucusa from injury during acupuncture. In our study, acupuncture at Stomach Meridian could increase EGF in gastric mucosa of experimental gastric ulcer rabbits, suggesting that acupuncture at Stomach Meridian can promote the healing of gastric mucosal lesion by regulating EGF.

SS is the main inhibitory hormone in digestive tract and can inhibit release and activity of the gastrointestinal hormones as well as gastric acid secretion and participates in protecting gastric mocusa[21-23]. Somatostatin receptors exist in gastrointestinal tract and central nervous system. Among the 5 subtypes of SS-R, SS-R_1 is highly expressed in gastric mucosa and can inhibit secretion of gastrin, histamine and gastric acid and plays an important role in adjusting gastric acid secrection. SS can also inhibit upper gastrointestinal hemorrhage and epithelial hyperplasia[24-26]. There are reports on the change of SS in digestive ulcer[27-29]. Lin *et al*[30] found that stomach perfusion with alcohol can result in gastric mucosal lesion and increase SS, but after electro-acupuncture at Foot-Yangming Meridian, SS in gastric antrum and medulla is decreased. Wang *et al*[31] showed that under psychological stress, gastric mocusal lesion of rats is associated with level of SS, but after electro-acupuncture at Zusanli, the degree of gastric mucosal lesion is reduced and SS in blood plasma tends to decrease. Chen *et al*[32] showed that Codonapsis pilosula, one of the Chinese herbs, can increase SS in stomach and duodenum mucosa, thus promoting healing of alimentary tract ulcer, suggesting that the level of SS and expression

of SS-R_1mRNA are increased in rabbits with experimental gastric ulcer due to the process of compensation feedback.

Konturek *et al*[33] found that SS may play a role in inhibiting epithelial proliferation and mocusal healing in stomach. However, Pfeiffer *et al*[34] found that epithelial growth factor receptors and SS receptors can increase the healing process of experimental gastric ulcer. Our study showed that the level of SS and expression of SS-R_1mRNA in Foot-Yangming Meridian group were significantly lower than those in model group, suggesting that the effect of SS on cell proliferation is weakened during the process of ulcer healing[35].

The protecting effect of acupunture at Foot-Sanyang Meridian on gastric mocusal lesion indicates that the action of acupuncture at Foot-Yangming Meridian is the strongest, suggesting that Foot-Yangming Meridian is closely related with stomach. This result accords with traditional meridian theory and clinical practice of acupuncture and has confirmed once again the relative specificity between Foot -Yangming Meridian and stomach.

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RAPID COMMUNICATION

Cyclin D1 antisense oligodexoyneucleotides inhibits growth and enhances chemosensitivity in gastric carcinoma cells

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Abstract

AIM: To examine the effects of cyclin D1 antisense oligodexoyneucleotides (ASODN) on growth and chemosensitivity of gastric carcinoma cell lines SGC7901 and its mechanism.

METHODS: Phosphorothioate modified cyclin D1 ASODN was encapsulated by LipofectAMINE2000 (LF2000) and transfected into cells, the dose-effect curves and growth curves were observed. 5-FU, MTX, CDDP of different concentrations were given after transfecting cells with cyclin D1 ASODN for 24 h，the dose-effect responses were observed and IC50s were calculated. The mRNA expression of cyclin D1, thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR) was detected by reverse transcription-PCR (RT-PCR) at 24 h and 48 h after transfection.

RESULTS: Dose-dependent inhibitory effect was caused by cyclin D1 ASODN in SGC7901 cells. Transfecting gastric carcinoma cells with 0.2 μmol/L cyclin D1 ASODN for 24 h could inhibit growth significantly and reduce expression of cyclin D1 mRNA. Cyclin D1 ASODN could increase the chemosensitivity to 5-FU, MTX, CDDP in cells, The IC50s of different chemotherapeutic agents in ASODN plus chemotherapy groups were significantly lower than those in controls. Transfection with cyclin D1 ASODN leaded to an increase in TS and DHFR mRNA and a decrease in TP mRNA as determined by RT-PCR at 24 h, the alterations were more significant at 48 h.

CONCLUSIONS: Cyclin D1 ASODN can decrease mRNA expression of cyclin D1，inhibit growth and enhance the chemosensitivity by changing the expression of enzymes related to metabolism of chemotherapeutic agents in SGC7901 gastric carcinoma cells.

INTRODUCTION

Cyclin D1 involves in the regulation of G1 phrase in cell cycle, with three subtypes (D1, D2, D3), cyclin D1 located in 11q13, cDNA is 4.3 Kb, is identified as the proto-oncogene and overexpressed in breast, esophageal, and hepatic carcinoma. Cyclin D1 is an important cell cycle regulatory protein that is expressed in the early G1 phase. This protein, in association with the cyclin-dependent kinases CDK4 and CDK6, mediates the phosphorylation of pRb [1]. In this study, we transfected gastric carcinoma cells with cyclin D1 antisense oliogodeoxyneucletides，to observe the effects of it on growth and chemosensitivity to 5-fluorouracil (5-FU)，methotrexate (MTX) and cisplatin (CDDP), furthermore, we examined the expression of enzymes related to metabolism of chemotherapeutic agents, including TS, TP and DHFR, to find out the underlying mechanism.

MATERIALS AND METHODS

Cell culture and reagents

Human moderately differentiated gastric adenocarcinoma cell line SGC7901 was kindly gifted by the Prof. Daiming Fan (the Fourth Military Medical University, Xi'an, China). Cells were cultured in DMEM medium (GIBCO) with 10% newborn calf serum (GIBCO) at 37 ℃ in 50mL/L CO_2. Cyclin D1 antisense oliogodeoxyneucletide 5'-GGA GCT GGT GTT CCA TGG-3' (ASODN) was complementary to the translation start site of the cyclin D1 cDNA and sense oligomers 5'-CCA TGG AAC ACC AGC TCC-3' (SODN) was used as control[2], the two sequences were synthesized and phosphorothioate modified by Shanghai Shengong Biotechnology Corp. The lyophilized ODNs was diluted by sterile water and filtered, storaged in -20 ℃. Lipofectamine2000 (LF2000, Invitrogen)-ODN complexes were prepared as described in the instruction. 5-FU, MTX and CDDP were purchased from SIGMA.

Dose-effect curves

Cells were plated in 96-well plates, SODN and ASODN were mixed with LF2000 respectively, diluted to a increasing concentration (from 0.05 to 0.5 μmol/L) and added to plates, after transfection for 24 h, the media were replaced with DMEM contain serum and cells were continue cultured for 72 h. The viability was determined by MTT assay and survival rates were calculated. The optimal concentration, able to inhibit cell growth of at least 50% *vs* control, was selected for further experiments.

Growth Curves

Cells were plated in 12-well plates, treated with LF2000-SODN or ASODN complexes at concentration of 0.2 μmol/L, after transfection for 24 h, replaced with media with serum and cultured for 6 d, cell numbers were counted everyday and the growth curves were drawn.

Chemotherapy-induced cytotoxicity

Cells were plated in 96-well plates and divided into three groups: (1) chemotherapy group, (2) SODN + chemotherapy group, (3) ASODN + chemotherapy group. After transfection with 0.2 μmol/L LF2000-ASODN or LF2000-SODN complexes for 24 h, cells were incubated with medium contain chemotherapeutic agents (5-FU, MTX or CDDP) for 72 h. Cell variability was examined by MTT assay, and dose-effect curves were drawn. The IC50s were calculated by software Prism3 (Graphpad, USA).

Reverse transcriptase polymerrase chain reaction (RT-PCR) analysis

Cells were plated in 6-well plates, transfected and cultured as described, the cells were harvested at 24h and 48h after transfection. Total RNA was isolated by TRIZOL Reagent (GIBCO). Reverse transcription was carried out on 1 μg of total RNA, 20 μL reaction system contained oligo(dT) 0.1μg, 2 mmol/L dNTPs 4 μL, Rnasin 0.1μg, MMLV 200U, incubated at 42 ℃ for 1h and enzymes were inactivated at 95 ℃ for 5min. 2.5μL RT products were used for PCR reaction, the dNTPs, sense and antisense primers (100 pmol), Taq DNA polymerase were added into reaction system of 40 μL. G3PDH was used as the control, primers and PCR condition as follow(Table1)

PCR conditions After denaturation for 4 min at 94 ℃, thirty-five cycles were accomplished as follow: Cyclin D1, 94 ℃, 60 s → 65 ℃, 1.5 min→ 72 ℃, 1.5 min; TS, 94 ℃, 30 s → 55 ℃, 30 s → 72 ℃, 30 s; TP, 94 ℃, 3 0s→ 60 ℃, 30 s → 72 ℃, 30 s; DHFR, 94 ℃, 30 s → 60 ℃, 30 s → 72 ℃, 30 s; G3PDH, 94 ℃, 30 s → 54 ℃, 30 s → 72 ℃, 30 s.

RT-PCR products (cyclin D1:514 bp; TS：208 bp; TP:353 bp; DHFR:231 bp; GAPDH: 309 bp) were analyzed by 2% agarose gel electrophroesis and visualized by EB staining, densitometric scanning of the bands was carried on and relative amount of each gene mRNA expression was estimated by normalized to the G3PDH mRNA detected in the same sample.

Statistical analysis

Results were expressed as mean±SD and the Student's *t* test was used for statistical analysis (two sides). $P < 0.05$ was taken as level of significance.

Table 1 Sequences of primers (bp)

	Primers	Length of amplification
Cyclin D1	5′-GGA TGC TGG AGG TCT GCG AGG AAC-3′	514
	5′-GAG AGG AAG CGT GTG AGG CGG TAG-3′	(332--845)
TS	5′-CAC ACT TTG GGA GAT GCA CAT ATT T -3′	208
	5′-CTT TGA AAG CAC CCT AAA CAG CCA T-3′	(853--1060)
TP	5′-ACA AGG TCA GCC TGG TCC TC-3′	353
	5′-TCC GAA CTT AAC GTC CAC CAC -3′	(491--834)
DHFR	5′-TCC ATT CCT GAG AAG AAT CGA CCT T-3′	231
	5′-CAC AAA TAG TTT AAG ATG GCC TGG G-3′	(657--887)
G3PDH	5′-TCC CTC AAG ATT GTC AGC AA-3′	309
	5′-AGA TCC ACA ACG GAT ACA TT-3′	

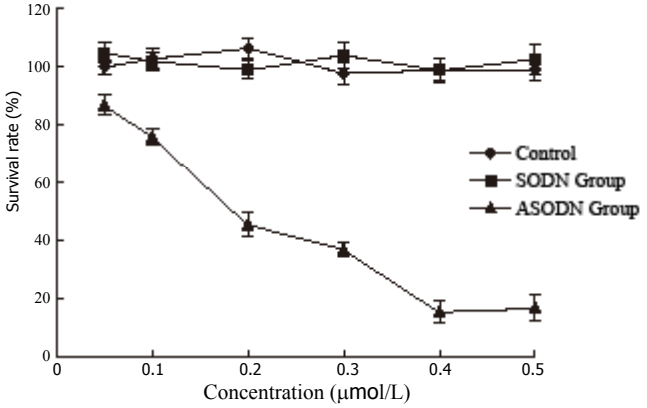

Figure 1 Dose-response curves of SGC7901 cell lines.

RESULTS

Cyclin D1 ASODN inhibits the growth of SGC7901 cells

Dose-dependent inhibitory effects were caused by cyclin D1 ASODN in SGC7901 cells， as shown in Figure 1. At concentration of 0.2 μmol/L, the inhibitory rate was 54.7% and 0.2 μmol/L was taken as optimal concentration for further experiments. Transfection with 0.2 μmol/L cyclin D1 ASODN significantly inhibited the growth of cells. IC50 in ASODN group was lower than that in the control from the third day as shown by growth curves, and there was no difference between SODN group and the control (Figure 2).

Cyclin D1 ASODN enhances the chemsensitivity of SGC7901 cells

After transfecting with cyclin D1 SODN or ASODN, cells were treated with 5-FU, MTX or CDDP in different concentrations. We found that transfection with cyclin D1 ASODN enhances the cytotoxicity of 5-FU, MTX and CDDP significantly, the IC50s in ASODN + chemotherapy groups to different chemotherapeutic agents were significantly lower than the those in chemotherapy group and SODN+ chemotherapy group. The dose-effect curves and IC50s to different treatment are shown in Figure 3 and Table 2.

Table 2 IC50 of *5-FU*, *MTX* and *CDDP* in each group ($\times 10^6$ mol/L)

	5-FU	MTX	CDDP
Chemotherapy Group	3.95 ± 0.92	1.27 ± 0.32	2.12 ± 0.22
SODN+Chemotherapy Group	4.43 ± 0.41	1.25 ± 0.10	1.88 ± 0.07
ASODN+Chemotherapy Group	0.98 ± 0.15 [ac]	0.38 ± 0.04 [ac]	0.52 ± 0.07 [ac]

[a]P<0.05 *vs* Chemotherapy Group; [c]P<0.05 *vs* SODN+ Chemotherapy Group.

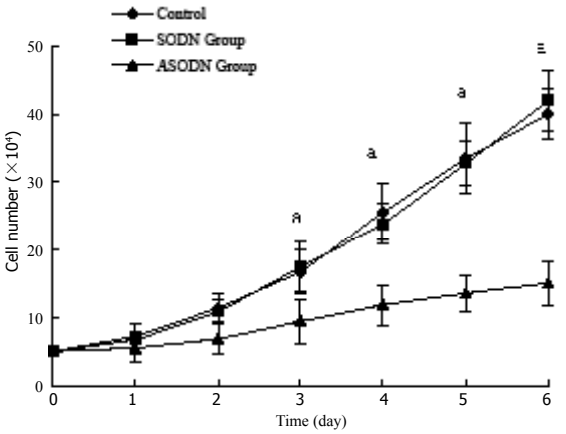

Figure 2 Growth curves of SGC7901 cell lines. [a]$P<0.05$ *vs* control.

Effect of cyclin D1 ASODN on mRNA expression of cyclin D1 and enzymes related to chemotherapy

Relative cyclin D1, TS, TP and DHFR mRNA expression is quantified by densitometric analysis, and illustrated in Figure 4. Comparing with the control, the cyclin D1 mRNA expression reduced to 26.3% and 50.0% at 24 h and 48 h, respectively. In comparison with the control, TS mRNA levels were reduced to 83.5% and 48.4% at 24 h and 48 h, respectively. TP mRNA levels were significantly increased to 124.6% and 148.3% at 24 h and 48 h, respectively. DHFR mRNA levels were reduced to 52.3% and 37.5% at 24 h and 48 h, respectively.

DISCUSSION

The use of antisense oligonucleotides as selective inhibitor of gene expression has become an important tool in current laboratory research and clinical trails. In our study ASODN were phosphorothioate modified and encapsulated by LipofectAMINE2000, to enhance the efficiency of transfection and prolong the time of action. We found dose-dependent inhibitory effects were caused by cyclin D1 ASODN in SGC7901 cells, the percentage of inhibition were 54.7% in cells treated with 0.2 μmol/L cyclin D1 ASODN for 24 h, and the growth of cells in ASODN group were also inhibited as shown by growth curves. Furthermore, cyclin D1 ASODN markedly reduced mRNA expression of cyclin D1. It suggested that cyclin D1 ASODN could inhibit the growth of gastric carcinoma cells through decreasing the cyclin D1 expression. The expression of cyclin D1 mRNA at 48h elevated a little, the lost of inhibitory effect of ASODN may be due to digestion by nucleases existed in cells.

5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), metabolic product of 5-FU, forms a tight-binding complex with TS and thereby blocks DNA synthesis process. Inhibition of TS by FdUMP is one of main mechanism underlying 5-FU action, the degree of inhibition of TS and the persistence of inhibition are essential factors for maximal in vivo growth inhibition by 5-FU[3]. TP is an enzyme that involved in pyrimidine nucleoside metabolism, and able to catalyze the conversion of 5-FU to 5-fluoro-2'-deoxyuridine (5-FdUR), which is the first step in one pathway for the metabolic activation of 5-FU. The 5-FdUR can

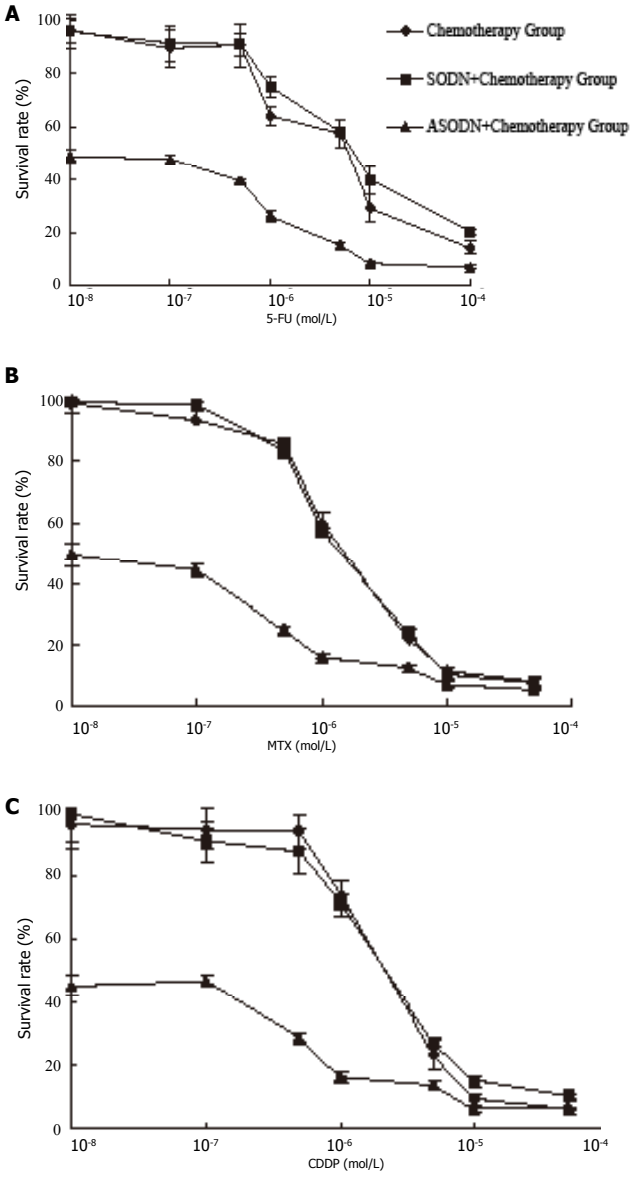

Figure 3 Effect of Cyclin D1 ASODN on chemosensitivity in SGC7901 cells. **A**: 5-FU; **B**: MTX; **C**: CDDP.

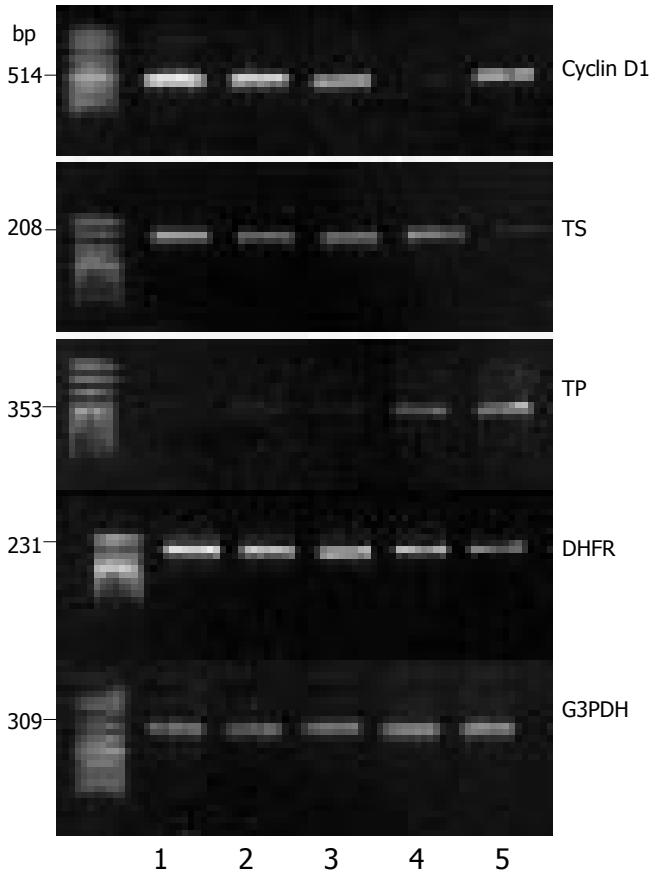

Figure 4 Effect of Cyclin D1 ASODN on mRNA expression of Cyclin D1, TS, TP and DHFR. 1, the Control; 2, SODN group at 24 h; 3, SODN group at 48 h; 4, ASODN group at 24 h; 5, ASODN group at 48 h.

be activated to 5-FdUMP, which blocks TS activity, and exerts antiproliferative effect.[4] The major target for MTX is the enzyme DHFR, which is important in biosynthesis of RNA and DNA. MTX has a chemical structure similar to folic acid, and can competitively inhibit DHFR activity. At 48 h after cyclin D1 ASODN transfection, the mRNA expression of TS, TP, DHFR in SGC7901 cells changed significantly in accompany with enhancement of chemosensitivity as shown in our study.

E2F may play an important role in this alteration. E2F is a cellular transcription factor, which can induce a number of genes important in the passage of cell cycle through the G1/S phase transition as well as in the initiation of DNA synthesis. When E2F binds the promoter and pRb simultaneously, the pRb-E2F complex acts as a transcriptional suppressor and completely silences the transcription of target genes. Interaction with E2F is the means through which pRb exerts its antiproliferative effect[5]. It would be expected that decreased expression of cyclin D1 by ASODN might affect pRb phosphorylation and might lead to change of level of E2F protein as a consequence of alteration in their interactions with pRb, thus change the expression of TS, TP and DHFR and enhance the chemosensitivity to 5-FU and MTX.

Warenius *et al* [6] firstly found that high cyclin D1 expression is related to CDDP resistance in the 16 human cell lines. Furthermore, CDDP resulted in significantly higher rates of cell killing in the antisense cyclin D1 transfected laryngeal squamous cell carcinoma cell lines CCL23 than parental cells, and ID50 decreased, which suggest the decreased expression of cyclin D1 may enhance the DNA-damaging effects of CDDP [7]. It is possible that the blockade of autocrine mitogenic signals by inhibiting cyclin D1 expression might also be a mechanism for increase in CDDP chemosensitivity[8]. As shown in our study, cyclin D1 ASODN can increase the cheosensitivity to CDDP in SGC7901 cells, but the exact mechanism needs further research.

Cyclin D1 ASOND could inhibit the growth of gastric carcinoma cells and change the expression of enzymes related to metabolism of chemotherapeutic agents through the influence on expression of cell cycle regulators, and enhance the chemosensitivity to 5-FU, MTX and CDDP, indicating that ASODN technology is a feasible way to enhance the efficiency of chemotherapeutic agents and provide a new strategy for combination therapy of gastric cancer.

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RAPID COMMUNICATION

Effect of drug treatment on hyperplastic gastric polyps infected with *Helicobacter pylori*: A randomized, controlled trial

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Abstract

AIM: To study the effects of drug treatment on hyperplastic gastric polyps infected with *Helicobacter pylori* (*H pylori*).

METHODS: Forty-eight patients with hyperplastic gastric polyps (3-10 mm in diameter) infected with *H pylori* were randomly assigned to a treatment group (n = 24) which received proton-pump inhibitor (omeprazole or lansoprazole), clarithromycin, bismuth citrate and tinidazole, and a control group (n = 24) which received protective agent of gastric mucosa（tepretone）. Patients underwent endoscopy and *H pylori* examination regularly before enrollment and 1-12 mo after treatment.

RESULTS: Twenty-two patients in the treatment group and 21 in the control group completed the entire test protocol. In the treatment group, polyps disappeared 1-12 mo (average, 6.5 ± 1.1 mo) after the treatment in 15 of 22 patients (68.2%) and *H pylori* infection was eradicated in 19 of the 22 patients (86.4%). However, 12 months after the study, no change in polyps or *H pylori* status was seen in any controls ($^bP < 0.01$).

CONCLUSION: Most hyperplastic gastric polyps disappear after eradication of *H pylori*.

