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EDITORIAL

Co-localization hypothesis: A mechanism for the intrapancreatic activation of digestive enzymes during the early phases of acute pancreatitis

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Abstract

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment. This review focuses on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. The findings, summarized in this review, support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

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Key words: Acute pancreatitis; Digestive enzymes; Pancreatic acinar cells

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INTRODUCTION

Acute pancreatitis is an inflammatory disease of the pancreas which, in its severe forms, leads to varying degrees of pancreatic necrosis. Passage of a biliary tract stone into or through the terminal biliopancreatic ductal system is the most frequent inciting cause for clinical acute pancreatitis and studies performed using an opossum model of biliary acute pancreatitis have suggested that the offending stone triggers pancreatitis by obstructing drainage from the pancreatic duct[1]. Other studies performed using that same model have indicated that the earliest changes of pancreatitis occur within the acinar cells of the pancreas and that periductal as well as perilobular changes, including cell injury and inflammation, occur at later times[2].

The morphological changes of acute pancreatitis suggest that an autodigestive process has occurred and, indeed, activated digestive enzymes can be detected within the pancreas during clinical acute pancreatitis. Activated digestive enzymes can also be detected within the pancreas during the very early stages of most experimental pancreatitis models. Since the pancreas normally synthesizes and secretes many potentially harmful digestive enzymes, it is generally believed that it is the premature, intrapancreatic activation of those pancreas-derived enzymes which, ultimately, leads to cell injury and acute pancreatitis. However, for the most part, the potentially harmful digestive enzymes that are synthesized and secreted by the pancreas are present, within the gland, as inactive pro-enzymes or zymogens and activation of those zymogens normally occurs only when they reach the duodenum. How, then, might these zymogens be prematurely activated within the pancreas during the early stages of acute pancreatitis and how might that activation lead to acinar cell injury/necrosis?

Not surprisingly, these questions have prompted the performance of many studies, using various experimental models of acute pancreatitis, over the past several decades and several hypotheses have been advanced which address these questions. One of those hypotheses is the so called "co-localization hypothesis" which suggests that, during the early stages of acute pancreatitis, pancreas derived

digestive zymogens become co-localized with lysosomal hydrolases in acinar cell cytoplasmic vacuoles and that, as a result of this co-localization, lysosomal hydrolases such as cathepsin B, activate trypsinogen. According to this hypothesis, trypsin then activates the other digestive enzyme zymogens and the activated digestive enzymes gain access to the cell interior leading to the cell injury/necrosis which typify acute pancreatitis[3].

This review will summarize current evidence that supports the co-localization hypothesis. To place this hypothesis in its proper context, we will begin by briefly summarizing current concepts regarding the physiological synthesis, intracellular trafficking, and secretion of certain types of proteins by the exocrine pancreas. This will be a highly selective review which will primarily draw upon work performed by many collaborators in the authors' laboratory over the past 25 years and the contributions of those individuals are gratefully acknowledged.

PANCREATIC ACINAR CELL BIOLOGY

Protein assembly

On a "gram of protein-per-cell" basis, pancreatic acinar cells synthesize more protein than any other cell in the body. Digestive enzymes, which are secreted from the pancreas and designed to act within the gastrointestinal tract, account for most of the protein mass synthesized by acinar cells but acinar cells also synthesize many other types of proteins, including the hydrolytic enzymes that are designed to digest un-needed material within intracellular lysosomes. Both types of enzymes, secretory digestive enzymes and lysosomal hydrolases, are assembled on ribosomes attached to the rough endoplasmic reticulum (ER). As they are assembled, they elongate within the cisternae of the rough ER until a signal peptide is added and, at that point, they are cleaved freeing them to assume their tertiary structure within the ER. Many secretory digestive enzymes are synthesized as pro-enzymes or zymogens which are inactive until they are proteolytically processed following secretion. Many of the lysosomal hydrolases are also synthesized as inactive pro-enzymes but their post-translational processing and activation is accomplished within the cell.