Key words: *Helicobacter pylori*; Hyperplastic gastric polyps; Therapy

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INTRODUCTION

The risk of conversion of hyperplastic gastric polyps is very low, only accounting for 1.5%-3% [1]. Patients with gastric polyps may present with bleeding of the upper gastrointestinal tract, abdominal pain, or gastric outlet obstruction. Large gastric polyps or polyps associated with complications can be removed endoscopically or surgically. *Helicobacter pylori* (*H pylori*) infection is closely associated with hyperplastic gastric polyps and *H pylori* is present in 100% of hyperplastic gastric polyps[2-4]. Hyperplastic gastric polyps may disappear in 40-71% patients after eradication of *H pylori*[5, 6]. Since the pathogenicity of *H pylori* is different in various regions, we conducted this randomized, controlled trial to see whether hyperplastic gastric polyps disappear after eradication of *H pylori*.

MATERIALS AND METHODS

Selecting and grouping of patients

Forty-eight patients were from the Gastroenterology Department, First Affiliated Hospital of Zhejiang University and Red Cross Hospital of Hangzhou (26 men, 22 women, age range from 21 to 73 years, average 47 years). All patients did not receive antibiotics, bismuth, steroid and non-steroid drugs before their enrollment in the study. Hyperplastic gastric polyps (3-10 mm in diameter) were diagnosed on the basis of the examination results at least three histological samples. Patients were considered to have multiple gastric polyps (at least 5) when they were easily cut. Our criteria for hyperplastic gastric polyps included hyperplasia of the foveolar epithelium on histologic examination and infiltration of inflammatory cells into the stroma in biopsy specimens [7, 8]. Hyperplastic gastric polyps were diagnosed by two blinded pathologists. Patients with adenomatous gastric polyps, Peutz-Jegher syndrome and juvenile polyps were excluded. The diagnosis of *H pylori* infection was based the positive results of staining with Giemsa and ^{14}C-urea breath test. The patients were randomly assigned to two groups and sequentially numbered. In the treatment group (n = 24), patients received proton-pump inhibitor (omeprazole 20mg/d or lansoprazole 30 mg/d), clarithromycin (1g/d), bismuth citrate (440 mg/d) and tinidazole (1g/d), 2 weeks a course. In the control group (n = 24), patients had endoscopic examination and received protective agent of gastric mucosa (tepretone 150 mg/d). Two patients of the treatment group did not take the drugs for one course. In the control group, 2 patients lost follow-up and 1 patient took both proton-pump in-

hibitor and amoxicillin. These 5 patients exited from our study. If polyps progressed and were accompanied with malignant transformation, the study was stopped and the polyps were removed endoscopically. After completion of our study, endoscopic removal of polyps or eradication of *H pylori* was proposed for those who failed in *H pylori* eradication.

Endoscopy, histologic examination and assessment of H pylori eradication

Patients in the treatment group underwent endoscopy every 3 months after the treatment. On each occasion, biopsy specimens were taken from the same areas (three from the antrum and three from the body) for histologic examination. If patients without eradication of *H pylori* were not adapted to endoscopic examination very well, controls underwent endoscopy every 3 months after enrollment. Biopsy specimens for histologic examination were stained with Giemsa and evaluated for the presence of *H pylori*. Histologic diagnosis of the biopsied mucosa of the antrum and body was made by two blinded pathologists. The severity of activity, inflammation, atrophy, and metaplasia was graded on a scale from 1 to 4 and expressed as the histologic index according to the updated Sydney System: 1: normal, 2: mild, 3: moderate, and 4: marked [9]. Eradication of *H pylori* was confirmed by the negative results of these two tests 1-3 months after the treatment and endoscopic examination. The size and number of polyps were measured at each endoscopic examination using biopsy forceps (GIF XQ240 or GIF140, Olympus) placed near the polyp (open size: 6 mm in diameter; closed size: 2 mm in diameter). The endoscopic data on the disappearance and regression of polyps were reviewed independently by two blinded endoscopists.

Statistical analysis

All data were analyzed by unpaired *t* test (for age), Wilcoxon rank-sum test and Fisher's exact test. $P < 0.05$ was considered statistically significant. All data were coordinated by SPSS RDS and statistical analyses were done by SPSS software.

RESULTS

Comparison of baseline clinico-pathological characteristics between treatment and control groups

Twenty-two patients in the treatment group and 21 patients in the control group completed the entire study. The two groups were similar with respect to the number, age, sex, coexisting disease, as well as the number, size and distribution of polyps, histologic findings (Table 1). The two groups were comparable.

Analysis of curative effects between treatment and control groups

In the treatment group, *H pylori* was eradicated without serious side effects in 19 of 22 patients (86.3% [95% CI, 63%-99%]), and polyps disappeared in 15 of 22 patients (68.2% [95% CI, 54%-91%]) 1-12 months after treatment (Figure 1). Hyperplastic gastric polyps in the other 4 patients with successful *H pylori* eradication regressed to a certain extent, decreasing in size or number. However, in the remaining 3 patients without *H pylori* eradication, no polyps showed regression and no diminution of inflammation in the gastric mucosa 12 months after the treatment. In the control group, all patients showed no change of *H pylori* infection (0% [95% CI, 0%-21%]), no hyperplastic gastric polyp regression or disappearance (0% [95% CI, 0%-21%]) and no significant diminution of inflammation (Table 2). Polyps were enlarged or increased in number in 5 of the 21 patients. The rates of eradication of *H pylori* and disappearance of polyps in the treatment group were significantly higher than those in the control group ($P < 0.01$).

Table 1 Clinico-pathological characteristics of treatment and control groups

Characteristics	Treatment groups (n = 22)	Control groups (n = 21)
Mean age(mean±SD，yr)	49 ± 9	47 ± 8
Men, n(%)	13(59.1)	11(52.4)
Coexisting disease, n(%)		
Chronic atrophic gastritis	10(45.4)	12(57.1)
Duodenal ulcer	2(9.1)	2(9.5)
Gastric ulcer	0(0)	1(4.8)
Mean number of polyps	5.6	4.2
Mean size of polyps(mm)	6.6	7.8
Distribution of polyps, n (%)		
Body	79(64.2)	61(69.3)
Antrum	19(15.4)	15(17.0)
Angle	8(6.5)	5(5.7)
Fundus	9(7.3)	4(4.5)
Cardia	8(6.5)	3(3.4)
Histologic findings		
Inflammation	2.7, 2.4	2.6, 2.4
Activity	2.5, 2.5	2.5, 2.3
Atrophy	2.4, 2.6	2.5, 2.4
Metaplasia	0.9, 0.6	1.0, 0.6

DISCUSSION

In our study, *H pylori* was successfully eradicated in 19 of the 22 patients. The regression or disappearance of hyperplastic gastric polyps was seen in the 19 patients. The polyps disappeared in 15 of the 19 patients 1-12 months (average, 6.5 ± 1.1 mo) after the treatment. However, none of the polyps in any of the controls or in patients without *H pylori* eradication showed regression. These results strongly suggest that eradication of *H pylori* leads to regression and disappearance of hyperplastic gastric polyps. *H pylori* is the main cause of chronic active gastritis and can produce multi-virulence agents, damage the gastric mucosa, stimulate gastric body to release inflammation medium, activate various cytokines and promote inflammation reaction. *H pylori* infection damages the gastric mucosa and glandular cells, stimulates crypt epithelia and muscularis mucosa hyperplasia with eminent mucus. If the damage factors continue their existence, the pathological changes can progress to intestinal metaplasia or atypical hyperplasia and even

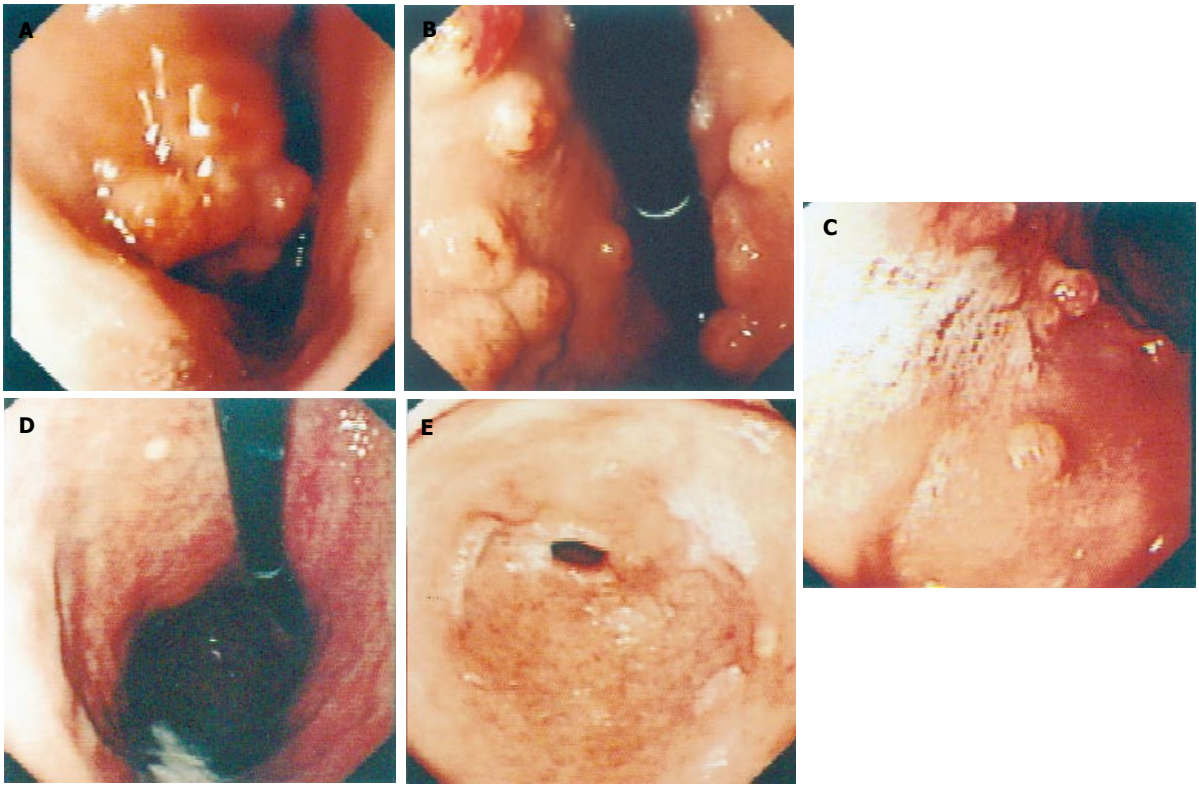

Figure 1 Effect of drug treatment on hyperplastic gastric polyps infected with *H pyori*. **A** and **B**: Multiple gastric polyps of the antrum and body; **C**: Decreased size of gastric polyps 7 mo after treatment; **D** and **E**: Disappearance of gastric polyps 11 mo after treatment.

Table 2 Inflammation status in treatment and control groups

Variable	Treatment groups (n = 22)	Control groups (n = 21)	P
Histologic findings			
Inflammation	1.6, 1.5	2.4, 2.3	<0.01
Activity	1.1, 1.3	2.4, 2.5	<0.01
Atrophy	2.2, 2.1	2.4, 2.4	>0.05
Metaplasia	0.8, 0.6	0.9, 0.7	>0.05

carcinoma. Yasunaga *et al* [10] reported that increased production of interleukin-1 beta and hepatocyte growth factor due to *H pylori* infection may contribute to thickening of the stomach by stimulating epithelial cell proliferation and foveolar hyperplasia in patients with enlarged fold gastritis, leading to formation of hyperplastic polyps.

Although hyperplastic gastric polyps did not accompany malignant transformation during the 9-12 month follow-up period in our study, *H pylori* infection is closely associated with gastric carcinoma. We therefore recommend that when hyperplastic gastric polyps are detected during endoscopy, serologic and pathologic tests should be done to detect and eradicatw *H pylori*.

Though the risk of hyperplastic gastric polyps conversing into cancer is very low, large hyperplastic gastric polyps should be snared and removed completely. Because of the malignant potential, all gastric polyps (0.5 cm or larger in diameter) should be removed. In addition, about 1 year after eradication of *H pylori* (4 patients in this study), any remaining hyperplastic polyps should be removed endoscopically because of the potential for development of cancer.

In conclusion, *H pylori* infection is related with hyperplastic gastric polyps and inflammatory cell infiltration, eradication of *H pylori* can prevent formation of hyperplastic gastric polyps.

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RAPID COMMUNICATION

Melanoma differentiation-associated gene-7, MDA-7/IL-24, selectively induces growth suppression, apoptosis in human hepatocellular carcinoma cell line HepG2 by replication-incompetent adenovirus vector

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Abstract

AIM: To investigate the effect of replication-incompetent adenovirus vector expressing MDA-7/IL-24 on tumor growth and apoptosis in human hepatocellular carcinoma (HCC) cell line HepG2 and normal liver cell line L02.

METHODS: We constructed the recombinant replication-incompetent Ad.mda-7 virus vector and infected it into the human HCC cell line HepG2 and normal liver cell line L02. RT-PCR was performed to detect the mRNA expressing in cells. by ELISA was used to detect MDA-7/IL-24 protein expression in the culture supernatant. The effect of apoptosis induced by Ad.mda-7 was confirmed by Hoechst staining and flow cytometry assay with Annexin-V and PI staining. MTT assay was used to determine growth inhibition of HepG2 cells, and cell-cycle and hypodiploidy analyses were performed by flow cytometry.

RESULTS: Recombinant replication-defective virus expressing MDA-7/IL-24 was constructed successfully. RT-PCR showed that the Ad.mda-7 could mediate the expression of the exogenous gene MDA-7/IL-24 into HepG2 and L02. The concentration of MDA-7/IL-24 protein in supernatant was 130 pg/mL and 110 pg/mL in Ad.mda-7-infected L02 and HepG2 cells, respectively. Ad.mda-7 infection obviously induced apoptosis (from 2.60±0.72% to 33.6±13.2%, $P = 0.00012$) and growth suppression in HepG2 (inhibition ratio IR = 68%) and an increase in the percentage of specific cancer cell types at the G2/M phase of the cell cycle (from 6.44% to 32.29%, $P < 0.01$), but not in L02 cells.

CONCLUSION: These results confirm selectively induction of apoptosis and growth suppression by the mda-7/IL-24 gene with replication-incompetent adenovirus vector in human hepatocellular carcinoma cell line HepG2.

Key words: Cancer gene therapy; Hepatocellular carcinoma (HCC); Apoptosis; Growth suppression; MDA-7/IL-24

Wang CJ, Xue XB, Yi JL, Chen K, Zheng JW, Wang J, Zeng JP, Xu RH. Melanoma differentiation-associated gene-7, MDA-7/IL-24, selectively induces growth suppression, apoptosis in human hepatocellular carcinoma cell line HepG2 by replication-incompetent adenovirus vector. *World J Gastroenterol* 2006; 12(11): 1774-1779

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common lethal malignant tumors in the world[1], causing an estimated 1 250 000 deaths every year worldwide. Unfortunately, about 50% new cases are from China. The clinical therapies for HCC include surgical resection and liver transplantation, but only few HCC patients can receive these treatments. Moreover, the recurrent rate is very high even the patients received surgical treatments. Besides clinical therapies, the gene therapies for HCC are running, including transgenic therapy by antioncogenes such as p53 and Rb, anti-sense nucleotide technique, drug gene therapy such as suicide gene-like HSV-TK, tumor vaccine, and so on. However, the clinical effect of gene therapies was limited because these genes were not specific to tumor cells, which means they kill the normal cells and the tumor cells simultaneously. The research on the treatment protocol selectively killing tumor cells but not influencing normal cells has become a hot topic of research on tumor treatment[2]. Melanoma differentiation-associated gene-7 (MDA-7)/IL-24 was identified by a combination of recombinant fibroblast interferon (IFN-b) and the protein kinase C activator mezerein (MEZ) subtraction hybridization by Fisher in 1995[3], according

to the chromosomal location on 1q32, the presence of a secretory signal, its association with specific cells of the immune system, and the ability of MDA-7 protein to act as an immune modulator. Now mda-7 has been renamed IL-24[4-7].

Some studies indicated that over-expression of mda-7/IL-24 by a replication-defective adenovirus vector results in growth suppression and apoptosis in a broad range of different carcinoma cell lines, including mesotheliomas[8], osteosarcoma[8], melanoma[2,3,9,10], and carcinomas of the lung[11,12], breast[13], pancreas[14], glioblastoma[15,16] and prostate[17]. The anti-tumor effects were independent of the genomic status of p53, RB, p16[2]. Although MDA-7/IL-24 has the properties potentiality kill many different types of cancer cells, it has not any harmful effects in many kinds of normal cells. These unique potentiality of mda-7/IL-24 suggested that this gene could prove beneficial for cancer gene therapy[2]. In the present study, we investigated the impact of Ad.mda-7 on growth, cell cycle and survival of human HCC cell line HepG2 and normal liver cell line L02, resulting in selectively induction of apoptosis and growth suppression by the mda-7/IL-24 gene with replication-incompetent adenovirus vector. In these contexts, the study provides important support to the use of Ad.mda-7 for selective cancer gene therapy for HCC.

MATERIALS AND METHODS

Cell lines, transfection and culture conditions

Human HCC cell line HepG2 and normal human liver cells line L02 (gift from Dr Guanjian) were cultured in high glucose DMEM supplemented with 100 mL/L fetal bovine serum (FBS) at 37℃ in a humidified incubator containing 50 mL/L CO_2 95% in air.

Virus construction, identification and purification

The recombinant replication-defective Ad.mda-7 virus was created in our laboratory. Briefly, human MDA-7/IL-24 cDNA was directionally cloned into pSGCV to produce pSGCMV-MDA7. By using plasmid transfection method, the pSGCMV-MDA7 and adenovirus skeletal plasmid were co-transfected to HEK293 cells to construct the recombinant adenovirus vector Ad.mda-7, carrying MDA-7/IL-24 gene, by intracellular homologous recombinant. The genomes were analyzed to confirm the recombinant structure and then the virus was plaque purified and amplificated in 293 cells.

RNA isolation and RT-PCR

After cells infected with 1 000 VP/cell (virus particle/cell) of Ad.vec and Ad.mda-7, respectively, were harvested at 48 h, total RNA was extracted from cells using the Qiagen RNeasy mini kit (USA) according to the manufacturer's protocol. Primers used in PCR were designed according to the reported IL-24 cDNA sequence. The primer sequences were 5'-GGGCTGTGAAAGACACTAT-3' (forward) and 5'-GCATCCAGGTCAGAAGAA-3' (reverse). The primer sequences of β-actin were 5'-CCTTCCTGGGCAATGGAGTCCT-3' (forward) and 5'-GGAACAATGATCTTGATCTT-3' (reverse). The reaction mixture with corresponding primers was amplified through 30 cycles, each cycle consisting of denaturation at 94℃ for 30 s, primer annealing at 55℃ for 30 s, and extension at 72℃ for 30 s. Cycles were preceded by incubation at 95℃ for 3 min to ensure the full denaturation of the target gene and also an extra incubation at 72℃ for 5 min to ensure full extension of the product. The products of PCR were analyzed on 10 g/L agarose gel electrophoresis.

ELISA assay

The cell culture supernatant was collected and stored at -20℃ until use. A total of 100 μL of the supernatant was added to Microplate wells, and 200 μL of anti IL-24 (Biotin USA) was added 10 s later. After incubation at 37℃ for 30 min, the sample was washed 5 times with wash buffer, followed by addition of 200 μL of HRP to each well following an incubation at 37℃ for 30 min. Then the wells were washed 5 times, followed by addition of 100 μL of TMB and incubation in room temperature with protection from light. Twenty minutes later, 100 μL of stop solution was added, and the absorbance was read on a microplate reader at 450 nm. All experiments were performed in duplicate. Finally, we calculated the value of results with standard curve.

MTT assay to determine cell growth

Cells were seeded in 96-well tissue culture plates ($1x10^3$ cells/well) and treated with PBS, 1 000 VP/cell of Ad.mda-7 and Ad.vec (1 000 VP/cell), respectively, at the next day. At the indicated time points, the medium was removed, and fresh medium containing 0.5 mg/mL MTT (Roche Diagnostics GmbH Co., Germany) was added to each well. The cells were incubated at 37℃ for 4 h, followed by addition of about 150 μL of solubilization solution (0.01 mol/L HCl in 100 g/L SDS) to each well, and incubation of cells for a further 10 min at 37℃ with gentle shaking. The optical density of the plates was read on a microplate reader at 540 nm.

Hoechst staining test (fluorescent microscopy evaluation of cell apoptosis)

After 48 h of infection, cells were washed once with PBS and fixed in 40 g/L paraformaldehyde for 30 min at room temperature. After two washes with PBS, cells were stained for 30 min in the dark at room temperature with 0.05 mg/mL Hoechst 33258 (Sigma USA) in PBS. Nuclear fragmentation was visualized using a fluorescence microscope equipped with a UV-2A filter and Olympus BX60 photographic camera. Apoptotic cells were identified by condensation of nuclear chromatin and its fragmentation.

Apoptosis and necrosis assay (Annexin-V-PI assay)

Cells were trypsinized and washed once with complete media. Aliquots of cells (5×10^5) were resuspended in complete media (0.5 mL) and stained with FITC-labeled Annexin-V (Jinmei Co., China) according to the manufacturer's instructions. Propidium iodide (PI) was added to the samples after staining with Annexin-V

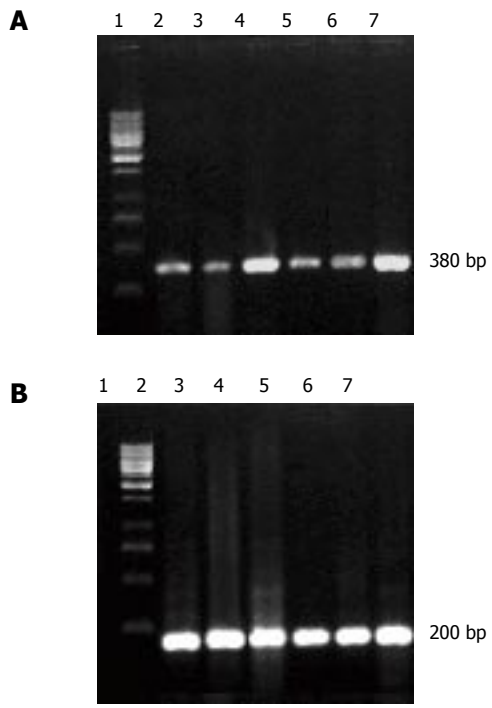

Figure 1 Expression of mda-7/IL-24 mRNA. Infection of normal human liver cells and HCC with Ad.mda-7 resulted in an expression of mda-7/IL-24 mRNA. Cells infected with 1 000 VP/cell of Ad.vec or Ad.mda-7 were harvested at 48 h, treated as described in "Materials and Methods". The total RNA was extracted and the RT-PCR was performed. (**A**) Lane 1: marker; lane 2: control L02 cells; lane 3: Ad.vec-infected L02 cells; lane 4: Ad.mda-7-infected L02 cells; lane 5: control HepG2 cells; lane 6: Ad.vec-infected HepG2 cells; lane 7: Ad.mda-7-infected HepG2 cells. (**B**) Expression of β-actin.

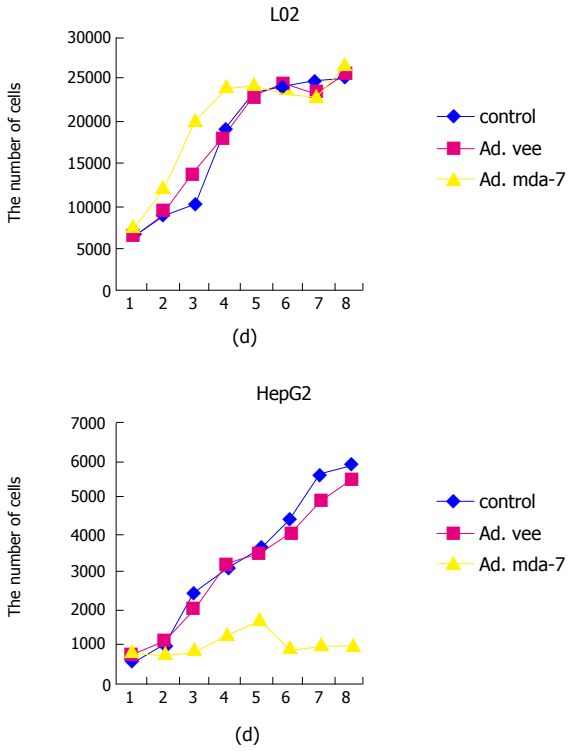

Figure 2 Ad.mda-7 expression causing in vitro inhibition of growth of HCC cells, but not normal liver cells. The various cell types were uninfected (control) or infected with 1 000 VP/cell of Ad.vec or Ad.mda-7. Cell numbers were determined over an 8-d period. Cells were seeded in 96-well plates and treated the next day as described in 'Materials and Methods'. After 24 h, the medium was removed, and cells were stained with MTT. Experiments were repeated three times in quadruplicates, and the results were presented as mean±SE.

to distinguish late apoptotic and necrotic cells. Flow cytometry (Becton Dickinson, San Jose, CA, USA) was performed immediately after staining.

Cell-cycle and hypodiploidy analyses

Cells were cultured as aforementioned. After reaching 30% confluence, the cells were treated with DMEM without FCS for 24 h for synchronization. At the next day, cells were treated with PBS, Ad.vec and Ad.mda-7, respectively. Forty-eight hours later, cells were trypsinized, washed with PBS and fixed in 700 mL/L ethanol overnight at -20℃. Cells were then washed with PBS, and aliquots of $1x10^6$ cells were resuspended in 1 mL of PBS containing 1 mg/mL of RNase A and 0.5 mg/mL of PI. After 30 min of incubation, cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

All the experiments were performed at least three times. The results were expressed as mean ± SE. Statistical comparisons were made using an unpaired two-tailed Student's t test. A $P<0.05$ was considered statistically significant.

RESULTS

mRNA expression of MDA-7/IL-24 gene

To determine the efficiency of transgenic expression, HepG2 and L02 cells were infected with Ad.vec and Ad.mda-7, and mRNA expression was detected using RT-PCR (Figure 1). The results suggested that the expression of MDA-7/IL-24 mRNA could be detected in both HepG2 and L02 cell lines infected by Ad.mda-7 but not by Ad.vec or control team. MDA-7/IL-24 gene could be expressed in cells with infected with Ad.mda-7.

Protein expression of MDA-7/IL-24

Secreting MDA-7/IL-24 protein was confirmed by ELISA assay after Ad.mda-7 infection. After 48 h of L02 and HepG2 cells infection with Ad.mda-7, the concentration of MDA-7/IL-24 protein in the supernatant was detected 130 pg/mL and 110 pg/mL, respectively, whereas no MDA-7/IL-24 protein expression was detected in L02 and HepG2 cells treated with PBS and Ad.vec.

Growth suppression and viability assays

HCC cell line HepG2 and normal liver cell line L02 were infected with Ad.mda-7 and proliferation and cell viability were determined by MTT. As shown in Figure 2, no proliferation arrest effect was observed on normal liver cell line L02 of Ad.vec, Ad.mda-7 or control groups. However, the anti-proliferative activity of Ad.mda-7 was readily apparent in HCC cell line HepG2, and the inhibition ratio was 68%.

Apoptotic effect determined by Hoechst staining

We observed that Ad.mda-7 infection induced apoptosis of human HCC cell line HepG2. As shown in Figure 3, a

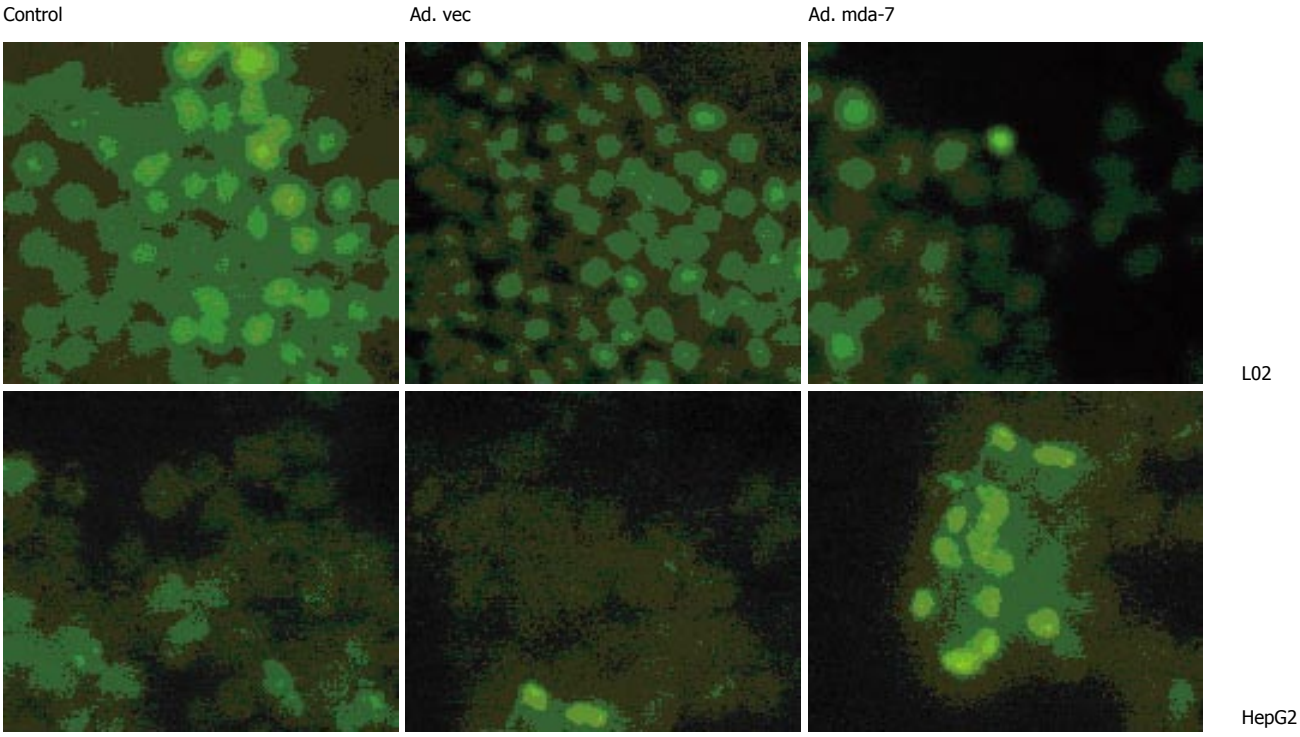

Figure 3 Over-expression of mda-7 causes apoptosis selectively in HCC cells (400×). Cells were infected with 1 000 VP/cell of Ad.mda-7 and all assays were performed 48 h after infection as described in 'Materials and Methods'. To evaluate characteristic apoptotic morphology with fluorescence microscopy, the cells were seeded on glass slides and fixed with 40 g/L paraformaldehyde in PBS for 1 h at room temperature. After washing twice with PBS, cells were stained for 30 min in the dark at room temperature with 0.05 mg/mL Hoechst 33258 in PBS. The nuclear fragmentations were visualized by using a fluorescence microscope equipped with a UV-2A filter and Olympus BX60 photographic camera. Apoptotic cells were recognized by condensation of nuclear chromatin and its fragmentation.

Table 1 Over-expression of mda-7 induces apoptosis selectively in HCC cells

	L02			HepG2		
	Control	Ad.vec	Ad.mda-7	Control	Ad.vec	Ad.mda-7
Early apoptotic cells	110±23	120±36	100±11	50±37[b]	300±85[d]	1 990±430
Late apoptotic cells	60±14	100±21	130±15	10±40[b]	200±63[d]	1 370±902
Total cells	170±28	220±45	230±16	60±72[b]	500±150[d]	3 360±132

The table shows the apoptotic cells in 10 000 cells. The percentage of early apoptotic cells (stained with Annexin-V only) and late apoptotic and necrotic cells (stained with PI) was calculated using the CellQuest software (Becton Dickinson, San Jose, CA, USA). [b]$P<0.01$ *vs* Ad.mda-7 group; [d]$P<0.01$ *vs* Ad.mda-7 group.

significantly higher apoptotic rate was observed in HepG2 cells infected with Ad.mda-7 (48%) compared to those infected with Ad.vec (1.6%) and the control group (1.1%) (counted 1 000 cells consecutively) ($P<0.005$), whereas no apparent changes were observed in normal liver cell L02, the rate of apoptosis being 1.7%, 1.9% and 2.1%, respectively ($P>0.05$). These data indicated that MDA-7/IL-24 could induce apoptosis in HepG2.

Evaluation of apoptosis effect by flow cytometry

Annexin-V and PI staining assays with flow cytometry quantified the effect of Ad.mda-7 on apoptosis induction in human HCC cell HepG2 and normal liver cell L02 (Table 1). We observed a significantly increased percentage of apoptotic HCC cells HepG2 infected with Ad.mda-7 as compared to HepG2 infected with Ad.vec and the control cells. In contrast, L02 cells did not show significantly increased apoptotic rate after being infected with Ad.mda-7. Thus, these results suggested that Ad.mda-7 infection could kill HCC cells but not normal liver cells.

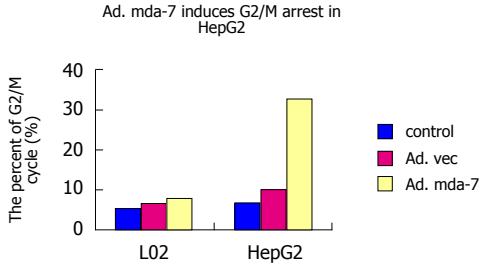

Figure 4 Induction of G2/M arrest in HCC cells HepG2, but not in normal liver cells L02 by Ad.mda-7 infection.

Cell cycle analysis by flow cytometry

Cell cycle phase assayed by flow cytometry after the fixed cells were stained with PI. As shown in Figure 4, Ad.mda-7 markedly induced a G_2/M accumulation in HepG2 cell lines, showing that the rates of G_2/M phase cells were 32.29% in Ad.mda-7 group, 10.30% in Ad.vec and 6.44% in control groups ($P<0.01$). However, the

rates of G_2/M phase cells were 7.95%, 6.65% and 5.54% in L02 cells of Ad.mda-7, Ad.vec and control groups, respectively ($P > 0.05$), demonstrating only minimal G_2/M accumulation in normal liver cells L02. Thus, these results suggested that Ad.mda-7 infection could significantly induce an increase in the percentage of HCC cell line HepG2 in the G2/M phase of the cell cycle, but not in normal liver cell line L02.

DISCUSSION

A study by Jiang *et al*[3] demonstrated that melanoma differentiation-associated gene-7 (MDA-7/IL-24) was highly expressed in melanocytes, but low expressed in melanoma cells, and because of the inducing capacity of growth arrest and differentiation in human melanoma cell line, named MDA-7. After the MDA-7/IL-24 was transfected into malignant melanoma cells, it could obviously block the growth, and promote apoptosis and differentiation, even reverse the malignancy of the melanoma[3]. Now it is clear that the MDA-7/IL-24 gene has been localized to chromosome 1q32 and its exon/intron structure has been established. Mda-7 is composed of seven exons with the first exon being noncoding. The chromosomal assignment and gene structure have been independently confirmed[4-6]. The region includes IL-10, IL-19, IL-20 and mda-7[4]. Moreover, based on structure of protein and the receptor, the gene has been reclassified and grouped into a newly recognized family of IL-10-related interleukins, IL-24[7]. Several independent studies have demonstrated that over-expression of the MDA-7/IL-24 gene, using vectors either plasmid or a replication-defective adenovirus, resulted in growth arrest and induction of apoptosis in a broad range of cancer cells, including lung cancer[11,12], breast cancer[13], pancreatic cancer[14], glioma[15,16], and prostate cancer[17]. Some signal transduction pathway and molecules have been reported as being regulated during mda-7-induced tumor suppression, including activation of the caspase cascade, PKR, p38, STAT3, PI3K, GSK-3, ILK-1, BAX, BAK, Fas, DR4, TRAIL, inducible nitric oxide synthase (iNOS), IRF-1, IRF-2 and p53[8]. Treatment of tumor cells with Ad-mda7 resulted in an increase in cells in the G2/M cell cycle phase[2]. In comparison to the antioncogenes, such as p53, the inhibitory effect of MDA-7/IL-24 on the growth of cancer cells was not related with the state of antioncogenes in these cancer cells (p53, Rb, or p16ink4)[2,11,14], so it could be used in cancer treatment more effectively without the influence in the expression of these anticancer genes in various kinds of cell lines. Surprisingly, the previous studies revealed that MDA-7/IL-24 gene had no any toxic and side effects on the normal cells, such as epidermal cells, lung fibroblasts, breast cells, prostate and lung epithelia, astrocytes, endothelia, melanocytes and so on[2,3], suggesting its selective property to malignant tumors. Therefore, MDA-7/IL-24 is considered a unique cytokine –tumor suppressor in the IL-10 family and there is no any toxic effect in normal cells, suggesting it as a perfect gene for use as a human cancer gene therapy.

In this study, the replication-incompetent adenovirus vector carrying MDA-7/IL-24 was successfully constructed and transfected into human normal liver cell line L02 and HCC cell line HepG2. Its effects on the two kinds of cells were observed, which provided the theoretical foundation for its application in the gene therapy for HCC in clinical practice. RT-PCR indicated MDA-7/IL-24 was successfully transfected into L02 and HepG2 cells with Ad.mda-7, but the control and Ad.vec groups could not show the mRNA expression. The protein expression was confirmed by ELISA assay and the effect was very different, although protein expression was seen both in L02 and HepG2. MTT assay revealed the capability of Ad.mda-7 in tumor growth arrest of HCC cell line HepG2, but not of normal liver cell line L02, indicating that MDA-7/IL-24 induces growth arrest only in HCC cells. Like the pervious studies, Ad.mda-7 induced a G_2/M accumulation in HCC cell line HepG2, but not in normal liver cell line L02. Moreover, infection with Ad.mda-7 could increase the percentage of apoptotic cells apparently in HCC cells. On contrary, no increased percentage of apoptotic cells appeared in L02 cells.

In conclusion, Ad.mda-7 can induce the gene MDA-7/IL-24 expression in normal liver cells and hepatocellular carcinoma cells. Over-expression of MDA-7/IL-24 obviously induces the apoptosis and growth suppression in hepatocellular carcinoma cell line HepG2, without any toxic effect on normal liver cell line L02. These findings provide support for future clinical applications of MDA-7/IL-24 in the gene therapy of hepatocellular carcinoma.

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CASE REPORT

Tubulovillous adenoma of anal canal: A case report

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Abstract

Tumors arising from the anal canal are usually of epithelial origin and are mostly squamous cell carcinoma or basal cell carcinoma. We present a case of benign anal adenomas arising from the anus, an extremely rare diagnosis. A 78-year-old white man presented with rectal bleeding of several months duration. Examination revealed a 4 cm friable mass attached to the anus by a stalk. At surgery, the mass was grasped with a Babcock forceps and was resected using electrocautery. Microscopic examination revealed a tubulovillus adenoma with no areas of high grade dysplasia or malignant transformation. The squamocolumnar junction was visible at the edges of the lesion confirming the anal origin of the tumor. We believe the tubulovillus adenoma arose from either an anal gland or its duct that opens into the anus. Although seen rarely, it is important to recognize and treat these tumors at an early stage because of their potential to transform into adenocarcinoma.

Key words: Tubulovillous adenoma; Anal canal

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INTRODUCTION

The anal canal is lined by transitional mucosa in its proximal one-half and by stratified squamous epithelium in the distal portion. Anal glands and ducts arise from this area and are lined by stratified columnar epithelium. The anal glands have secretary functions and help lubricate the anal canal[1]. Tumors of the anal canal are uncommon and are classified according to their tissue of origin. These consist of tumors of epithelial origin (squamous cell carcinoma, basal cell carcinoma, adenosquamous carcinoma), lymphoid tissue (lymphoid polyp, malignant lymphoma), mesenchymal tissue (fibroma, fibrosarcoma, leiomyoma, leiomyosarcoma), neural tissues (neurofibroma, ganglioneuroma), and vascular tissue (hemangioma, angiosarcoma)[2].

The most common tumor of the anal canal is squamous cell carcinoma, which arises from the squamous epithelium of the anal canal. Rarely, adenocarcinoma of the anus has been recognized and is believed to arise from the anal glands or ducts. There is a single report of multiple adenomatous polyps arising in the transitional zone of the anus in a patient with familial adenomatous polyposis (FAP), seven years after colon resection and ileo-anal anastomosis[3].

We describe a patient with a tubulovillus adenoma arising from the anal canal. These tumors are rarely encountered in patients without predisposing risk factors, such as FAP, ulcerative colitis or Crohn's disease.

CASE REPORT

The patient was a 78 years old white man who presented with history of rectal bleeding for several months. Bleeding occurred with almost every bowel movement and consisted of passage of fresh blood. The patient was otherwise healthy with no recent change in bowel habit, weight loss or reduced appetite. The patient was referred to the gastrointestinal service for work-up of rectal bleeding. His past history was positive only for hypertension. General physical examination was unremarkable. At the time of colonoscopy, rectal examination revealed a 4 cm friable mass attached to the anus by a definite stalk. The mass was located entirely outside the anal canal, with no extension into the rectum. Colonoscopy showed polyps in transverse (tubular adenoma), descending (tubulovillus adenoma with high grade dysplasia) and sigmoid colon (villous adenoma), which were removed endoscopically by the snare technique. At surgery, the anal polyp was found to be attached to the anus by a stalk. The mass was grasped with a Babcock forceps and was resected using electrocautery.

Gross examination of the specimen showed a tan-red, soft tissue mass, measuring 4.0 cm x 1.4 cm x 1.2 cm (Figure 1A). Microscopic examination revealed a tubulovillus adenoma (TVA), with nearly 30% of the surface of the polyp showing villous architecture (Figure 1B). There were no areas of high grade dysplasia or malignant transformation. The squamocolumnar junction was visible

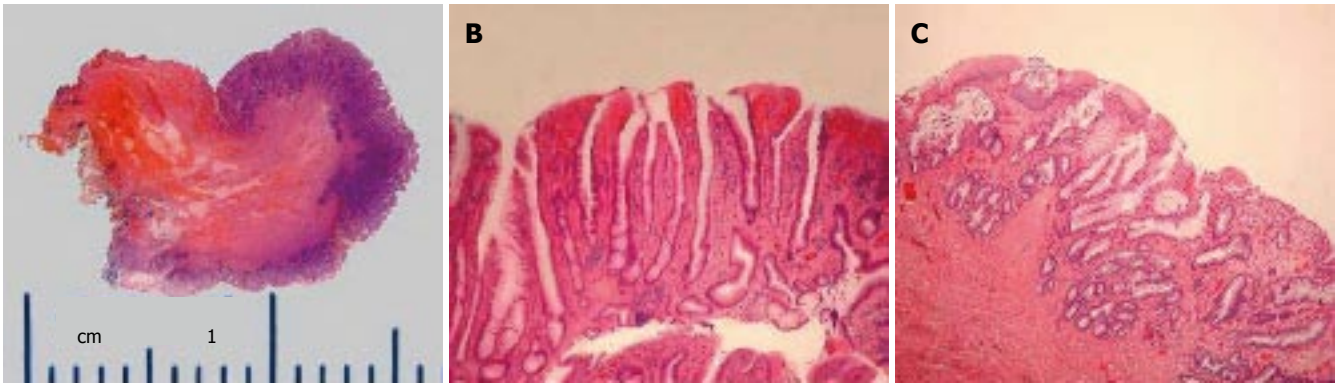

Figure 1 A: Gross appearance of the tubulovillus adenoma mounted on a glass slide. **B:** Areas with villous architecture were apparent (Hematoxylin and Eosin, original magnification x 400). **C:** The squamocolumnar junction is seen at the upper left (arrow). No atypical features were noted in the squamous component. (Hematoxylin and Eosin, original magnification x 400).

at the edges of the lesion and did not show any atypical changes (Figure 1C).

DISCUSSION

The anal canal is lined by stratified squamous epithelium and the most common tumor arising from this site is squamous cell carcinoma. Rarely, adenocarcinomas have been reported and these are believed to arise form the anal glands or their ducts which open into the anus. The current concept of the etiology of colorectal adenocarcinoma is based on the multistep genetic sequence of events which lead to the transformation of normal mucosa to adenoma and finally carcinoma[4]. That a similar sequence may occur in the development of adenocarcinoma of the anus was shown in a recent study which described the development of an invasive apocrine adenocarcinoma arising from a benign adenoma in the perianal region of a 45 year old women[5]. In our patient, the polyp was attached to the anus by a narrow stalk. Moreover, at histopathology, squamocolumnar junction was visible at the edges of the histological specimen confirming that the polyp arose from the anus and not from the rectum. We believe the site of origin of the tubulovillus adenoma in our patient was from one of the anal glands which are the only adenomatous elements in the anus. It is unclear why such tumors are not diagnosed more frequently. It is possible that because of the submucosal location of the anal glands, such adenomas are not easily visible and only become apparent when malignant transformation takes place. Clinically, it is important to recognize and treat these tumors at an early stage because of their potential to transform into adenocarcinoma.

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CASE REPORT

Acute biliary pancreatitis and cholecystolithiasis in a child: One time treatment with laparoendoscopic "Rendez-vous" procedure

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Abstract

Acute biliary pancreatitis (ABP) is rare in childhood and endoscopic sphincterotomy should be avoided in the child due to the risk of both early and late complications but, when necessary, the optimal timing between endoscopic procedure and cholecystectomy is still uncertain. A nine years old child with acute biliary pancreatitis underwent successfull laparo-endoscopic "Rendez-Vous" procedure in which endoscopic drainage of the common bile duct and laparoscopic cholecystectomy were performed simultaneously. This is the first case reported of laparo-endoscopic Rendez-Vous in a child. The excellent outcome of this patient and the review of the literature concerning other available options for the treatment of such cases suggest that this procedure offers great advantages, especially in children, of reducing the required number of treatments, the risk of ineffectiveness, the number of anaesthesia, the length of hospital stay and the risk of iatrogenic morbidity.

Key words: Acute biliary pancreatitis; Gallstones; ERCP; Laparoscopic cholecystectomy; Endoscopic sphyncterotomy

La Greca G, Di Blasi M, Barbagallo F, Di Stefano M, Latteri S, Russello D. Acute biliary pancreatitis and cholecystolithiasis in a child: One time treatment with laparo-endoscopic "Rendez-vous" procedure. *World J Gastroenterol* 2006; 12(11): 1782-1785

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INTRODUCTION

Acute biliary pancreatitis (ABP) is rare in childhood and endoscopic sphincterotomy should be avoided in the child due to the risk of both early and late complications but, when necessary, the optimal timing between endoscopic procedure and cholecystectomy is still uncertain. In the child the literature concerning therapeutic ERCP with ES for ABP is scarce, and ERCP, combined with ES or not, carries also the well known risk of iatrogenic acute pancreatitis. The optimal diagnostic and therapeutic approach and the timing of the procedures in children with cholecystolithiasis will often depend on the level of expertise of both the biliary endoscopist and laparoscopist. A nine years old child with acute biliary pancreatitis underwent the first successfull laparo-endoscopic "Rendez-Vous" procedure in which endoscopic drainage of the common bile duct and laparoscopic cholecystectomy were performed simultaneously. The favourable outcome of the reported case led us to deepen this issue.

CASE REPORT

A nine-year-old girl, weighing 52 kilograms, BMI of 27, was urgently admitted to our department because of pain in the right upper abdomen associated with vomiting. The only abnormal laboratory tests were the serum ALT 163 u/L (normal value 10-55), amylase 131 U/L (normal value < 110), lipase 482 U/L (normal value < 300), and ALP 168 U/L (normal value 38-126). Abdominal ultrasonography revealed multiple gallstones (3-5 millimeter) and sludge in the gallbladder and a normal common bile duct (CBD). The patient was kept NPO with intravenous fluids and analgesics and was discharged from the hospital after being asymptomatic for 24 h against medical advice, because her parents refused laparoscopic cholecystectomy (LC). The patient was followed in the paediatric outpatient clinic where metabolic or haematologic causes of cholecystolithiasis were excluded.