Intracellular trafficking of secretory proteins[4]

Following their assembly within the ER, the enzymes destined for secretion are carried within small transport vesicles to the Golgi stacks where they are sorted and some are post-translationally modified. Those that are destined to be secreted in a constitutive manner are then transported to the cell surface where they exit the cell. Most of the secreted digestive enzymes and their zymogens, however, are subject to regulated discharge - i.e. they are stored within the cell until their secretion is accelerated in response to extracellular stimuli. Following their assembly and transport to the Golgi, these proteins traverse the Golgi stacks and they are then packaged within condensing vacuoles which evolve into zymogen granules as they mature and migrate towards the luminal surface of the cell. There, by mechanisms which are regulated by neurohormonal secretagogue stimuli, fusion/fission of the zymogen granule membrane with the luminal plasmalemma occurs and this leads to the formation of a fusion pore which permits the granule contents to be discharged into the extracellular (i.e. ductal) space. In an overall sense, this regulated pathway allows for the massive and rapid discharge of intracellularly stored proteins which, in the case of pancreatic acinar cells, consists primarily of digestive enzymes[5].

Intracellular trafficking of lysosomal hydrolases[6]

The lysosomal hydrolases are a group of more than 50 dissimilar acid hydrolases which function to degrade un-needed cellular material as well as material taken up by cells via either endocytosis or phagocytosis. As noted for other proteins, lysosomal hydrolases are assembled within the cisternae of the rough ER and, in common with other newly synthesized proteins, the nascent lysosomal hydrolases are transported to the Golgi complex which they traverse from its *cis* to its *trans* surface, passing through the *medial* and *trans*-Golgi subcompartments. In contrast to proteins destined for either secretion or transport to other intracellular sites, the lysosomal hydrolases are sorted from other newly synthesized proteins as they traverse the Golgi stacks by a complex mechanism in which *N*-acetylglucosamine phosphates are added to mannose residues and then the *N*-acetylglocosamine groups are removed, leaving the remaining mannose residues phosphorylated at the 6-position. Those mannose-6-phosphorylated lysosomal hydrolases are then bound to receptors in the *trans*-Golgi that specifically recognize mannose-6-phosphate residues. The mannose-6-phosphate receptors along with their associated lysosomal hydrolases are then shuttled to the pre-lysosomal compartment where, as a result of the acidic pH of this compartment, dissociation of the hydrolase/receptor complexes occurs, thereby liberating the hydrolases within the pre-lysosomal compartment and allowing the now unoccupied receptors to return to the Golgi where they are free to bind and transport additional mannose-6-phosphorylated lysosomal hydrolases. Of potential significance however, is the fact that, even under physiological conditions, sorting of lysosomal hydrolases from secretory proteins in the Golgi is incomplete and, as a result, a fraction of the newly synthesized lysosomal hydrolases enters the secretory pathway[7-9]. This fraction of the lysosomal hydrolases is subject to regulated secretion from the cell along with other secretory proteins[10]. Another fraction of lysosomal hydrolases may also be secreted from the cell by being enclosed within so-called secretory lysosomes[11]. Lysosomal hydrolases may also be constitutively secreted from the cell without ever being packaged within lysosomes.

Many of the lysosomal hydrolases are initially synthesized as inactive or only partially activated pro-enzymes. Their complete activation is achieved through post-translational processing of the pro-peptide as it undergoes intracellular transport to pre-lysosomes[12]. Together, pre-lysosomes, lysosomes, endosomes, phagosomes, and autophagosomes function as an

interconnected network of organelles which contain a wide variety of acid hydrolases capable of degrading nucleic acids, proteins, carbohydrates, and lipids.

Activation of digestive enzyme zymogens

Some of the digestive enzymes (e.g. amylase, lipase, DNAase, RNAase) are synthesized and secreted from pancreatic acinar cells as active enzymes but others, including most of the potentially harmful digestive enzymes (e.g. trypsin, chymotrypsin, phospholipase, elastase, carboxypeptidase) are synthesized as inactive pro-enzymes or zymogens. Under physiological conditions, activation of these zymogens does not occur until they reach the duodenum where the brush border enzyme enterokinase (enteropeptidase) catalytically activates trypsinogen and trypsin then catalyzes the activation of the other zymogens. In most instances, activation involves cleavage of the zymogen and release of an "activating peptide" which, prior to its release, had maintained the zymogen in its inactive state. Thus, quantitation of free activating peptide levels may provide information regarding the extent of zymogen activation prior to that measurement[13].