Three weeks later the patient was again urgently admitted because of a recurrence of abdominal pain, vomiting and a fever (38.6℃). Ultrasonography showed gallstones, a dilated CBD (8 mm) and pancreatic edema. Laboratory test showed a nine fold increased serum amylase of 998 U/L and of serum lipase 2430 U/L. Total and direct bilirubin were both increased to 2.81 mg/dl and 1.58 (normal

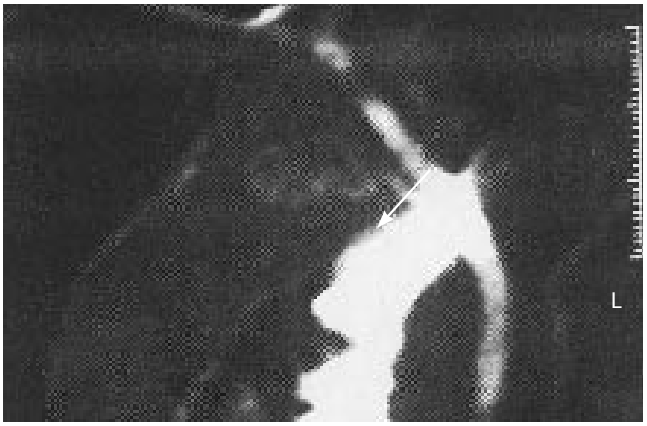

Figure 1 Cholangio-MRI showing a persistent filling defect (arrow) in the extrahepatic bile duct suspected to be biliary sludge or a stone.

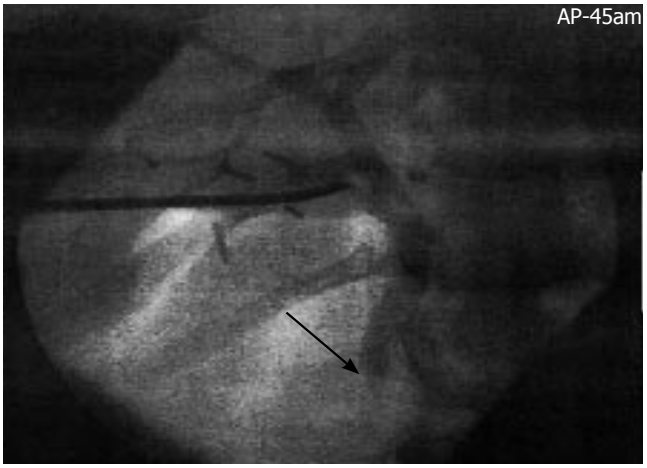

Figure 2 Intra-operative transcystic cholangiography 30 min after contrast medium injection showing the stop of contrast medium passage into the duodenum and a vanished image of the papilla of Vater (arrow) due to occluding biliary sludge.

value 1.3 and 0.5) respectively and other pathologic parameters were: gamma GT 479 U/L (normal value 78), ALT 1032 u/L, AST 584 U/L (normal value 10-45), LDH 1855 U/L (normal value 313-618), ALP 283 U/L and C reactive protein (CRP) 6.8 mg/d (normal value <1). White blood cell count (WBC) was 13 200 (normal value 6000-9000) with 91% polymorphonucleates, and blood glucose was 132 mg/dL. According to the Atlanta classification and Ranson's criteria[1] the diagnosis of mild acute biliary pancreatitis was given and a conservative treatment was started with fluids, analgesics and imipenem. The main symptoms disappeared after 12 h so that the conservative treatment was continued. After 48 h a CT scan showed a pancreatic and peripancreatic oedema that was classified as Balthasar stage B[2]. The bilirubin remained slightly abnormal (1.62 total and 0.74 direct) but serum amylase and lipase were increased to 1300 and 30560 U/L respectively, whereas CRP reduced to 4.4 mg/dL. A cholangio-MRI was performed (Figure 1) showing a filling defect in the upper portion of the CBD that was highly suspicious for a gallstone, sludge or mucinous aggregate.

Based on increased experience in adults with cholecystocholedocolithiasis, we decided to perform a laparoendoscopic "Rendez-vous" for both the endoscopic drainage of the CBD and treatment of gallstones despite the fact that there was no evidence in the literature of this procedure being performed on a child that was considered suitable, without contraindication and useful. Laparo-endoscopic "Rendez-Vous" procedure was as follows: The whole laparo-endoscopic procedure was carried out to maintain the patient in the same supine "french" position used for the laparoscopic cholecystectomy. Three trocars (10-10-5 mm) were introduced with open technique. The first manoeuvre was the preparation, encircling and clipping of the cystic duct to avoid migration of stones in the CBD. An intra-operative cholangiography was performed to introduce a Pedinelli catheter (8 fr) in the cystic duct. This showed a dilated CBD and a delayed passage of the contrast medium in the duodenum (Figure 2), but could not confirm the filling defect. The X-ray picture of the whole biliary tree remained unmodified without any flow of the contrast medium in the duodenum, up to 30 minutes after the injection and despite intravenous administration of a dose of antispastic drug (cymetropio bromide). This led us to suspect an underlying sphincter of Oddi dysfunction but a manometry was considered unnecessary because the indication for retrograde exploration of the papilla was given. During the time waiting to control the passage of the contrast medium in the duodenum, the cholecystectomy was carried out. The endoscopist performed the procedure using an adult lateral view duodenoscope. The papilla appeared normal but without any flow of bile. A guide wire could be introduced through the papilla and its correct position inside the still contrasted CBD could be confirmed. In this way the standard and more dangerous unpreventable retrograde injection of the Wirsung duct could be avoided. A paediatric endoscopic balloon catheter Olympus Swift ® (4 mm diameter) was then introduced in the CBD, inflated, and retrieved trough the papilla. Some biliary sludge could be retrieved and immediately bile flowed. This procedure was repeated twice and a gentle dilatation of the papilla was also performed for 2 minutes. The endoscope was then extracted and the laparoscopic procedure ended after clipping of the cystic duct. The post-operative laboratory controls showed the normalization of amylase, lipase and bilirubin the day after. On day two after the procedure a control US showed a normal CBD. The patient was discharged three days after the procedure. Neither recurrence of symptoms, nor abnormality in laboratory tests or US controls occurred up to the last 24 mo follow-up.

DISCUSSION

Acute biliary pancreatitis in the child is rare and the main causes are congenital disorders or malformations of the pancreatic-biliary ductal system. In children, the main indication of diagnostic ERCP with or without endoscopic sphincterotomy (ES) are biliary atresia, intrahepatic cholestasis, anomalous arrangement of the pancreatic-biliary ductal system including the dilatation of the CBD.[3,4,5] In the adult, ES is a part of the treatment of ABP when this is caused by stones or sludge impacted in the papilla.[6,7] Concerning the timing of ERCP +/- ES, early ap-

proach is not more beneficial than conservative treatment in patients without jaundice.[8] In the child the literature concerning therapeutic ERCP with ES for ABP is scarce, especially because the pancreatitis, according to Hsu and Nowak[9,10] is often not "biliary" in the "adult" meaning of the term. In the reported case the pancreatitis was truly "biliary", because related to micro-lithiasis and sludge and also combined with an underlying sphyncter of Oddi dysfunction which can often be related to recurrent pancreatitis also in children.[11] The treatment of ABP in childhood needs a multidisciplinary approach[12] to optimize laparoscopic treatment,[13,14] and intraoperative cholangiography is considered mandatory to minimize unnecessary ERCP.[15] In fact ERCP, combined with ES or not, carries the well known risk of iatrogenic acute pancreatitis because of papilla manipulation, pancreatic intraductal hyperpression and/or chemical damage all factors related to unpreventable retrograde injection of the pancreatic duct.[16,17] When ABP in children is related to gallstones there is a relevant risk of recurrence and therefore the standard treatment is endoscopic treatment sequentially combined days or weeks before or after cholecystectomy.[18,19] The sole case of urgent ERCP-ES in a 9-year-old patient with ABP due to a stone impacted in the papilla was reported recently[10] and was performed under anaesthesiological control of sedation. This patient was discharged after ES, with a planned laparoscopic cholecystectomy and was therefore still at risk of recurrent pancreatitis in the possible event of an incomplete sphincterotomy. There is the well known problem of the optimal timing between ERCP and LC, also considered " The bilateral interface…" between the two procedures[20] but the optimal diagnostic and therapeutic approach in children will often depend on the level of expertise of both the biliary endoscopist and laparoscopist.[19,21,22] All these considerations and the favourable outcome of the case convinced us that simultaneous laparoendoscopic treatment carries great advantages especially in children. The literature shows that comparable good success rate of ERCP between adults and children (98% *vs* 97.5%) with a comparable low incidence of complications (2.5% *vs* 3.4%) are obtainable only by very experienced teams[22] but unfortunately complications can reach up to 33%.[18,21,23] Therefore ERCP in the paediatric population should be minimized because stones often pass spontaneously[19] and also because the only sure way to avoid post-ERCP complications is to avoid ERCP itself.[24] This is certainly possible with the combined laparo-endoscopic approach because ERCP is avoided and ES is limited only to those patients with stones or abnormal cholangiography that cannot be resolved laparoscopically via the cystic duct or when a sphyncter of Oddi dysfunction can not be resolved through a simple dilatation. The balloon papilla dilatation carries comparable effectiveness but with a discordant incidence of relevant complications like pancreatitis that can be related to unnecessary retrograde injection of the pancreatic duct or manipulation, but anyway in children dilatation should be preferred to preserve the papilla.[25,26] The dilatation is also effective in early stone retrieval during the attack of ABP.[27]

Other favourable considerations concerning effectiveness of this combined approach can be found in the literature concerning the standard sequential approach. In a large prospective randomized study[28] the effectiveness of ERCP/ ES to clear CBD stones was only 84%, when performed prior to laparoscopic cholecystectomy and therefore 16% can be ineffective. Unfortunately, these patients often also have to undergo urgent surgery, with exposure to a second X-Ray for the mandatory cholangiography and often the need for a surgical opening of the CBD. This carries the need of relevant surgical skill but carries also an increased risk of complications, often need of T tube or other biliary drain, and prolonged hospitalization[29,30]. In the child both open and laparoscopic surgery and especially suturing of the small duct requires a high degree of surgical expertise to avoid the risk of late stenosis of the CBD. On the other hand, laparoscopic clearance of CBD stones in a large prospective study[28] of adults was impossible in 15% of the cases, despite all being operated on by experienced surgeons, and conversion to open surgery or post-operative ERCP carried a relevant incidence of biliary complications, but the post-operative ERCP can itself be related to risk of ineffectiveness.

A recent analysis of management of suspected common bile duct stones in children[31] shows clearly the poor results and pitfalls of the two common pathways of the sequential treatment with ERCP-ES and laparoscopic cholecystectomy. If performed prior to cholecystectomy a total of 71% of ERCP were unnecessary and 7% failed, if performed after cholecystectomy 50% of ERCP were unnecessary because stones were not found. Despite a reported incidence of 0% complication rate, 9 of 12 ES (75%) were unnecessary based on findings of an intraoperative cholangiography, some days prior. Therefore, a large number of unnecessary invasive procedures, all potentially related to morbidity and mortality, are often ineffective especially because they are out of synch with the evolving pathophysiology of gallstone disease.[19] On the other hand, the simultaneous approach of RV is tailored to a "real time" picture of the CBD and the compliance of the papilla, eliminating all previous and subsequent unnecessary procedures, and also minimizing the factors of ineffectiveness. Concerning ABP, endoscopy and laparoscopy, a recent review[32] pointed out that the sequential approach of ERCP-ES first followed by LC is the least efficient, and despite simultaneous treatment is theoretically the best option, the laparoscopic CBD clearance is considered difficult and time consuming.[32] The main experiences with the simultaneous laparoendoscopic approach[33,34] were always extremely positive, showing a high effectiveness in stone clearance of 97%-100%, a low morbidity and hospital stay comparable to simple laparoscopic cholecystectomy.[35] It is also important to consider that for a child younger than 10 years of age the ERCP usually requires general anaesthesia[5,22] and an additional administration is certainly needed for the successive cholecystectomy. On the other hand, to perform the laparoendoscopic RV only one administration of anaesthesia and related psychological trauma is necessary for the child. The outcome of the case and the analysis of the literature, with a comparison of combined laparoendoscopic Rendez-Vous to the other available options convinced us that considering the technical aspects, the related risk of iatrogenic complications, optimization

of the timing and the effectiveness of the procedure, the laparo-endoscopic Rendez-Vous in these cases is the best option for the child, perhaps also for the adult, the surgeon, the endoscopist and the hospital itself.

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CASE REPORT

Complete pancreatic heterotopia of gallbladder with hypertrophic duct simulating an adenomyoma

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Abstract

The gallbladder is an unusual location of pancreatic heterotopia, defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the main body of the gland. A 28-year-old man presented with anorexia, nausea and pain in the right upper abdomen. On physical examination, the abdomen was tender to palpation and Murphy sign was positive. The patient underwent a cholecystecomy. This case, in our opinion, is very interesting since it permits to consider a controversial issue in the pathology of the gallbladder. The histological appearance of ductal structure in pancreatic heterotopia resembles the histological picture of both Aschoff-Rokitansky (AR) sinuses and adenomyomas. This finding suggests that these lesions are linked by a common histogenetic origin. We suggest that the finding of an adenomyoma in the gallbladder should prompt an extensive sampling of the organ in order to verify the coexistence of pancreatic rests.

Key words: Pancreatic heterotopia; Gallbladder; Hypertrophic duct; Adenomyoma; Aschoff-Rokitansky sinuses

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INTRODUCTION

The gallbladder is an unusual location of pancreatic heterotopia, defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the main body of the gland[1]. About 75% of all pancreatic rests are located in the stomach, duodenum and jejunum, the appendix, diverticulum of Meckel and the ampulla of Vater[2] tract, ectopic pancreas has been found in the umbilicus, fallopian tube, mediastinum[2], spleen[3] and omentum[4]. Here, we describe a case of gallbladder pancreatic heterotopia associated with a hypertrophic duct simulating a small intrapancreatic adenomyoma.

CASE REPORT

A 28-year-old man presented with anorexia, nausea and pain in the right upper abdomen. On physical examination, the abdomen was tender to palpation and Murphy sign was positive. The patient underwent surgery, and a cholecystectomy was performed. The gallbladder was 10.5 cm in length and 3.5 cm in width, with a mural thickness of 0.3 cm. No stones were present. An intramural, firm, yellow nodule measuring 0.6 × 0.5 cm was seen in the gallbladder body: it was well circumscribed and showed a central umbilication on the mucosal surface. Light microscopy revealed diffuse proliferation of the surface epithelium, resulting in numerous invaginations into a thickened wall and numerous Aschoff-Rokitansky (AR) sinuses. The muscular layers appeared hypertrophied and possibly hyperplastic, with frequent foci of adenomyomatosis. No inflammatory infiltrate was seen. The epithelial component had typical features of pancreatic tissue. Acini were composed of polygonal cells with basal nuclei and apical granular cytoplasm surrounding a minute lumen. Endocrine islands were occasionally detected. The ductal system consisted of intercalated, intralobular and interlobular ducts: the major duct, surrounded by bundles of smooth muscle, appeared to drain into the gallbladder lumen. Ductal epithelium was surrounded by a smooth muscle component and displayed a typical pancreatic duct-like immunophenotype, characterized by immunoreactivity for cytokeratins 7, 8, 18, 19 and CA19-9. No reactivity against CEA and cytokeratin 20 was found. Acinar cells arranged in a single layer with typical exocrine differentiation were focally positive for α1-antitrypsin, chimotrypsin and cytokeratins 8 and 18. They were negative for CEA, CA19-9 and cytokeratins 7, 19 and 20. The patient, after cholecystectomy, had an uneventful recovery and normal development at follow-up.

DISCUSSION

This case, in our opinion, is very interesting since it

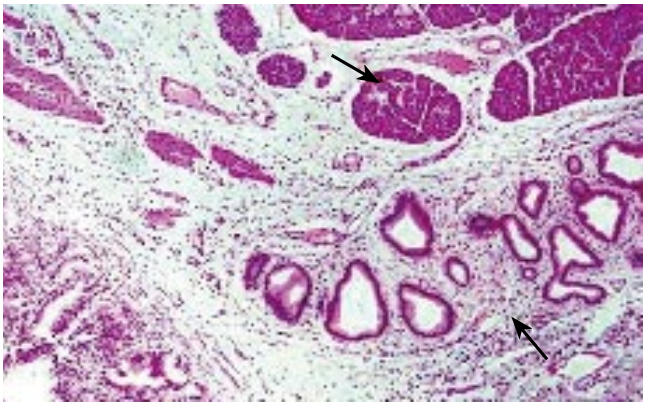

Figure 1 Complete pancreatic ectopia of gallbladder: red arrow indicates acinar components, green arrow indicates ductal structures surrounded by smooth muscle bundles (HE, original magnification, x100).

permits to consider a controversial issue in the pathology of the gallbladder. The histological appearance of ductal structures in pancreatic heterotopia resembles the histological picture of both AR sinuses and adenomyomas. This finding suggests that these lesions are linked by a common histogenetic origin.

In fact, AR sinuses are structures consisting of down growth of the gallbladder mucosa into the wall[5]. They are thought to be the consequence of increased intraluminal pressure, analogous to colonic diverticula[5]. Sometimes, mucosal proliferation is so exuberant as to result in formation of branching sinuses and cystic structures. In many cases, the muscular layer is hypertrophic and hyperplastic, thus resembling focal adenomyomatosis[5]. The latter feature is seen in our case (Figure 1), where the major duct, surrounded by a thickened muscular layer, shows morphological features overlapping those of adenomyomas. Analogously, it is not precisely defined when florid AR sinuses become adenomyomatosis[5].

Likewise, adenomyomas consist of glandular formations lined with cuboidal-to-columnar epithelium and surrounded by bundles of smooth muscle[6,7], similar to the prominent duct seen in our case.

Also heterotopic pancreas, when it includes only ducts, without acinar cells (canalicular or abortive form), shows the same morphological appearance[5].

On the basis of such morphological observations, we believe that AR sinuses, adenomyomas (when focal), adenomyomatosis (when diffuse), and abortive forms of pancreatic heterotopia can be considered variable manifestations of the same pathological process and be grouped into the same nosological category.

Consistent with this hypothesis, several authors have suggested that adenomyomas associated with pancreatic heterotopia found in other organs (stomach and duodenum) should be considered as an abortive variant of pancreatic heterotopia missing an acinar component[7,8]. This hypothesis emphazises the concept of a common pathogenetic origin of these lesions[7,8].

In contrast, Ryan *et al* believed that the adenomyomas of the gallbladder, in spite of histology, arise from diverticular disease of the gallbladder and are not pathogenetically related to heterotopic pancreas[9].

To our knowledge, only 30 cases of ectopic pancreas in the gallbladder have been reported; if the association between adenomyosis and pancreatic rests is true, as we propose, this finding should be more frequently observed. Therefore, we suggest that the finding of an adenomyoma in the gallbladder should prompt an extensive sampling of the organ in order to verify the coexistence of pancreatic rests. Unfortunately, it is not possible to provide immunohistochemical support to our hypothesis, since the gallbladder and the pancreas have the same embryologic origin, and thus there are no specific markers allowing unequivocal differentiation between pancreatic tissue and gallbladder epithelium. Recently, Ko *et al*[10] reported that proliferating duct cells of the pancreas express vimentin. Accordingly, we used immunohistochemistry to detect the expression of vimentin in order to verify their observations, but in our hands duct cells of both normal pancreas and pancreatic adenocarcinoma were negative for this marker. Further studies are thus required, apart from morphology, to support an unequivocal link between adenomyomas and pancreatic heterotopia in the gallbladder.

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CASE REPORT

Fatty liver in H63D homozygotes with hyperferritinemia

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Abstract

To study the clinical correlates of the H63D mu-tation we have analysed the phenotype of H63D homozygotes identified through mutation analysis in a referral laboratory. A total of 366 blood samples referred for *HFE* analysis were screened for C282Y and H63D mutations. Four H63D homozygotes were identified. All had raised serum ferritin but normal transferrin saturation. They were negative for hepatitis B and C and only one patient consumed excess alcohol. In all 4 cases ultrasonography revealed fatty liver. In two patients a liver biopsy was done and showed mild siderosis with an unusual distribution and macrovesicular steatosis. These data confirm the association between fatty liver, hyperferritinemia and increased hepatic iron, but do not clarify whether siderosis was related to steatosis rather than homozygosity for the H63D mutation. Patients with fatty liver may complicate the interpretation of data in population studies of the expression of H63D homozygosity.

Key words: Hyperferritinemia; *HFE* gene; H63D homozygosity; Fatty liver

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INTRODUCTION

Hereditary haemochromatosis (HH) is a common autosomal recessive disorder of iron metabolism with an incidence of up to 1 in 200 and an estimated frequency of carriers of 1 in 10 among people of Northern European descent[1-3]. The disease is characterized by enhanced gastrointestinal absorption of iron to excessive accumulation in tissue, which may result in damage to liver and other target organs[4]. C282Y missense mutation in the *HFE* gene was found to be strongly related to the occurrence of HH[2]. In a UK study, homozygosity for the C282Y mutation was found to account for 91% of HH[5]. A second missense mutation in the *HFE* gene, H63D, is found in around 4% of patients with HH, but its role in iron overload is still debated[2,6-8]. The H63D mutation is variably distributed worldwide. It is more prevalent than the C282Y mutation so that approximately one in five of the European population are H63D heterozygotes[3,9]. Individuals who are compound heterozygous for C282Y and H63D can have iron overload in the range diagnostic of haemochromatosis, although the penetrance of the genotype is low[2,10,11]. Similarly, homozygosity for H63D has been associated with iron overload, ranging from asymptomatic subjects to patients with typical haemochromatosis. As with compound heterozygosity for C282Y/H63D, the penetrance is low and the phenotypic presentation of this genotype varied considerably[11,12]. The aim of this study was to analyse the phenotypic expression of H63D homozygotes identified through the genetic screening of patients referred to our Centre for *HFE* mutation analysis.

366 consecutive blood samples, referred to the Centre for Hepatology at the Royal Free and University College Medical School (UCL), were analysed for *HFE* mutations. Mutation analysis was requested on the basis of biochemical or clinical suspicion of HH, family screening or known diagnosis of haemochromatosis. Samples were obtained after informed written consent, where appropriate. C282Y and H63D mutations were detected by polymerase chain reaction (PCR) amplification of total genomic DNA followed by restriction digestion with RsaI and MboI enzymes respectively, as previously described[5].

Table 1 Clinical, biochemical and serological features of the four H63D homozygous patients

	Case 1	Case 2	Case 3	Case 4
Age (yr)	36	35	44	66
BMI (kg/m^2)	30	24	32.5	31
Blood pressure (mmHg)	140/100	130/80	160/100	150/95
Alcohol intake (g/wk)	8	0	60	0
Blood sugar (mmol/L)	4.2	4.6	5.6	9.5
Total cholesterol (mmol/L)	4.1	4.3	5.5	6
Triglicerides (mmol/L)	1.6	1.5	3.6	2.9
AST (U/L)	46	69	44	15
ALT (U/L)	117	175	112	18
γGT (U/L)	50	45	70	30
Ferritin (μg/L)	454	350	568	423
HCV/HBV serology	neg	neg	neg	neg

Legend: BMI=body mass index; wk=week; neg=negative. Normal ranges: BMI 18-25 kg/m^2; blood pressure max<120mmHg, min<80mmHg (see the seventh report of the Joint National Committee on high blood pressure, NIH publication no 03-5233, December 2003); blood sugar<6mmol/L.

CASE REPORTS

Four males were found to be homozygous for the H63D mutation. Their main features are summarised in Table 1.

Case 1

English male patient aged 36 years with suspected iron overload indicated by a serum ferritin concentration of 454 μg/L (reference range: 39-340). Serum iron, transferrin saturation and total iron binding capacity (TIBC) were normal. He was referred because of abnormal levels of liver enzymes and elevated serum ferritin found during investigation for dyspepsia. He consumed 8 g of alcohol per week. He had a positive family history for obesity and maturity onset diabetes mellitus. There was no family history of haemochromatosis. On examination he was well, overweight and mildly hypertensive (140/100). Abdominal examination was normal. Liver function tests showed: ALT 117 U/L, AST 46 U/L and γGT 50 U/L. Viral markers for hepatitis B and C were negative. Ultrasonography revealed a large liver, with diffuse hyperechogenicity, characteristic of fatty change. Since the liver enzymes and the ferritin remained persistently elevated, a liver biopsy was performed. It showed moderate macrovesicular steatosis (grade 2 on a scale of 0 to 3) with grade 1 siderosis (on a scale of 0 to 4)[13] in periportal hepatocytes and Kupffer cells, with evidence of pericellular fibrosis of zone 3 (stage 1 of steatohepatitis according to Brunt *et al*[14]). The sinusoidal cells had unusual granular siderosis (Figures 1A, 1B, 1C). The hepatic iron concentration was increased being 185 μg/100g dry weight (reference range: 35-136). The patient was seen every three months for 2 years to monitor transaminases and ferritin. On the basis of the result of the liver biopsy, venesection therapy was started, together with dietary restrictions. Over a period of two years, he was treated with seven phlebotomies and approximately 1.3 g of iron were removed. The serum ferritin concentration returned to normal at 95 μg/L as did liver function tests except for minimal elevation of ALT at 45 U/L.

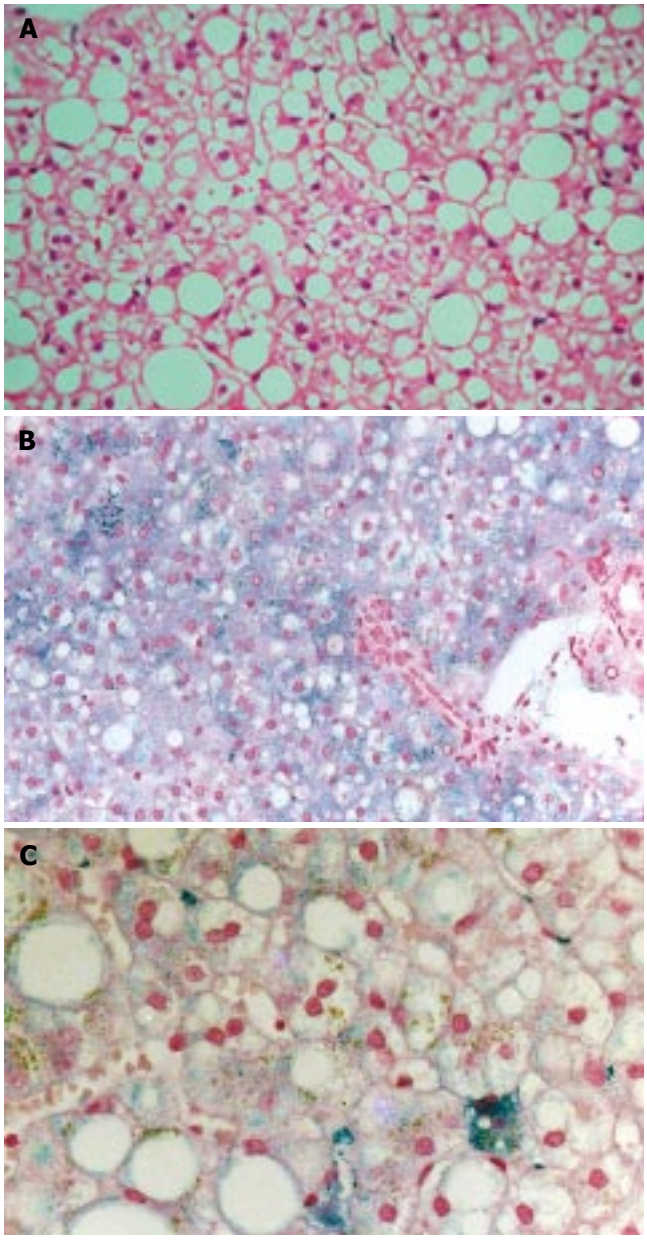

Figure 1 Case 1 liver histology. **A**: Hematoxylin and eosin staining shows grade 2 hepatic steatosis; **B**: Perls' staining shows grade 1 hepatocyte siderosis with predominant periportal distribution; **C**: Higher power of Perls' staining shows clustered Kupffer cell siderosis (right lower field) and also irregular large granular deposits in sinusoidal cells.

Case 2

Male patient aged 35 years from Lebanon with mild abnormality of iron indices. In 1996 he had a routine check-up and was found to have abnormal ALT (175 U/L) and AST (69 U/L), together with a minimally elevated serum ferritin (350 μg/L). Serum iron, TIBC and transferrin saturation were normal, as were the other liver function tests. The family history was negative for haemochromatosis. The patient did not drink alcohol. Viral markers for hepatitis B and C were negative. Between 1989 and 1995 he had been a blood donor giving approximately 2 units (approximately 450 mL each) of blood every year. On examination he had a normal build with gynaecomastia. Blood pressure was normal. Abdominal examination showed a palpable liver

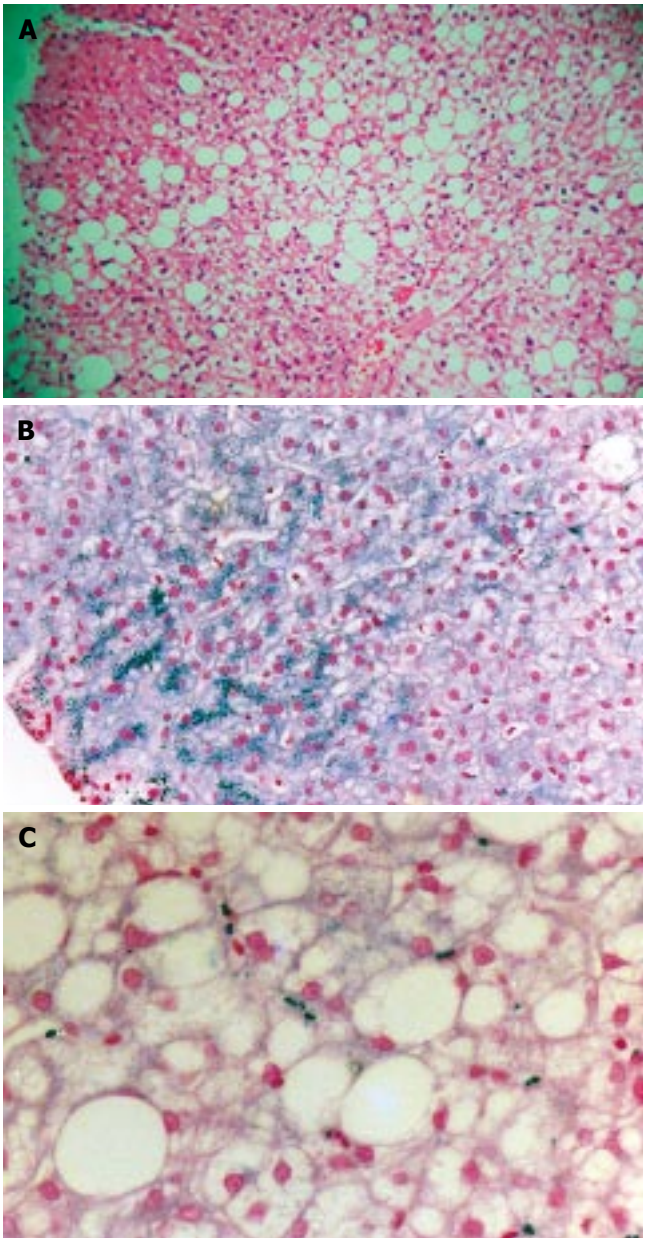

Figure 2 Case 2 liver histology. **A**: Hematoxylin and eosin staining shows grade 2 hepatic steatosis; **B**: Perls' staining shows grade 2 hepatocyte siderosis with predominant periportal distribution; **C**: Higher power shows focal Kupffer cell siderosis and more granular irregular sinusoidal siderosis.

on deep inspiration (2 cm) but no other abnormalities. The alpha-1-antitrypsin level was 1.0 g/L (normal range 1.2-2.6 g/L). Analysis showed him to have phenotype MZ. Ultrasound examination revealed a hyperechogenic liver compatible with hepatic steatosis. A liver biopsy was performed and showed moderate macrovesicular steatosis (grade 2) without fibrosis (stage 0 of steatohepatitis[14]). There was grade 2 siderosis in periportal hepatocytes, and focal Kupffer cell iron. The sinusoidal cell iron had a granular pattern similar to that seen for case 1 (Figures 2A, 2B, 2C). No cholestasis or alpha-1-antitrypsin staining was detected.

The patient was seen every three months for 4 years to monitor transaminases and ferritin, which remained persistently elevated.

Case 3

Irish male patient aged 44 years referred with possible iron overload indicated by a ferritin concentration of 568 μg/L. Serum iron, transferrin saturation and TIBC were normal. He was referred to our clinic in November 1998 to investigate raised levels of liver transaminases: ALT was 112 U/L, AST 44 U/L and γGT 70 U/L. The family history was positive for obesity and maturity onset diabetes mellitus and was negative for haemochromatosis. Physical examination showed a middle aged obese man (103 kg/178 cm; Body Mass Index (BMI) =32.5 kg/m^2). Blood pressure was elevated in the supine position (160/100) and the hypertension was treated with Tenif one per day (atenolol 50 mg, nifedipine 20 mg) and Valsartan (80 mg/day). The patient had also a history of moderate alcohol abuse (alcohol intake: 60 g/week) and elevated triglyceride levels (3.61 mmol/L). Viral markers for hepatitis B and C were negative. Ultrasonography of the abdomen revealed a liver of normal size, but with echoreflectivity consistent with fatty liver. No liver biopsy was done. The patient was seen every three months for 2 years to monitor weight, transaminases and ferritin. He abstained from alcohol. Dietary therapy was started and the patient lost 6 kg in weight. No venesection was performed. During follow-up, liver function tests remained mildly abnormal with an ALT of 84 U/L. The other enzymes fell to normal levels and triglycerides fell to 1.73 mmol/L. Despite abstinence and losing weight, his ferritin remained elevated (482 μg/L). The possibility of iron overload was raised and the patient was genotyped for mutations in the *HFE* gene and found to be homozygous for H63D.

Case 4

English male patient aged 66 years with an elevated serum ferritin concentration (423 μg/L) on a background of type 2 diabetes and peripheral neuropathy. Serum iron, transferrin saturation and TIBC were normal. He was diagnosed as hypertensive in February 1988 and he is currently treated with Enalapril. He also had atrial fibrillation for which he has been treated with warfarin. He was treated for diabetes mellitus with gliclazide, metformin and acarbose. Physical examination showed no features of chronic liver disease and no hepatomegaly. Liver function tests were normal. Viral markers for hepatitis B and C were negative. Abdominal ultrasonography revealed an enlarged and fatty liver. Since his ferritin levels remained in the region of 400 μg/L, the patient was tested for mutations in the *HFE* gene and found to be homozygous for H63D mutation.

DISCUSSION

Four H63D homozygotes were identified by screening 366 blood samples referred for genetic analysis in the *HFE* gene. All the four patients had high serum ferritin. It is noteworthy that in all the cases an abdominal ultrasonography showed fatty liver. In the two cases where liver biopsy was done and histology showed not only mild siderosis of hepatocytes, typical of iron overload of HFE-related haemochromatosis, but also siderosis of sinusoidal cells, with a granular pattern that could be related to nonalco-

holic steatohepatitis (NASH). Both patients had significant macrovesicular steatosis. One patient was treated with dietary restriction and venesection, after which liver function tests and serum ferritin concentration returned to normal. These findings suggest that hepatic steatosis, together with a biological effect of the H63D mutation, could be responsible for the hepatic siderosis in these patients. Recent evidence suggests that H63D homozygosity could lead to iron overload with variable penetrance and phenotype[12]. However, an association with fatty liver has not been previously reported. Another study of more than 10,000 blood donors suggested an effect of H63D homozygosity on iron metabolism[15], although the mean serum iron indices for this genotype were within the normal range, possibly reflecting the fact that blood donors are in general healthy and young (mean age was 38 years for men and 36 years for women). These findings have been reinforced experimentally by the demonstration that transgenic mice homozygous for the H63D mutation have elevated transferrin saturation and hepatic iron concentration compared to wild type mice[16].

H63D homozygosity could thus contribute to iron overload but the phenotypic expression may be influenced by cofactors. Sex, age, diet and modifier genes are likely to influence penetrance of the genotype. In this series all 4 cases were male and this reflects the relative protection of women from iron overload by menstruation and pregnancy. Furthermore, three of the cases had one or more metabolic disorders which are part of the insulin resistance syndrome (IRS)[17]. Case 1 was overweight (BMI>25 kg/m^2) and mildly hypertensive, Case 3 was obese (BMI>30 kg/m^2) and had hypertriglyceridemia and hypertension requiring antihypertensive treatment. Case 4 was obese, he had type 2 diabetes mellitus requiring therapy, hypertrigliceridemia and hypertension under therapy.

Although hyperferritinemia may be associated with hepatic steatosis per se[18,19], in two patients presented here hepatic siderosis with an unusual histological pattern was present. Homozygosity for H63D may have played a role, but the unusual cellular pattern raises the possibility that in some cases of hepatic steatosis there are other changes in cellular iron handling which result in iron accumulation. Alterations in cytokines and hence hepcidin may result in accumulation of iron in sinusoidal cells[20,21]. A role for the mutations in the *HFE* gene in other diseases, however, has been suggested by their over-representation in subjects with NASH and with the dysmetabolic iron overload syndrome (DIOS) characterized by an association between iron overload and insulin resistance[22-24]. The molecular mechanism explaining these associations is not clear. Hepatic steatosis has been recognised as the first of two "hits" in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is enough to trigger lipid peroxidation[25]. However, many patients with fatty liver do not progress to necroinflammation[26]. It has been suggested that the second hit for the development of NASH may be oxidative stress, leading to necroinflammation[27]. Several potential second hits have been suggested. Iron even in relatively low concentrations could synergize with lipid overload and induction of ethanol-inducible cytochrome P450 2E1 (CYP2E1) to increase oxidative stress in hepatocytes[28].

If iron leads to oxidative stress and to progression of non-alcoholic fatty liver to NASH, venesection therapy is theoretically beneficial, and this may have contributed to the normalisation of serum ferritin concentration and improvement of liver function tests in Case 1. In conclusion, our findings confirm a link between fatty liver and mild iron accumulation. Whether homozygosity for H63D contributed to the association is uncertain. Further studies of iron regulatory proteins are needed in hepatic steatosis.

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CASE REPORT

Hepatic encephalopathy with status epileptics: A case report

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Abstract

A 62-year-old male with decompensated liver cirrhosis due to hepatitis C virus developed severe hepatic encephalopathy with status epileptic us. The blood ammonia level on admission was more than twice the normal level. Brain computed tomography and magnetic resonance imaging were normal. In addition, electroencephalogram showed diffuse sharp waves, consistent with hepatic encephalopathy. The status epilepticus was resolved after antiepileptic therapy (phenytoin sodium) and treatment for hepatic encephalopathy (Branched chain amino acids). The blood ammonia level normalized with the clinical improvement and the patient did not have a recurrence of status epilepticus after the end of the antiepileptic treatment. Additionally, the electroencephalogram showed normal findings. Thus, we diagnosed the patient as hepatic encephalopathy with status epilepticus. We consider the status epilepticus of this patient to a rare and interesting finding in hepatic encephalopathy.

Key words: Status epilepticus; Hepatic encephalopathy; Decompensated liver cirrhosis; Electroencephalogram

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INTRODUCTION

Hepatic encephalopathy typically presents with alterations in behavior, impairment of consciousness, and alterations in motor tone[1]. Although, according to some reports, the incidence of seizures in patients with hepatic encephalopathy is unknown, reports of epileptic seizures in patients with hepatic encephalopathy during the clinical course are very rare, but not in the terminal stage of liver cirrhosis[2-4]. We present a rare and interesting case of a 62-year-old male with hepatic encephalopathy with status epilepticus accompanying decompensated liver cirrhosis.

CASE REPORT

A 62-year-old patient was diagnosed as having decompensated liver cirrhosis due to hepatitis C virus one year earlier. At that time he reported a ten-year history of mild psychosis but no history of diabetes mellitus, epilepsy, stroke, or brain trauma. He was admitted to our hospital because of generalized tonic-clonic seizures for several hours and loss of consciousness (status epilepticus) in October 2004. He had a history of recurrent episodes of hepatic encephalopathy. The results of further clinical examination, except for signs of decompensated liver cirrhosis, were normal. Laboratory data showed anemia, thrombopenia, increased transaminase, alkaline phosphatase and total bilirubin, and decreased albumin, total cholesterol and BTR (branched chain amino acid/tyrosine ratio). Furthermore, the plasma ammonia (NH_3) level on admission was twice the normal level (186 μmol/L; normal value is 23-76 μmol/L). Chest and abdominal roentgenograms, brain computed tomography (CT) and magnetic resonance imaging (MRI) were also normal, although abdominal echosonography and CT revealed atrophic cirrhotic liver and splenomegaly. Electroencephlogram (EEG) on day 2 after admission showed diffuse sharp waves, consistent with hepatic encephalopathy (Figure 1). According to the history and the results of EEG, in combination with laboratory and radiological data, hepatic encephalopathy with status epilepticus was suspected. Status epilepticus and loss of consciousness were resolved after antiepileptic therapy (phenytoin sodium) and treatment for hepatic encephalopathy (branched chain amino acids; BCAA). The plasma NH_3 level returned to a normal level concurrently with his clinical improvement. He did not have a recurrence of status epilepticus after the end of antiepileptic treatment and his conscious level returned to normal with continuous therapy for hepatic encephalopathy with BCAA. Another EEG on day 33 after admission showed a normal level (Figure 1). Thus, we diagnosed our patient as having hepatic encephalopathy with status epilepticus. In the follow-up, 7 mo later, he was in good condition, in spite of

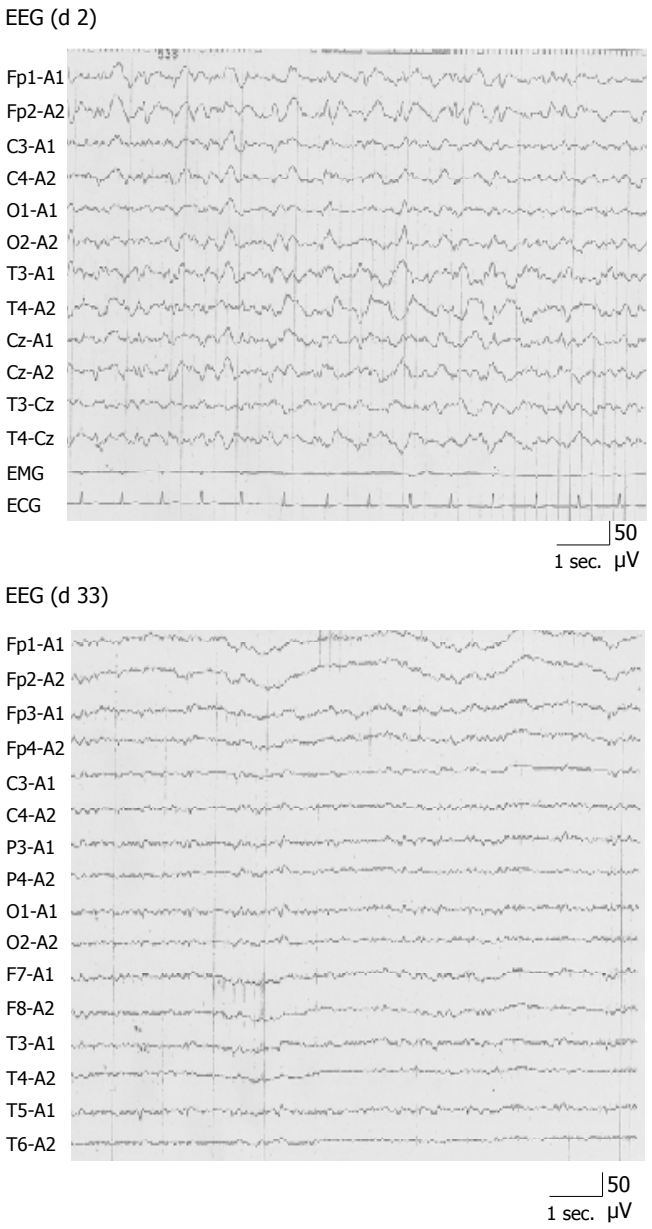

Figure 1 Diffuse EEG activity on day 2 after admission characterized by delta waves mixed with theta waves. Signs compatible with hepatic encephalopathy. The EEG activity on day 33 after admission returned to normal.

the recurrence of hepatic encephalopathy.

DISCUSSION

The present case was diagnosed as hepatic encephalopathy with status epilepticus. Although the patient had a history of mild psychosis, there were no psychological findings except for the loss of consciousness at the time the seizures occurred. In addition, he had a history of recurrent episodes of hepatic encephalopathy. Second, no focal brain lesion could be detected by brain CT and MRI, and the EEG was suggestive of metabolic dysfunction, especially hepatic encephalopathy. Third, the elevated plasma NH_3 level and EEG findings (diffuse sharp waves) on admission and its subsequent normalization with the clinical improvement after the end of antiepileptic treatment strongly suggested hepatic encephalopathy with status epilepticus.

Epileptiform abnormalities in EEG, seizures and status epilepticus may be seen in patients with hepatic encephalopathy, although their incidence is unknown. Ficker DM et al. reported in a retrospective study that the majority of cirrhotic patients with epileptiform changes in EEG either died or deteriorated[3]. However, reports of epileptic seizures in hepatic encephalopathy during the clinical course are very rare[5, 6].

The pathophysiology of seizures in hepatic encephalopathy remains unknown. Various metabolic factors may be suggested. The plasma NH_3 levels are consistently elevated, while other factors, such as short chain free fatty acids, phenols, mercaptanes and false neurotransmitters, have also been implicated. These factors may be responsible for the generation of epileptiform discharges as well as epileptic seizures in patients with hepatic encephalopathy[7, 8].

On the other hand, it has been reported that the focal hyperemia/hypermetabolism may be related either to the seizures or to the encephalopathy itself, based on intracranial ultrasonography and single-photon-emission computed tomography (SPECT)[6].

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CASE REPORT

Left paraduodenal hernia in an adult complicated by ascending colon cancer: A case report

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Abstract

Paraduodenal hernia is the most common internal hernia. The clinical symptoms of paraduodenal hernia may be intermittent and nonspecific. Therefore, it is difficult to diagnose preoperatively. Abdominal computed tomography (CT) scan currently plays an important role in the evaluation and management of paraduodenal hernia before surgical operation. We report one unique case of preoperatively diagnosed left paraduodenal hernia complicated by advanced ascending colon cancer and reviews of Japanese literature.

Key words: Paraduodenal hernia; Internal hernia; Colonic malrotaion

Kurachi K, Nakamura T, Hayashi T, Asai Y, Kashiwabara T, Nakajima A, Suzuki S, Konno H. Left paraduodenal hernia in an adult complicated by ascending colon cancer: A case report. *World J Gastroenterol* 2006; 12(11): 1795-1797

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INTRODUCTION

Paraduonenal hernia is an uncommon cause of small bowel obstruction. It has been reported that the most common type of internal hernia, reported in more than 50% of internal hernia cases. We report a case of left paraduodenal hernia complicated by ascending colon cancer, which was diagnosed correctly with abdominal CT. This case was confirmed at surgical operation and the clinical and imaging findings of these hernias were reviewed in Japanese literature.

CASE REPORT

A 47-year-old woman was admitted with right lower quadrant pain and nausea. An elastic hard mass was palpable in the right lower quadrant abdomen. The body temperature was 36.8℃. Arterial blood pressure and cardiac rate were 96/80 mmHg and 70/min respectively. Laboratory tests detected only an anemia. Plain abdominal X-ray showed air-fluid levels of the small intestine loop in the upper abdomen (Figure 1). Abdominal CT scans with intravenous administration of contrast medium showed ascending colon with enhanced mass and ileum dilatation (Figure 2). Abdominal CT scan also showed an encapsulated small bowel loop with no dilatation in the left upper abdomen and the inferior mesenteric vein (IMV) was located in the anterior of the encapsulated small bowel loop formation (Figure 3). Colonoscopy revealed advanced ascending colon cancer with obstruction. We preoperatively diagnosed as ascending colon cancer with left paraduonenal hernia.

Laparotomy revealed the hernia orifice and hernia sac was located in left mesocolon (Figure 4). IMV was located in the upward of the anterior portion of the hernia sac. Approximately 40 cm of jejunum was within a hernial sac. There were no volvulus or ischemic changes of the jejunum. The patient underwent right hemicolectomy with an end-to-end ileocolonic anastomosis. Anterior hernial sac incision was also performed. The postoperative course was uneventful.

DISCUSSION

Paraduonenal hernia has been reported that the most common type of internal hernia, reported in more than 50% of internal hernia cases[1,2]. The clinical symptoms of paraduodenal hernia may be intermittent and nonspecific. It is difficult to diagnose preoperatively. Therefore, paraduodenal hernia was found incidentally at laparotomy, autopsy or during radiological investigation for unrelated disease[2-5].

Based on the published cases of paraduodenal hernia in Japan, approximately 68% (82/120 cases) were left sided, and 32% (38/120 cases) were right sided. 70% of these cases were man, and 30% cases were woman[6,7]. The mean age of the onset was 39.5 years. These frequencies of

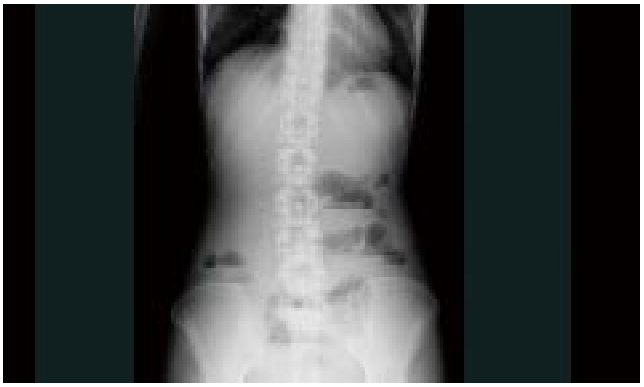

Figure 1 Plain abdominal X-ray showed air-fluid levels of the small intestine loop in the upper abdomen.

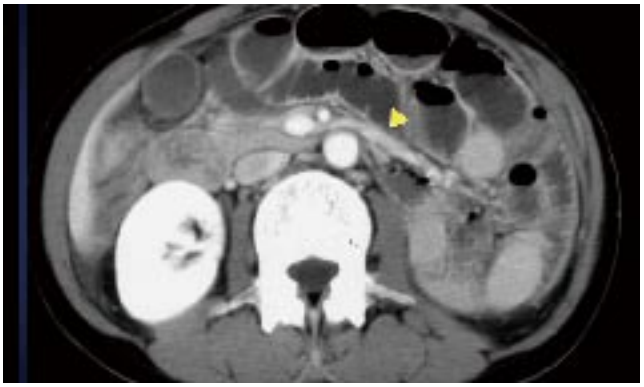

Figure 3 Inferior mesenteric vein (IMV) was located in the anterior of the encapsulated small bowel loop formation (yellow arrow head).

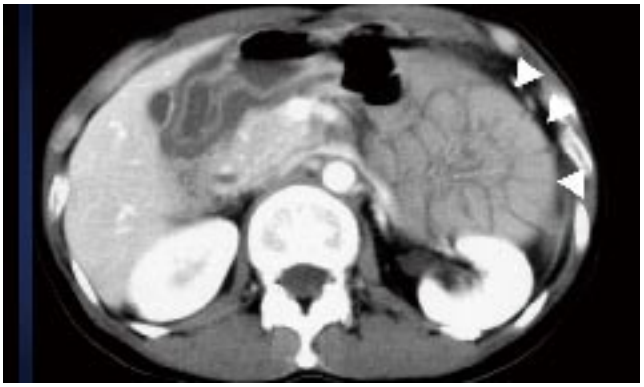

Figure 2 Abdominal CT scan also showed an encapsulated small bowel loop with no dilatation in the left upper abdomen (white arrow head).

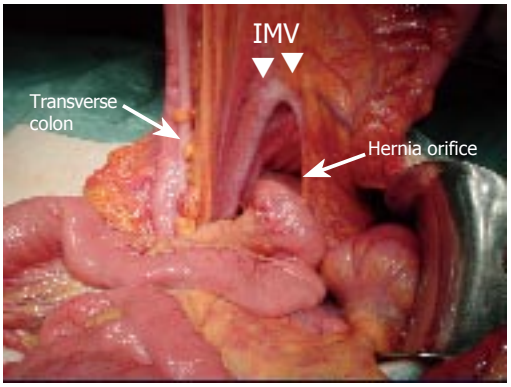

Figure 4 Large orifice of the hernia sac (white arrow) in the transverse mesentery and IMV formation. The small intestine had herniated through hernia orifice.

paraduonenal hernia were almost similar to the incidence in English literature.

Internal hernia complicated by colorectal cancer was reported only 3 cases. In our patient case, the origin of the bowel obstruction was not caused by left paraduonenal hernia but obstructed by advanced ascending colon cancer. Clinical findings of paraduodenal hernias are variable, ranging from mild digestive complaints to acute or chronic symptoms of obstruction, sometime asymptomatic.

Many of the paraduodenal hernia can be diagnosed incidentally at laparotomy, autopsy or during radiological investigation for an unrelated disease. Therefore, diagnosis of asymptomatic paraduodenal hernia has been difficult. On the other hand, recently reported cases of symptomatic paraduodenal hernia, 80% (8/10 cases) were diagnosed before operation[6,7]. Especially, abdominal CT currently plays an important role in the evaluation and management of internal hernias[2,3,8,9].

Enhanced CT is helpful in establishing a preoperative diagnosis of the presence of paraduonenal hernia. The specific findings of paraduonenal hernia by CT scan show clustering of small bowel loops, well-circumscribed edge that corresponds to the hernia sac, stretched and engorged mesenteric vessels. In the case of left paraduodenal hernia, IMV were usually located in the upward and anterior displacement of the hernia sac. Abnormalities in the mesenteric vessels including engorgement, crowding and stretching, and displacement of the main mesenteric trunks to the left are important clues to this diagnosis[1,10].

Paraduodenal hernias are believed to occur due to a congenital defect in the descending mesocolon. The small bowel may occur to invaginate into this space, the fossa of Landzert, which lies to the left of the fourth portion of the duodenum. The herniated small-bowel loops may become trapped within this mesenteric sac.

Treatment for the duodenal hernia is based on reduction of the hernia sac and closure of the defect or incision of the hernia sac[11,12]. Paraduodenal hernia induces an over 50% lifetime threat of incarceration, leading to bowel obstruction and strangulations. Therefore, surgical operation should be performed to avoid these complications. Indeed, 20% cases of internal herinas needed for small intestinal resection caused by bowel necrosis[6,7]. In general, postoperative course was uneventful, recurrence case was rare. Recently, laparoscopic surgery for left paraduodenal hernia without bowel necrosis may be useful surgical method because of its minimal invasiveness and aesthetic advantage[13,14]. In our case, we performed anterior hernia sac incision following the right hemicolectomy. Surgeons should pay attention to the mesenteric vessels located near the hernia sac orifice. The displacement of the main mesenteric trunks may

cause vessel injury during surgical treatment.

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CASE REPORT

Destructive granuloma derived from a liver cyst: A case report

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Abstract

We herein report the case of an idiopathic liver cystic mass which aggressively infiltrated the thoraco-abdominal wall. A 74-year-old woman who had a huge cystic lesion in her right hepatic lobe was transferred to our hospital for further examinations. Imaging studies revealed a simple liver cyst, and the cytological findings of intracystic fluid were negative. She was followed up periodically by computed tomography (CT) scans. Seven years later, she complained of a prominence and dull pain in her right thoraco-abdominal region. CT revealed an enlargement of the cystic lesion and infiltration into the intercostal subcutaneous tissue. We suspected the development of a malignancy inside the liver cyst such as cystadenocarcinoma, and she therefore underwent surgery. A tumor extirpation was performed, including the chest wall, from the 7th to the 10th rib, as well as a right hepatic lobectomy. Pathologically, the lesion consisted of severe inflammatory change with epithelioid cell granuloma and bone destruction without any malignant neoplasm. No specific pathogens were evident based on further histological and molecular examinations. Therefore the lesion was diagnosed to be a destructive granuloma associated with a long-standing hepatic cyst. Since undergoing surgery, the patient has been doing well without any signs of recurrence.

Key words: Destructive granuloma; Liver cyst

Kawashita Y, Kamohara Y, Furui J, Fujita F, Miyamoto S, Takatsuki M, Abe K, Hayashi T, Ohno Y, Kanematsu T. Destructive granuloma derived from a liver cyst: A case report. *World J Gastroenterol* 2006; 12(11): 1798-1801

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INTRODUCTION

A liver cyst is a common lesion that tends to be mostly asymptomatic, however, once it is associated with other events such as bleeding, infection and rupture, it often becomes symptomatic and treatment is thus usually needed. Granulomas can be seen in any part of the body and they reflect a host inflammatory responses elicited by a wide variety of stimuli[1, 2], however, it is uncommon that a granulomatous reaction develops in a liver cyst[3].

We experienced the case of highly destructive, non-malignant, granulomatous lesion that originated from a long-standing hepatic cyst that infiltrated the thoraco-abdominal wall mimicking hepatic cystadenocarcinoma. To the best of our knowledge, this is the first known occurrence of a case of a hepatic granuloma that developed secondary to a liver cyst with a highly invasive capacity.

CASE REPORT

A 73-year-old Japanese woman presented at our hospital with right. hypochondralgia on September 1997. Computed tomography (CT) showed a huge liver cyst measuring 10cm in the right lobe of the liver without any solid component (Figure 1A). Subsequently, 2.4L of serous fluid were percutaneously drained, without any sclerosing agent administered into the cyst. A cytological examination of the aspirated fluid was negative for neoplastic cells. Since her symptom had subsided after the fluid drainage, she was carefully followed-up without any further treatment. On May 2003, she was again referred to us with abdominal distention and pain in the right thoraco-abdominal region. The results of laboratory tests showed slight elevations in the C-reactive protein (CRP) level (0.5mg/dL, normal range < 0.17). Serological tests for hepatitis B and C virus were negative. The serum levels of carcinoembryonic antigen, carbohydrate antigen 19-9 and α-fetoprotein were all within the normal ranges. Other tests including her liver function were within the normal limits. A CT scan showed a cyst in the right. lobe of the liver similar to the one observed 6 years previously. In addition, a calcified lesion was also found around the cyst (Figure 1B). An axial and coronal view of an MRI showed a multilocular cyst with a thick wall and solid component extending into the subcutaneous tissue (Figure 2A and B). MR cholangiopancreatography demonstrated no dilatation in the biliary system, thus indicating that there was most likely no communication with the liver cyst (Figure 2C). These findings closely resembled hepatic cystadenocarcinoma.

After admission, the cystic tumor gradually increased

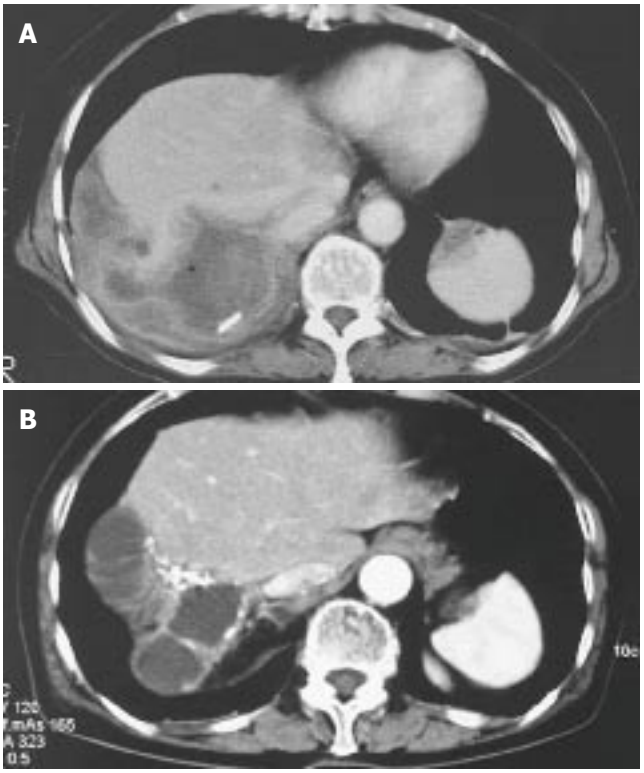

Figure 1 Computed tomography revealed a huge multilocular cyst measuring 10 cm in the right lobe of the liver on September 11th, 1997 (**A**). A similar cyst with a calcified lesion was found in the right lobe of the liver on May 15th, 2003 (**B**).

in size and finally her skin above the invasion site became reddish and swollen (Figure 2D), thus suggesting malignant tumor invasion. After obtaining the patient's informed consent, we performed an *en bloc* resection of the hepatic right lobe including the whole cyst, along with the diaphragm, rib bone, and skin. After completing the resection, the defect of the diaphragm was covered with the great omentum, and the thoraco-abdominal wall defect was reconstructed using a musculo-cutaneous flap including the anterior sheath and the left rectus abdominis muscle which both receive the blood supply from the superior abdominal artery (Figure 3A and B). Macroscopically, the cystic tumor directly invaded the intercostal space through the diaphragm (Figures 4A and 4B). No microorganism was found in the cyst fluid or the resected materials according to the results of microbilological culture examinations. A pathological examination revealed that the bone had been destroyed by the invasion of the granuloma, which was composed of epithelioid cells with necrotic areas (Figure 4C). No malignancy or microorganism was detected in any tissue samples. Because the histology was similar to tuberculosis, Ziehl-Neelsen staining and DNA-PCR using three different primers for a tubercle bacillus were performed with negative results. Hepatic hydatid disease was excluded by the fact that she had no history of living or traveling to the epidemic area such as Hokkaido prefecture in Japan and her blood samples did not show the presence of the antibodies against ehinococcus. From these findings, the cystic tumor was therefore histologically diagnosed to be a hepatic epithelioid granuloma. (Figure 4D)

The patient had an uneventful postoperative course and was discharged on postoperative day 27. She remains asymptomatic until 24 mo postoperatively, without any signs of recurrence.

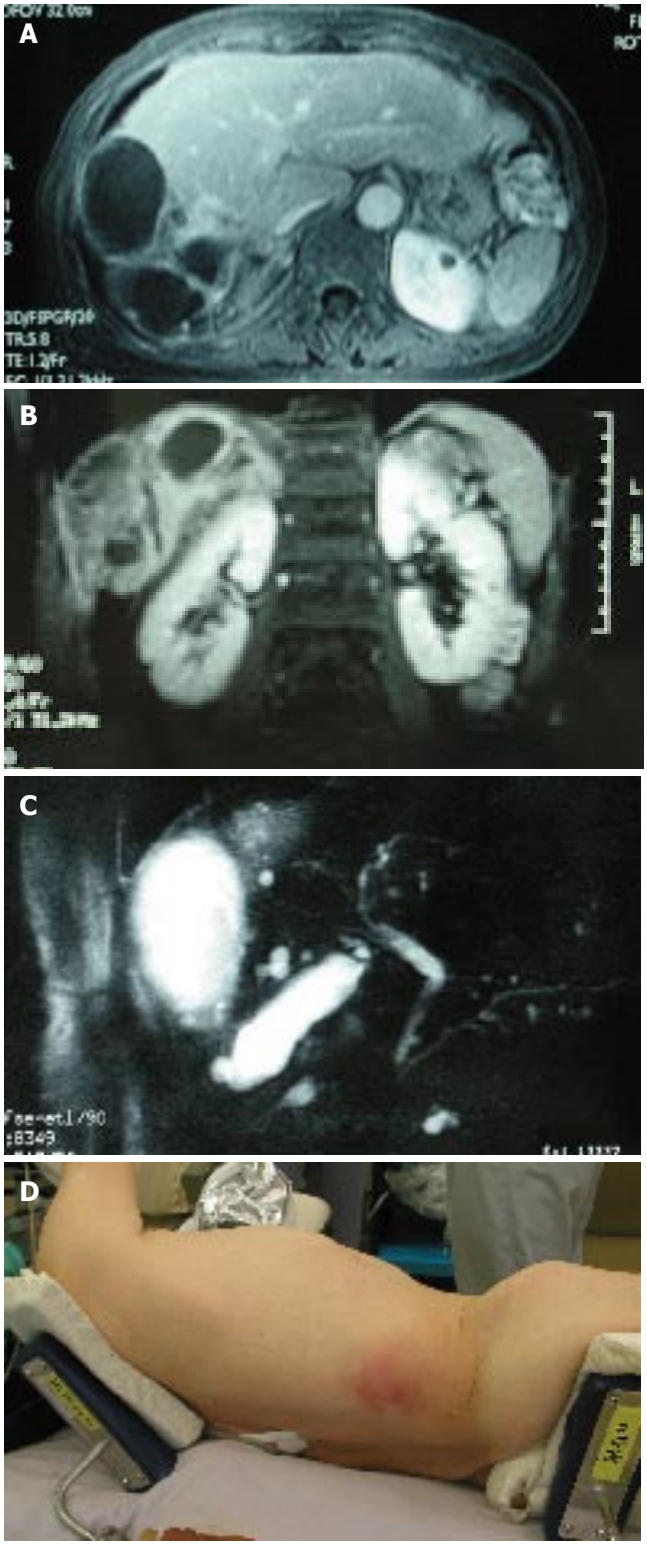

Figure 2 An axial (**A**) and coronal (**B**) view of magnetic resonance imaging (MRI) showed a multilocular cyst with a thick wall with a solid component extending into the subcutaneous tissue. MR cholangio-pancreatography showed no dilatation in the biliary system, and most likely no communication with the liver cyst (**C**). The skin showed red swelling by the subcutaneous extension of the hepatic cyst (**D**).

DISCUSSION

There has been a great progress in the diagnostic modali-

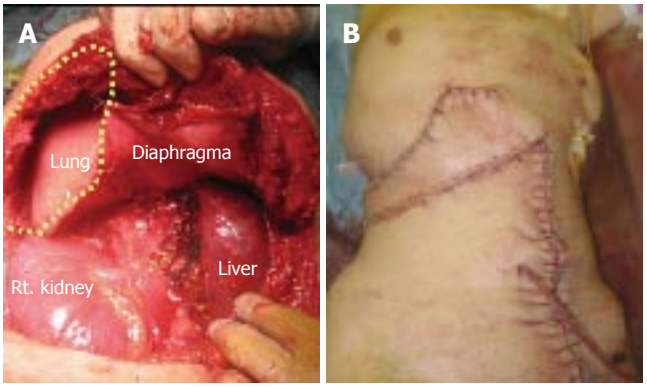

Figure 3 An en bloc resection resulted in a huge defect (**A**, yellow dotted circle). The defect of the diaphragm was covered with the great omentum, and the thoraco abdominal wall defect was reconstructed by a musculo-cutaneous flap including the anterior sheath and the left rectus abdominis muscle which both receive the blood supply comes from the superior abdominal artery (**B**).

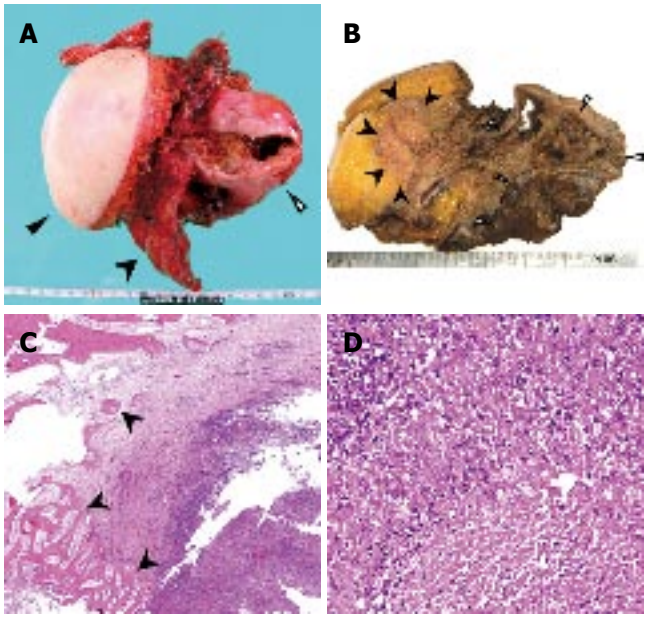

Figure 4 Macroscopically, the cystic mass directly invaded the intercostal space through the diaphragm (**A**. Resected crude specimen, black triangle; skin, black arrow head; ribs, white triangle; cystic tumor; **B**. Cut surface of the specimen, black arrow head; subcutaneous invasion, white triangle; cystic tumor). Microscopically, the bone was destroyed by an invasion of granuloma tissue (**C**). The granuloma was composed of epithelioid cells with necrotic areas (**D**).

ties for hepatic lesions including ultrasonography, CT, magnetic resonance imaging (MRI) and angiography[4]. Cystic lesions of the liver, which occupy a large part of hepatic lesions and are seen in up to 5% of the population, can be classified as developmental, neoplastic, inflammatory, or miscellaneous lesions. If a solid component develops inside the cyst, the possibility of a malignant transformation should be considered[5]. However, even routine imaging procedures such as CT or MRI generally fail to differentiate hepatic granulomas from other neoplasms[2].

Aspiration cytology can help in making a preoperative diagnosis, however, it is sometimes difficult to accurately hit the target tumor under US guidance, and this procedure also carries a risk of potentially causing needle-track and peritoneal seeding if the lesion is a malignant tumor[6, 7].

A wide variety of underlying conditions such as sarcoidosis[8], malignant lymphoma[9], tuberculosis[2], and HCV infection[10,11] can cause hepatic granulomas, with resulting prognostic and therapeutic implications. However, the histological features of such granulomas are not distinctive, while a specific etiological agent often cannot be identified despite serological, immunological microbiological, and radiological investigations, thus often resulting in a diagnosis of "idiopathic" hepatic granulomas. Although the exact etiology and pathogenesis of this aggressively infiltrating granuloma seen in the present case remains unclear, we speculated that an unidentified organism infection possibly occurred via two different routes: (1) the endogenous route, via the blood stream; (2) the exogenous route: a reverse infection through a drainage catheter when aspiration had previously been performed.

A spontaneous regression occurs in some cases of hepatic granulomas, therefore, patients can be treated by simple observation or conservative therapy with anti-inflammatory drugs including antibiotics or steroids in most cases. In the present case, the fact that destroyed and necrotic tissue caused inflammatory reactions suggested that empirical antibiotic therapy would be ineffective, to make matters worse, such conservative treatment could have possibly induced the development of such antibiotic-resistant bacteria as Methicillin-Resistant Staphylococcus Aureus (MRSA). In addition, steroid treatment is generally effective for sarcoidosis, however, it risks exacerbating tuberculosis and an earlier paper described a patient who died from miliary TB after the administration of empirical steroids [12].

Great improvements in operative techniques, perioperative patient management, and patient selection criteria have not made a hepatic resection one of the standards and effective methods for liver cysts[13]. As a result, a hepatectomy is recommended for cystic lesions when a tumor of the liver with a potential malignancy cannot be ruled out[14,15].

In the present case, the clinical course in which a solid component developed during the follow-up of a liver cyst, while infiltrating into the subcutaneous tissue with the destruction of the ribs was highly suggestive of the development of a malignancy, such as cystadenocarcinoma. The pathological diagnosis turned out to be a cancer-free lesion, namely, benign granuloma. Despite the highly invasive capacity of this lesion, the good clinical outcome in this case supports the suitability of a hepatic resection with the total removal of the affected areas as the treatment of choice.

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CASE REPORT

Acute pancreatitis associated with peroral double-balloon enteroscopy: A case report

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Abstract

A 58-year-old Japanese man had tarry stool and severe anemia. Neither upper nor lower gastrointestinal (GI) endoscopy showed any localized lesions. Thus, the source of his GI bleeding was suspected to be in the small intestine, and he underwent peroral double-balloon enteroscopy (DBE) using EN-450T5 (Fujinon-Toshiba ES System Co., Tokyo, Japan). There were no lesions considered to be the source of GI bleeding. After the procedure, the patient began to experience abdominal pain. Laboratory tests revealed hyperamylasemia and abdominal computed tomography revealed an inflammation of the pancreas and the peripancreas. He was thus diagnosed to have acute pancreatitis. Conservative treatments resulted in both clinical and laboratory amelioration. He had no history of alcohol ingestion, gallstone disease or pancreatitis. Magnetic resonance cholangiopancreatography demonstrated no structural alterations and no stones in the pancreatobiliary ductal system. As his abdominal pain started after the procedure, his acute pancreatitis was thus thought to have been related to the peroral DBE. This is the first reported case of acute pancreatitis probably associated with peroral DBE.

Key words: Enteroscopy; Small intestine; Pancreatitis; complication

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INTRODUCTION

The small intestine is located quite far from both the mouth and the anus, and thus it has been difficult to be examined by endoscopy until recently. However, capsule endoscopy and double-balloon enteroscopy (DBE), new methods of enteroscopy, have been introduced in recent years which have now made the observation of the entire small intestine possible.

DBE is a novel technique which was developed by Yamamoto *et al*, in Japan[1-3], and is spreading also in other countries. Both a 200-cm enteroscope and a 145-cm overtube which have a soft latex balloon at their tips are included in the system. Both balloons can be inflated or deflated by a balloon-pump controller which thus allows for a shortening of the proximal intestine, thus allowing the enteroscope to be inserted into the deep small intestine. Currently, two different diameter enteroscopes, EN-450P5/20 (Fujinon-Toshiba ES System Co., Tokyo, Japan) with an outer diameter of 8.5 mm (an overtube with an outer diameter of 12.2 mm) and EN-450T5 with an outer diameter of 9.4 mm (an overtube with an outer diameter of 13.2 mm) are available. With a larger accessory channel, EN-450T5 allows the performance of a variety of enteroscopic treatments including argon plasma coagulation/ablation, coagulation/biopsy (hot biopsy) and balloon dilatation. The indications of DBE are for the diagnosis and the treatment of small intestinal diseases. DBE is quite useful for the diagnosis of the origin of obscure gastrointestinal (GI) bleeding in the small intestine and for the endoscopic evaluation and histopathological diagnosis from the biopsy samples of small intestinal lesions such as ulcers, tumors and strictures. It is also useful for the treatment of small intestinal diseases, for example, the management of active GI bleeding and polypectomy of the small intestinal polyps. The technique of DBE is considered to be safe as only few complications related to sedation and original diseases have been reported previously[4,5].

In this paper, we report a case that developed acute pancreatitis after peroral DBE. To our knowledge, this is the first report which describes a case of acute pancreatitis probably associated with peroral DBE.

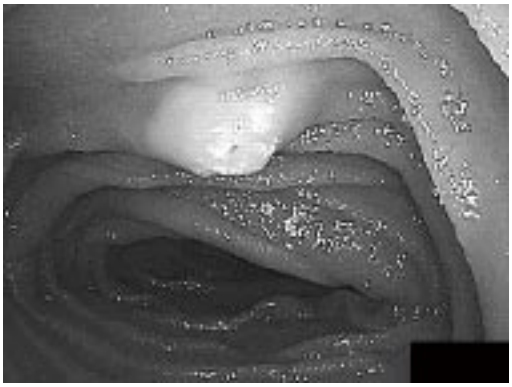

Figure 1 Peroral double-balloon enteroscopy revealed a small yellowish submucosal tumor with white specks on the surface in the upper jejunum.

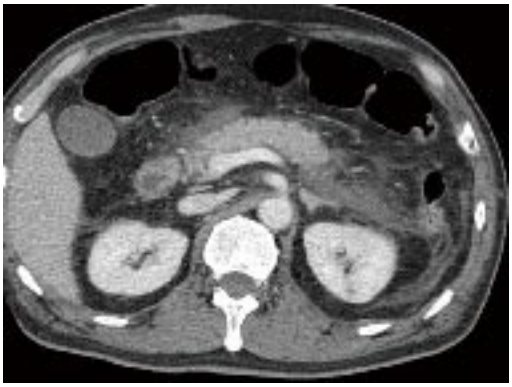

Figure 2 Abdominal CT showed inflammation and swelling of the pancreas, and a blurring of the fat planes in both the peripancreas and the left anterior pararenal space.

CASE REPORT

A 58-year-old Japanese man had tarry stool and syncope and was hospitalized by ambulance during the previous month. Since laboratory tests revealed severe anemia (hemoglobin level: 77 g/L, normal 140-180 g/L), he received red blood cell transfusion. Neither upper nor lower gastrointestinal GI endoscopy showed any localized lesions. Barium meal radiography of the small intestine and computed tomography (CT) also failed to show any lesions. As a result, the source of his GI bleeding was suspected to be a small lesion in the small intestine such as angiodysplasia, small ulcer or erosion, and he was thus referred to our hospital. Thirty days had already passed from the day when he had tarry stool and his hemoglobin level recovered up to 114 g/L. On the day of his admission, written informed consent was obtained from him and he underwent peroral DBE using EN-450T5. The total time of the procedure was 100 min. At the most distal site, the small intestinal mucosa was marked with a tattoo by a submucosal injection of sterilized ink through an injection catheter. There was a small submucosal tumor, which had a yellowish surface with white specks, located in the upper jejunum (Figure 1), which was considered to be a lymphangioma because of its typical image. But it was not considered to be the source of GI bleeding because there was no ulceration or erosion on its surface. After finishing the procedure, the patient began to experience abdominal pain. An abdominal examination revealed moderate epigastric tenderness and silent bowel sounds. His laboratory findings were as follows: white blood cells, 13 100/μL (normal 3 500-9 000 /μL); C-reactive protein, 2.37 mg/dL (normal < 0.10 mg/dL); serum amylase, 702 U/L (normal 50-159 U/L). Abdominal CT revealed an inflammation of the pancreas and the peripancreas (Figure 2). He was thus diagnosed to have acute pancreatitis. The patient was treated by total parenteral nutrition with intravenous rich fluids and gabexate. These treatments resulted in both clinical and laboratory amelioration and he started oral intake on day 16. Since then, his condition was good and he underwent peranal DBE on day 26. No lesions were observed on the anal side of the small intestine although the mark with a tattoo was not detected. It probably disappeared since it has been 26 d from the day of peroral DBE. Hence, his GI bleeding source remained unknown. He was discharged on day 30. His hospitalization was extended for more than 10 d because of pancreatitis after peroral DBE. The grade of his pancreatitis was diagnosed as severe according to the criteria of Cotton *et al*[6].

He had no history of alcohol ingestion, gallstone disease or pancreatitis. Magnetic resonance cholangiopancreatography on day 18 demonstrated no structural alterations and no stones in the pancreatobiliary ductal system. As his abdominal pain started after the procedure, his acute pancreatitis was thus thought to have been related to the peroral DBE.

DISCUSSION

Newly developed methods of enteroscopy, capsule endoscopy and DBE, are quite useful for the detection of the origin of GI bleeding in the small intestine. Capsule endoscopy causes minimum discomfort to patients because the patients are free after loading a recorder and taking a capsule orally. But in Japan, experience with capsule endoscopy is limited as it has not yet been approved for the clinical use at the present time and thus it can be used in only limited facilities as clinical studies. DBE is currently available in Japan. We therefore performed DBE in this case but we could not find a lesion which caused GI bleeding. We might have a greater chance to find a responsible lesion if we could perform DBE when the patient had a active bleeding. We chose EN-450T5 as we considered the possibility of the treatment of a lesion with hemorrhage by argon plasma coagulation and hemostatic clipping. The patient unexpectedly developed acute pancreatitis after the DBE procedure.

Regarding the safety of DBE, May *et al*[4] reported that the only complications observed were related to sedation in 248 enteroscpies (including 153 peroral enteroscopies). Yamamoto *et al*[5] demonstrated that two complications, mainly associated with the original diseases, occurred among 178 procedures (including 89 peroral enteroscopies). One was multiple perforations occurred in a patient with intestinal lymphoma who received chemotherapy and the other was in a patient who was discovered to have Crohn's disease and had postoperative fever and abdomi-

nal pain. In the above two studies, EN-450P5/20 was used and no instances of acute pancreatitis were reported. Up to now, we have performed peroral DBE using EN-450P5/20 on 13 cases (one case reached to the ascending colon), and no previous cases demonstrated pancreatitis. This was the first case in which we performed peroral DBE using EN-450T5. The thicker diameter of EN-450T5 might therefore be associated with the development of pancreatitis.

A few cases developing pancreatitis have been reported as a complication of upper or lower GI endoscopy without ampullary cannulation[7-9] although GI endoscopy is not known to be associated with an increased risk for pancreatitis. The underlying mechanism of pancreatitis in these cases is unclear. In case of upper GI endoscopy, it was possible that trauma to the papilla during duodenal intubation might have led to edema of the papilla and subsequent pancreatitis. In case of colonoscopy, it was possible that over insufflation of the colon might have caused pressure of the pancreas or compression of the colonoscope against the spine might have caused a direct trauma to the pancreas. The mechanisms of developing pancreatitis associated with peroral DBE were thought to be as follows: the one may be a pancreatic duct obstruction due to the direct oppression of the papilla by an overtube and the other may be an increase in the intralumenal pressure of the duodenum by a thick overtube and shortening technique.

Here we reported a case of acute pancreatitis that was thought to be associated with DBE. This case indicates that, when peroral DBE is performed, especially with EN-450T5, we have to keep in mind the possibility of developing pancreatitis. And if a patient complained a strong abdominal pain during and after the procedure, we have to rule out acute pancreatitis by examining serum amylase level and abdominal CT as soon as possible.

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CASE REPORT

Living-related liver transplantation for multiple liver metastases from rectal carcinoid tumor: A case report

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Abstract

A 42-year-old woman was admitted to our hospital because of multiple liver tumors detected by ultrasonography. Colonoscopy revealed submucosal tumor in the rectum, which was considered the primary lesion. Endoscopic mucosal resection followed by histopathological examination revealed that the tumor was carcinoid. The resected margin of the tumor was positive for malignant cells. Two courses to transcatheter arterial chemotherapy for liver metastasis were ineffective. Accordingly, the rectal tumor and metastatic lymph nodes were surgically resected. One month after the operation, she received liver transplantation (left lateral segment and caudate lobe) from her son. No recurrent lesion has been observed at two years after the liver transplantation. Liver transplantation should be considered as a treatment option even in advanced case of carcinoid metastasis to the liver. We also discuss the literature on liver transplantation for metastatic carcinoid tumor.

Key words: Carcinoid; Liver metastasis; Living related liver transplantation

Nakajima Y, Takagi H, Sohara N, Sato K, Kakizaki S, Nomoto K, Suzuki H, Suehiro T, Shimura T, Asao T, Kuwano H, Mori M, Nishikura K. Living-related liver transplantation for multiple liver metastases from rectal carcinoid tumor: A case report.

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INTRODUCTION

Carcinoid tumors are slow-growing neuroendocrine tumors, and often metastasize to the liver. There is no established treatment for liver metastases and the prognosis is poor[1, 2]. Liver transplantation for metastatic neuroendocrine tumor has already been reported worldwide[3-16], but the procedure is rarely performed in Japan[17]. We report here a case of living-related liver transplantation for liver metastases of rectal carcinoid tumor.

CASE REPORT

A 42-year-old woman was admitted to our hospital because of multiple liver tumors detected by ultrasonography. The medical history included bronchial asthma. There was no history of blood transfusion. Physical examination revealed a hard and swollen liver in the upper abdomen. Laboratory tests showed erythrocyte count of $367 \times 10^4/mm^3$ (normal: $400\text{-}500 \times 10^4/mm^3$), hemoglobin 10.5 g/dL (11.8-15.1 g/dL), leukocyte count $8200/mm^3$ ($4000\text{-}9600/mm^3$), platelet count $25.9 \times 10^4/mm^3$ ($16.0\text{-}35.0 \times 10^4/mm^3$), serum albumin 4.2 g/dL (3.9-5.0 g/dL), total bilirubin (T-Bil) 1.0 mg/dL (0.3-1.2 mg/dL), aspartate aminotransferase (AST) 30 IU/L(13-33 IU/L), alanine aminotransferase (ALT) 39 IU/L (6-27 IU/L), alkaline phosphatase (ALP) 311 IU/L (115-359 IU/L), γ-glutamyl transpeptidase (γ -GTP) 137 IU/L (10-47 IU/L), blood urea nitrogen (BUN) 20.0 mg/dL (8.0-20.0 mg/dL), and creatine (Cr) 0.9 mg/dL (0.6-1.0 mg/dL). Hepatitis B surface and hepatitis C virus antibody were negative. Serotonin and 5-HIAA in serum were within the normal range. Carcinoembryonic antigen, CA19-9, alfa-fetoprotein and protein induced by vitamin K antagonist (PIVKA)-II were normal but neuron-specific enolase (NSE) was elevated 46.1 ng/mL (0-10.0 ng/mL).

Abdominal ultrasonography (US) revealed multiple hyperechoic masses in both lobes of the liver (Figure 1A). Abdominal computed tomography (CT) also revealed multiple liver tumors enhanced mildly (Figure 1B). Abdominal angiography showed hepatomegaly and multiple liver tumors supplied by the hepatic artery. Colonoscopy showed

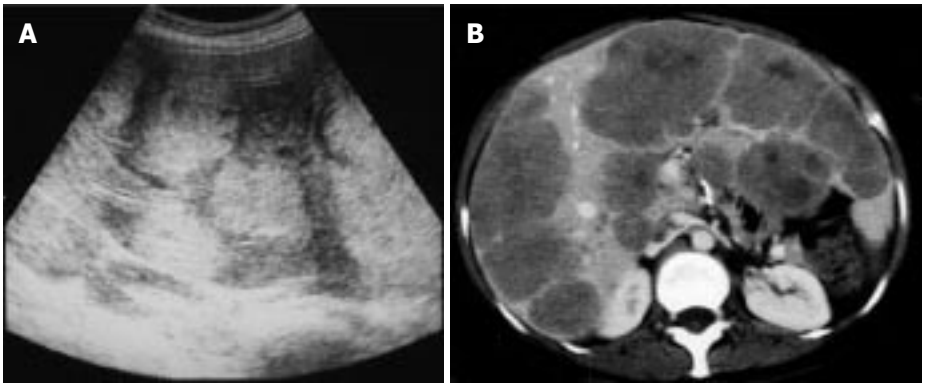

Figure 1 A: Ultrasonography showed multiple hyperechoic masses in the liver; **B**: Dynamic computed tomography showed multiple liver tumors. The surfaces of these tumors showed mild enhancement.

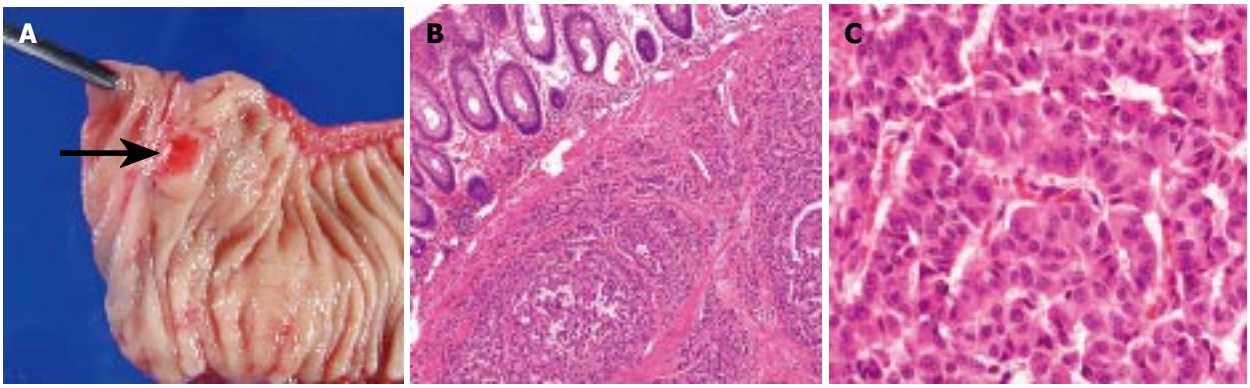

Figure 2 A: Macroscopic findings of the resected rectum. Arrow shows the primary lesion; **B**: The tumor cells showed tubular and alveolar formation, and their nuclei were slightly swelling (**C**). (**B**, **C**: H&E, original magnification, **B**: x 40, **C**: x 400).

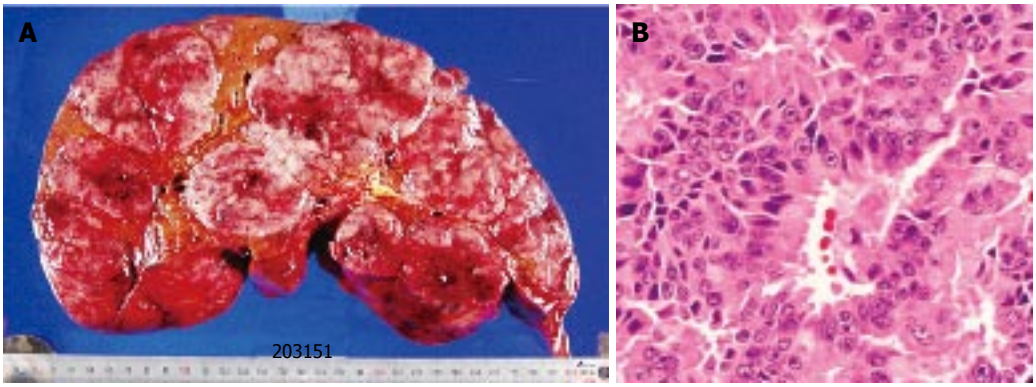

Figure 3 A: The cut surface of the resected specimen showed multiple tumors; **B**: Histopathological findings of the liver tumor were similar to those of the rectal tumor (H&E, original magnification, x 400).

submucosal tumor in the rectum. This tumor appeared as a low echoic mass by endoscopic ultrasonography (EUS). Then we performed endoscopic mucosal resection (EMR) for the submucosal tumor of the rectum, which was histopathologically diagnosed as carcinoid tumor. The resected margin of the tumor was positive for malignant cells. Transcatheter arterial chemotherapy for liver metastasis was applied twice (first: 5-fluorouracil [5-FU] + epirubicin [EPI] + mitomycin C [MMC], second: 5-FU + methotrexate [MTX]), but was ineffective because the liver tumors did not decrease in size and even grow 2 mo after the TACE. Accordingly, the rectal tumor and metastatic lymph nodes were resected surgically. Macroscopically, the rectal tumor was an elevated lesion with a central depression, measuring 58 mm in a diameter (Figure 2A). Histopathological examination showed atypical cells forming tubular and alveolar structures, with slightly swollen nuclei (Figures 2B and 2C). Lymph node metastases and blood vessel invasions were detected. Immunohistochemical examination revealed that most tumor cells were stained with chromogranin A, NSE and synaptophysin. The Ki-67 index was 6.1, but p53 protein was negative.

One month after the operation, there were no recurrence except for the liver. She received a liver transplantation (left lateral segment and caudate lobe) from her son. Standard liver volume (SLV) was 1014.4 g, graft volume (GV) was 450 g, GV/ SLV ratio was 44.4% and graft-to-recipient weight ratio (GR-WR) was 0.98. The volume of the resected liver was 4750 g, and multiple nodules of white and brown colors occupied the whole liver (Figure 3A). The histopathological findings of the liver tumor (Figure 3B) were similar to those of the primary lesion.

Table 1 Literature review of liver transplantation for metastatic neuroendocrine tumors

Reference	Number of patients	Median follow-up (m)	Survival rate (%)			Disease-free survival rate (%)	
			1-yr	3-yr	5-yr	1-yr	5-yr
Rosenau *et al* (2002)[3]	19	38	89	89	80	56	21
Coppa *et al* (2001)[4]	9	39	100	100	70	100	53
Lehnert *et al* (1998)[5]	103	-	68	54	47	60	24
Le Treut *et al* (1997)[6]	31	25	59	47	36	45	17
Florman *et al* (2004)[7]	11	30	73	48	36		
Cahlin *et al* (2003)[8]	10	28	80	80	-	-	-
Olausson *et al* (2002)[9]	9	22	89	89	-	-	-
Ringe *et al* (2001)[10]	5	18	80	80	-	-	-
Pascher *et al* (2000)[11]	4	42	100	75	50	-	-
Frilling *et al* (1998)[12]	4	54	50	50	50	-	-
Lang H *et al* (1997) [13]	12	49.5	83	83	83	-	-
Dousset *et al* (1996)[14]	9	29	33	33	33	-	-
Anthuber *et al* (1996)[15]	4	11	25	0	0	-	-
Routley *et al* (1995)[16]	11	-	82	-	57	-	-
Japan (2005)[17]	6	-	66.7	66.7	-	-	-

No invasion of the portal vein, hepatic vein, and bile duct was noted.

Her clinical course has been good and no recurrence has been demonstrated two years since the liver transplantation.

DISCUSSION

Neuroendocrine tumors have generally been classified by the site of origin. Furthermore, a new histopathological classification was reported by WHO[18]. The WHO classification has been considered by the size of the tumor, the depth of the tumor invasion, angiogenesis, lymphatic invasion, cellular atypia, necrosis, mitoses, Ki-67 index and p53 protein. Based on this classification, neuroendocrine tumors are divided into three types, well-differentiated endocrine tumor (carcinoid), well-differentiated endocrine carcinoma (malignant carcinoid), and poorly-differentiated endocrine carcinoma (small cell carcinoma)[18]. Although the standard therapy for liver metastasis of neuroendocrine origin is surgical resection[19], the prognosis of neuroendocrine tumor with liver metastasis is usually poor[1, 2]. When curative hepatic resection is difficult, transcatheter arterial chemo-embolization (TACE) and intra-arterial chemotherapy are performed and have been reported to be effective[20-23]. Our patient received two courses of intra-arterial chemotherapy but no satisfactory response was observed. Somatostatin analogue and interferon have been used for the treatment of carcinoid tumors[24-27]. These therapies are excellent for improvement of symptoms but the tumor response rate is usually low[24-27].

Liver transplantation has been widely performed in patients with end-stage liver disease and metastatic liver cancers from neuroendocrine tumors[3-6]. The 5-year survival rate of transplant recipients for neuroendocrine tumors metastases to the liver ranges from 0 to 83% (median, 50%) (Table 1). The main cause of death is recurrence of the carcinoid tumors. In Japan, the accumulative living related liver transplantations between 1996 and 2002 are more than 2000. Among these, transplantation was performed in only 6 cases of metastatic neuroendocrine tumors (0.2 %) and the 3-year survival rate is 66.7%[17]. Strictly speaking, the 5-year survival rate of liver transplantation for metastatic carcinoid tumor is 69% but is poor in noncarcinoid neuroendocrine tumors (4-year survival rate, 8%)[6]. Thus, histopathological discrimination is very important to predict the prognosis of neuroendocrine tumors. Our patient had a typical carcinoid tumor which is compatible with well-differentiated neuroendocrine carcinoma, with metastases in the liver and was considered to show good prognosis after transplantation. However, the case was advanced stage with lymph node metastasis, lymphatic and vascular invasion and extensive liver metastasis, and thus was considered a high recurrent risk requiring careful follow-up. Unexpectedly good prognosis of this case could be related to the radical resection of the tumor including primary and metastatic lesion and not classified in poor prognosis such as non-pancreatic primary lesion (rectum) and noncarcinoid apudoma[6]. The prognosis was markedly improved by transplantation and she remains well 2 years and 9 mo after surgery without local recurrence and metastasis. Although much longer follow-up period would provide more meaningful information to elucidate the prognosis of such unusual case, liver transplantation could be life-saving procedure for patients with metastatic neuroendocrine tumor resistant to alternative treatments.

Pelosi *et al*[28] reported that Ki-67 index is a significant predictor of prognosis and survival of patients with pancreatic neuroendocrine tumors. Furthermore, Moyana *et al*[29] reported that MIB-1 and p53 were associated with metastasis of the gastrointestinal carcinoid tumors. Rosenau *et al*[3] pathologically investigated patients who received a liver transplantation for metastatic neuroendocrine tumors. They reported that the survival rate of patients with high Ki-67 index (> 5%) or overexpression of the E-cadherin was low, and suggested that Ki-67 index and E-cadherin expression could be potentially useful prognostic markers after liver transplantation[3]. Our pa-

tient showed moderately positive Ki-67 index (6.1%, rate for carcinoid is around 2-3%) and should be followed as high recurrence risk case.

The Japanese medical insurance system covers liver transplantation for liver cirrhosis and hepatocellular carcinoma (HCC) based on Milan criteria[30]. However, the system does not cover metastatic liver cancer. Liver transplantation is a kind of special treatment for the end-stage liver disease and is also expensive. So, not all the patient in the end-stage liver disease has been covered by medical insurance in Japan. In our patient, distant and lymph node metastases were completely resected, and metastatic neuroendocrine tumors in the liver were removed through hepatectomy and liver transplantation even though the metastasis was far advanced within the liver though localized in the liver. We propose that metastatic neuroendocrine tumors of the liver should be classified as similar to HCC although cases beyond Milan criteria[30], like our case, could be also included in such classification because of its biological low malignant character.

In conclusion, we reported a female patient who underwent successful living liver transplantation for advanced liver metastases of rectal carcinoid tumor. She has been well for the last two postoperative years and remains alive without any recurrence in spite of positivity of poor prognostic parameters. Other parameters, such as oncogene, suppressor gene and cyclin shown in hepatocellular carcinoma[31], apart from those of histopathology and immunohistochemistry, are needed to help in clinical decision making with respect to the indications of transplantation.

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CASE REPORT

Autoimmune pancreatitis: Functional and morphological recovery after steroid therapy

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Abstract

Autoimmune pancreatitis, a recently recognized type of chronic pancreatitis, is not rare in Japan, but reports of it elsewhere are relatively uncommon. We report the first preoperatively diagnosed case of autoimmune pancreatitis in Hungary, which responded well to steroid treatment and provided radiographic and functional evidence of this improvement. A 62-year-old female presented with a 4-month history of recurrent epigastric pain and a 5-kg weight loss. The oral glucose tolerance test (OGTT) indicated diabetes mellitus and the result of the fecal elastase test was abnormal. Ultrasonography (US) and the CT scan demonstrated a diffusely enlarged pancreas, and endoscopic retrograde cholangiopancreatography (ERCP) an irregular main pancreatic duct with long strictures in the head and tail. Autoimmune pancreatitis was diagnosed. The patient was started on 32 mg prednisolone daily. After 4 wk, the OGTT and faecal elastase test results had normalized. The repeated US and CT scan revealed a marked improvement of the diffuse pancreatic swelling, while on repeated ERCP, the main pancreatic duct narrowing was seen to be ameliorated. It is important to be aware of this disease and its diagnosis, because AIP can clinically resemble pancreatobiliary malignancies, or chronic or acute pancreatitis. However, in contrast with chronic pancreatitis, its symptoms and morphologic and laboratory alterations are completely reversed by oral steroid therapy.

Key words: Autoimmune pancreatitis; Steroid therapy; Chronic pancreatitis; Pancreatic endocrine function; Pancreatic exocrine function

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INTRODUCTION

Autoimmune pancreatitis (AIP) is an increasingly recognized type of chronic pancreatitis that is clearly distinct from alcoholic chronic pancreatitis. It is characterized by its special morphology, immunologic features, pathology and steroid responsiveness[1-5]. Since Sarles *et al*[6] first described a case of pancreatitis with hypergammaglobulinaemia more than 40 years ago, several hundred cases have been reported, particularly in the Japanese literature[1-4,7-9]. Its prevalence in other populations is unclear.

We describe here the first preoperatively diagnosed case of AIP in Hungary and discuss the clinical features, diagnosis and treatment of this uncommon disease.

CASE REPORT

A 62-year-old teetotal woman with a 4-month history of recurrent epigastric pain and a 5-kg weight loss was referred to our hospital. There was no personal or family history of pancreatitis. On physical examination, an apple-sized, painless mass was palpated in the epigastrium. With the exception of elevated fasting glucose level, the results of detailed laboratory examinations, including Ca 19-9 antigen, were normal. The oral glucose tolerance test (OGTT) revealed diabetes mellitus. The fecal elastase test result was abnormal (120 μg/g faeces). The antinuclear antibody and rheumatoid factor tests were positive. Abdominal ultrasonography (US) revealed a diffusely enlarged pancreas, and the abdominal CT scan an enlarged, sausage-shaped pancreas (Figures 1A and 1B). On endoscopic retrograde cholangiopancreatography (ERCP), an irregular main pancreatic duct with long strictures in the head and tail was observed (Figure 2A). AIP was diagnosed. The patient was started on 32 mg prednisolone daily and enzyme replacement therapy. After 4 weeks she had become totally asymptomatic. The fasting glucose level, the results of OGTT and the fecal elastase test had normalized, and the repeated US and CT scan demonstrated a marked improvement of the diffuse pancreatic swelling (Figures 1C and 1D). On repeated ERCP, the main pancreatic duct narrowing was seen to be ameliorated (Figure 2B). We ad-

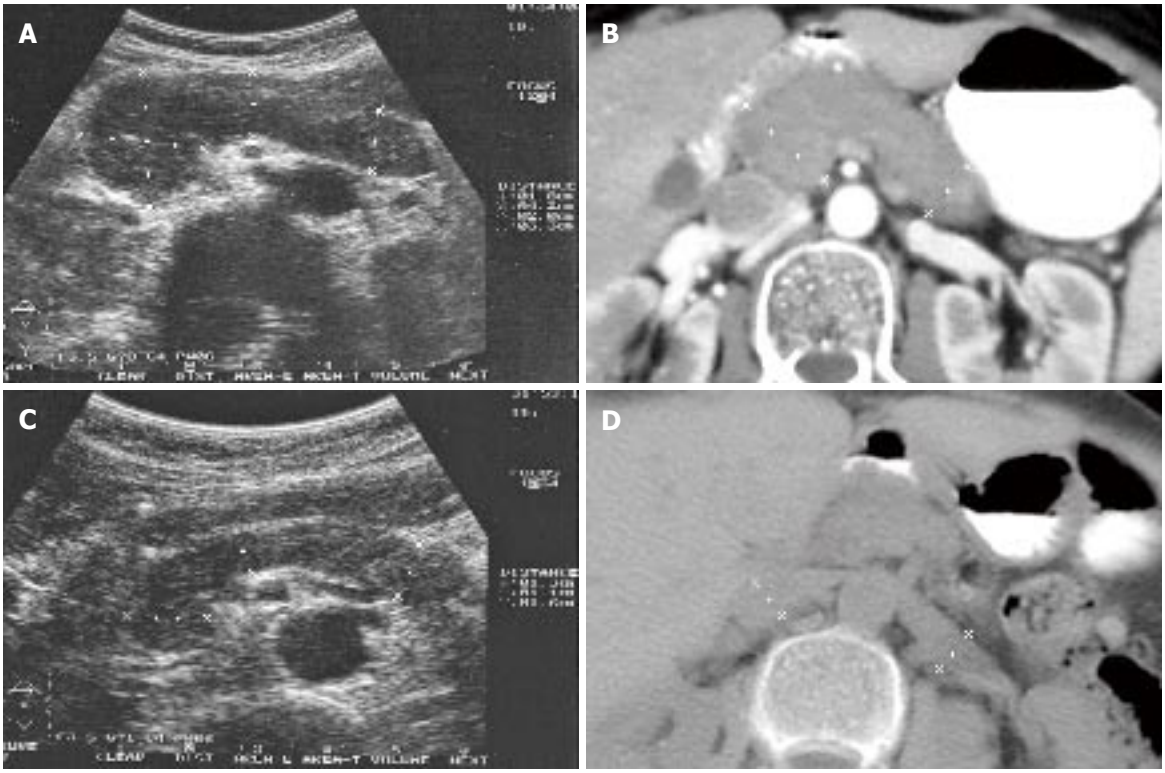

Figure 1 Abdominal ultrasonography (**A**) and CT (**B**) revealed a diffusely enlarged pancreas with sharp margins similar to sausage appearance. Four weeks after the steroid therapy the size of the pancreas became normal (**C** and **D**).

vised tapering and cessation of the steroid.

DISCUSSION

To date, several hundred cases of AIP have been reported, particularly in the Japanese literature[1-5,7-9]. This has led to the proposal of AIP as a distinct clinical entity, and AIP is currently categorized as an established etiology of chronic pancreatitis[10]. The disease has appeared outside Japan only very rarely; few cases have been published in Europe[11-16], most of which were diagnosed postoperatively. It is unlikely that AIP is a disease endemic to Japan. It appears likely that physicians in other countries may overlook this entity due to the lack of their familiarity with it, and regard such patients as cases of ordinary or idiopathic chronic pancreatitis, or pancreatobiliary malignancy.

The diagnosis of AIP is challenging as its symptoms are few and non-specific. Its clinical characteristics are jaundice, abdominal pain, weight loss and diabetes mellitus. Most of the patients are older than 50 years. In consequence of the symptoms and the age of these patients, they are often initially thought to have pancreatic carcinoma, and many of them actually undergo laparotomy[5,17]. AIP may be associated with a number of other autoimmune diseases, such as Sjögren's syndrome, primary sclerosing cholangitis, inflammatory bowel disease, systemic lupus erythematosus or retroperitoneal fibrosis. Laboratory findings suggestive of AIP include hypergammaglobulinaemia, increased levels of total IgG or IgG4, and the presence of autoantibodies such as antinuclear antibody, anti-lactoferrin antibody and anti-CA-II antibody[1-5]. Characteristic radiological features are an enlarged, sausage-shaped pancreas on abdominal US and CT, and narrowing of the main pancreatic duct on ERCP[18]. A common microscopic picture in AIP is periductal lymphoplasmacytic infiltration with pronounced acinar atrophy, marked fibrosis and obliterated phlebitis[19].

The diagnosis of AIP should be based on a combination of the clinical and laboratory findings, pancreatic imaging, and the exclusion of other conditions[4].

The principal treatment is corticosteroid at a recommended starting dose of 30 to 40 mg of prednisolone/day until the symptoms improve; this is followed by a taper of 5 to 10 mg. Steroid treatment usually leads to a rapid improvement in the clinical symptoms and the laboratory and morphological findings. Steroid therapy has also been reported to be beneficial as concerns the pancreatic fibrosis, exocrine and endocrine functions and associated diabetes mellitus[7-9,20]. It has been proposed that the response to steroid therapy should be included in the diagnostic criteria[21]. Steroid therapy may be tried empirically, when AIP is suspected on the basis of the typical pancreatic imaging results alone, if laboratory data and histologic findings are not available or do not fully meet the diagnostic criteria[5, 7] The response to steroid therapy can facilitate confirmation of the diagnosis of AIP.

In our patient, the diagnosis of AIP was indicated by the morphological alterations of the pancreas in US and CT, the narrowing of the main pancreatic duct on ERCP, and the autoimmune abnormalities. Histological findings were not available. The clinical symptoms and the abnormalities observed on US, CT and ERCP responded rapidly to steroid therapy, confirming the diagnosis of AIP.

It is very important to be aware of this disease, because

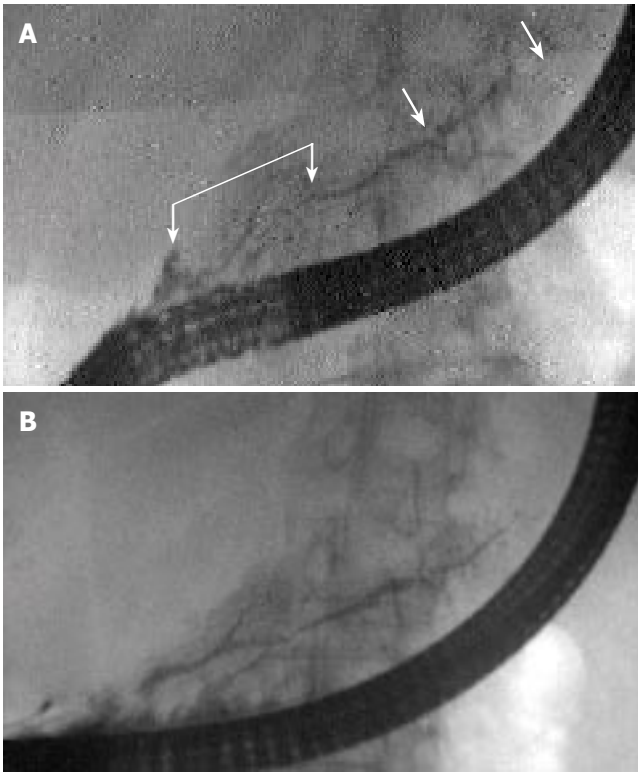

Figure 2 ERCP demonstrated multiple strictures of the Wirsung duct without posttenotic dilation (**A**). Four weeks after the steroid therapy the main pancreatic duct narrowing was seen to be ameliorated (**B**).

AIP may be mistaken for ordinary chronic pancreatitis or pancreatic cancer, leading to pancreatic resection instead of treatment with steroid. As awareness of this disease grows, the reported prevalence of AIP outside Japan may be expected to increase and the possibility of unnecessary surgery may be reduced.

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CASE REPORT

Sporadic somatic mutation of *c-kit* gene in a family with gastrointestinal stromal tumors without cutaneous hyperpigmentation

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Abstract

We described two members in a family with gastrointestinal stromal tumors (GISTs) without cutaneous hyperpigmentation. The patients were father and son who did not have cutaneous hyperpigmentation. Histological examination showed that these tumors were GISTs expressing CD34 and CD117. Tumor DNA extracted from paraffin-embedded specimens revealed somatic mutation with a deletion mutation at different codons in exon 11 of c-kit gene after direct sequencing analysis. No germline mutation was detected in DNA extracted from peripheral leukocytes obtained from the father and son. We propose that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and hyperpigmentation.

Key words: Sporadic GIST; Somatic c-kit mutation

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the human gastrointestinal (GI) tract, representing 0.1 to 3 % of all GI tract tumors[1]. It has been suggested that a mutation in the juxtamembrane (JM) domain of c-kit contributes to the development of GIST[2]. Furthermore, germline deletion or point mutation of the c-kit JM domain has been shown in a family with GIST and cutaneous hyperpigmentation[3,4]. GIST-cutaneous hyperpigmentation disease has been used to describe familial multiple GISTs with associated cutaneous hyperpigmentation[4]. Familial GIST is a rare autosomal dominant genetic disorder associated with kit germline mutations. We now report that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and cutaneous hyperpigmentation.

CASE REPORT

Patient 1

A 79-year-old man was referred to our hospital because of a gastric tumor (Figure 1). He complained of epigastria pain and fullness. There was no cutaneous hyperpigmentation. Gastroduodenal endoscopic examination and endoscopic ultrasonography (EUS) showed a gastric submucosal tumor (SMT) about 1.7 cm in size with small ulcer located at anterior wall, greater curvature side of high body. Proton pump inhibitor therapy was administered for 10 months. The SMT enlarged to 5cm in size demonstrated by upper gastrointestinal (GI) series, endoscopic ultrasonography (EUS), and abdominal computed tomography (CT) (Figure 2A). Wedge resection of the gastric SMT at the anterior wall, greater curvature side of high body with spleen preservation was performed with clear resected margin. Histological and immunohistochemical examination revealed low risk gastrointestinal stromal tumor (GIST) with strong positivity of c-kit and CD34 (Figure 2B). The patient lived free of disease for 9 months after surgery.

Patient 2

A 54-year-old man, the son of the patient 1, was referred to our hospital because of a gastric tumor (Figure 1). He complained of epigastria pain, abdominal fullness, body weight loss 7 to 8 kilograms for one month. There was no cutaneous hyperpigmentation. Gastroduodenal endoscopic examination and EUS showed a huge gastric SMT about 10 cm in size located at greater curvature side from high body to antrum with a deep ulcer and easily touch bleeding. Subsequent upper GI series and abdominal CT revealed a gastric SMT about 13×10 cm in size occupying the whole stomach (Figure 2C). Exploratory laparotomy revealed advanced gastric GIST with major vessel involvement. Biopsy of the gastric SMT and feeding jejunostomy

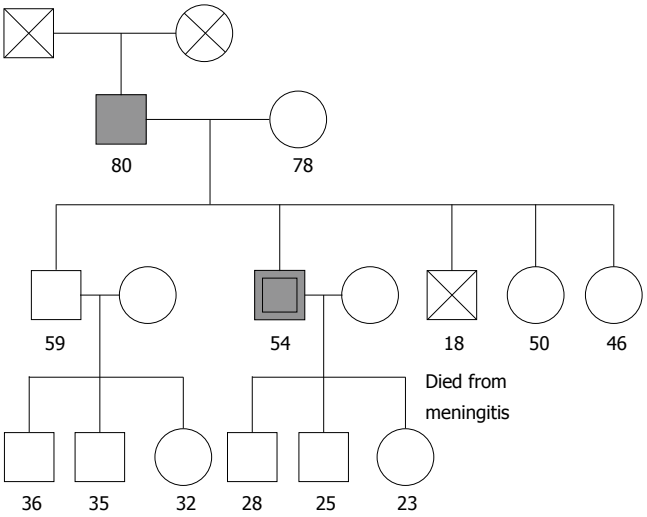

Figure 1 Pedigree of the family with GISTs without cutaneous hyperpigmentation. Hatched symbols indicate family members with GISTs. Double circles denote multiple GISTs. Squares indicate males and circles indicate females.

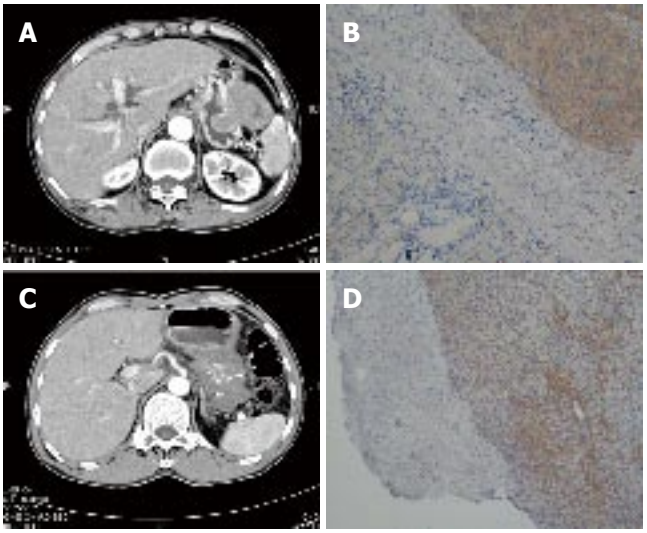

Figure 2 A: Abdominal CT revealed gastric GIST at the anterior wall and greater curvature side of high body of the stomach; **B**: Microscopic findings of the tumor resected. Tumor cells were composed of resicular spindle cells with mild nuclear pleomorphism but no necrosis, expressing strong positive c-kit staining immunohistochemically. (IHC staining, 200X); **C**: Abdominal CT revealed a gastric GIST measuring 13 cm x 10 cm in size occupying the whole stomach. **D**: Microscopic findings of the tumor resected. Tumor comprised proliferation of spindle cells with mild nuclear atypia in the mxyoid stroma but no necrosis, expressing positive c-kit staining. (IHC staining, 200X).

was performed. Histological and immunohistochemical examination revealed advanced high-risk GIST with strong positivity of c-kit and CD34 (Figure 2D). The patient received 400 mg Glivec for 7 months and partial response was illustrated by subsequent abdominal CT. The patient lived with disease for 7 months after surgery.

Genetic examination

To determine whether these GISTs showed a genetic defect, DNA was extracted from paraffin-embedded specimen of tumor tissues. Polymerase chain reaction amplification of genomic DNA for kit and PDGFRA was performed and amplification was analyzed for mutations as previously described[5]. Direct sequencing analysis of DNA from patient 1 showed deletion mutation at codon 560 in exon 11, causing a deletion mutant 560 del V (Figure 3B). While direct sequencing analysis of DNA from patient 2 revealed deletion at codons 557-559 in exon 11, resulting in replacement of WKV by C (Figure 3C). To determine if the mutation is familial, DNA was extracted from peripheral leukocytes obtained from patients 1 and 2. No mutation was detected in exon 11 of c-kit gene (Figure 3A).

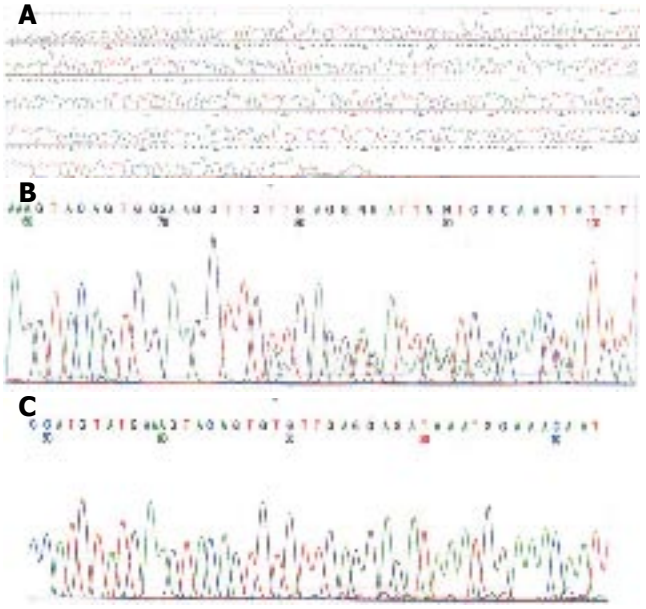

Figure 3 A. Direct sequencing analysis of DNA from peripheral leukocyte obtained from patients 1 and 2 revealed no mutation in exon 11 of c-kit gene; **B**. Direct sequencing analysis of DNA from patient 1 showed deletion mutation at codon 560 in exon 11, causing a deletion mutant 560 del V; **C**. Direct sequencing analysis of DNA from patient 2 revealed deletion at codons 557-559 in exon 11, resulting in replacement of WKV by C.

DISCUSSION

By histological, immunohistochemical examination, and molecular genetic analysis, this study has uncovered sporadic c-kit somatic mutation in a family with GIST without cutaneous hyperpigmentation.

GISTs appear to be related the interstitial cells of Cajal of the mesenteric plexus[7]. These cells are considered as GI pacemaker cells, from the interface between the automatic innervation of the bowel wall and its smooth muscle[8, 9]. GISTs express the cell-surface transmembrane receptor c-kit with a tyrosin kinase activity and kit oncoprotein. There are frequent gain-of-function mutations of c-kit in GISTs. These mutations result in constitutive activation of kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. It has been recently reported that kit activation occurs in all cases of GISTs, regardless of the mutation status of kit. Most GISTs express constitutively activated mutant isoforms of kit kinase or platelet-derived growth factor receptor alpha (PDGFRA), which are potential therapeutic targets for Imatinib mesylate (Glivec).

Gain of function mutations in the JM domain of c-kit contribute to the development of GIST[2]. Because normal kit gene is responsible to normal pigmentation, relationships between GISTs and cutaneous hyperpigmentation have been reported before. GIST-cutaneous hyperpigmentation disease has been used to describe familial multiple GISTs associated with cutaneous hyperpigmentation[3]. Furthermore, germline deletion mutation of the c-kit JM domain has been shown in tumors and normal somatic cells from a family with multiple GISTs who exhibited perineal hyperpigmentation[3]. A single-point germline mutation of c-kit has also been proposed to cause a familial GIST associated with systemic cutaneous hyperpigmentation[4]. A germline PDGRF missense mutation could be a second familial predisposing gene[5].

We described a rare case report regarding two members in a family with GISTs without cutaneous hyperpigmentation. No mutation was detected in DNA extracted from peripheral leukocytes obtained from the father and son. DNA extracted from paraffin-embedded specimens revealed different somatic mutation with a deletion mutation in the exon 11 of c-kit gene after direct sequencing analysis (deletion mutation at codon 560 versus deletion at codons 557-559 in exon 11). So mutation in the tumor is sporadic somatic but not germline. However, the cause of sporadic c-kit mutation in one family is unknown.

In summary, we propose that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and hyperpigmentation.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
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Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
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www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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Berlin
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
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cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
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International Conference on Surgical Infections, ICSI2006
6–8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
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10th World Congress of the International Society for Diseases of the Esophagus
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World Journal of Gastroenterology standard of quantities and units

Number	Nonstandard	Standard	Notice
1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku,18 500u or M_r18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μmol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD_{260}	A_{260}	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance instatistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If there is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	