Protective mechanisms

In a general sense, the acinar cells of the pancreas are protected from the injury which might be inflicted by premature, intracellular activation of trypsinogen and other digestive enzyme zymogens by virtue of 3 features. The first, as noted above, is the fact that most of the potentially harmful digestive enzymes are normally present within acinar cells as inactive zymogens. The second is the fact that potent inhibitors of trypsin are synthesized and co-transported through the cell along with trypsinogen and those inhibitors are available to dampen any trypsin activity that might arise as a result of intracellular trypsinogen activation. The third protective feature of pancreatic acinar cells is the fact that, throughout their intracellular trafficking within the cell, digestive enzymes and their zymogens are sequestered from the remainder of the cell by being enclosed within membrane bounded organelles. Very recently, two reports have appeared that suggest that acinar cells may have yet another protective feature. Pancreatic acinar cells express proteinase-activated receptor-2 (PAR2) which is a tethered ligand receptor that is activated by trypsin and, as shown by Namkung *et al*[14] and Sharma *et al*[15], activation of pancreatic PAR2 triggers events that protect the pancreas from pancreatitis.

THE CO-LOCALIZATION HYPOTHESIS

History

The so-called "co-localization hypothesis" grew out of studies, performed more than two decades ago, that employed the diet-induced and the secretagogue-induced models of acute experimental pancreatitis[16-20]. Those studies had shown that digestive enzyme synthesis and intracellular transport continue during the early stages of pancreatitis, but secretion of newly synthesized digestive enzymes from acinar cells is blocked. Those studies had also shown that, prior to the appearance of evidence reflecting acinar cell injury, the normal segregation of digestive enzyme zymogens from lysosomal hydrolases is perturbed and, as a result, both types of enzymes become co-localized within intra-acinar cell cytoplasmic vacuoles. That co-localization phenomenon could be demonstrated using techniques of immunolocalization as well as using techniques of subcellular fractionation. Finally, those studies had shown that digestive enzyme zymogens, including trypsinogen, are activated within acinar cells at very early stages during the evolution of experimental pancreatitis-i.e. prior to the appearance of cell injury. Since the lysosomal hydrolase cathepsin B is known to be capable of activating the digestive zymogen trypsinogen[21,22] and, since trypsin can activate the other digestive zymogens, these various observations suggested the following 3-tiered hypothesis: (a) that perturbation of normal intracellular trafficking of digestive zymogens and lysosomal hydrolases, is a very early event during the evolution of pancreatitis and that, as a result of this perturbation, digestive enzyme zymogens become co-localized with lysosomal hydrolases within acinar cell cytoplasmic vacuoles; (b) that, as a result of this co-localization phenomenon, the lysosomal hydrolase cathepsin B (and, most likely, other lysosomal hydrolases) activates trypsinogen and trypsin activates the other zymogens; and (c) that the organelles containing intracellulary activated digestive zymogens become fragile and they release their content of activated enzymes within the cell interior where those enzymes trigger changes leading to cell injury/death[23].

Challenges

Subsequent to its initial proposal, the co-localization hypothesis was challenged on two grounds. The first was the fact that, while most lysosomal hydrolases have a pH optimum of roughly 5.0, the site of co-localization during the early stages of pancreatitis appears to have a neutral pH. Subsequent studies characterizing the pH optimum for cathepsin B, however, indicated that considerable (although perhaps not optimal) activity was also present at pH 7.0[24] and, thus, this concern regarding the co-localization hypothesis appears to be unwarranted. The second challenge to the hypothesis, however, proved to be more substantial because it focused on the issue of causality. Its advocates suggested that the co-localization phenomenon, although real, might be a response to the early injury of pancreatitis. They argued that the co-localization of digestive enzymes with lysosomal hydrolases might simply be a reaction to injury, rather than causing this injury[25]. Some even suggested that cell injury-induced co-localization might be a protective response which could allow lysosomal hydrolases to inactivate and degrade those prematurely activated digestive zymogens[26].

THE EVIDENCE THAT CO-LOCALIZATION LEADS TO ZYMOGEN ACTIVATION IN PANCREATITIS

The remainder of this review will be devoted to an

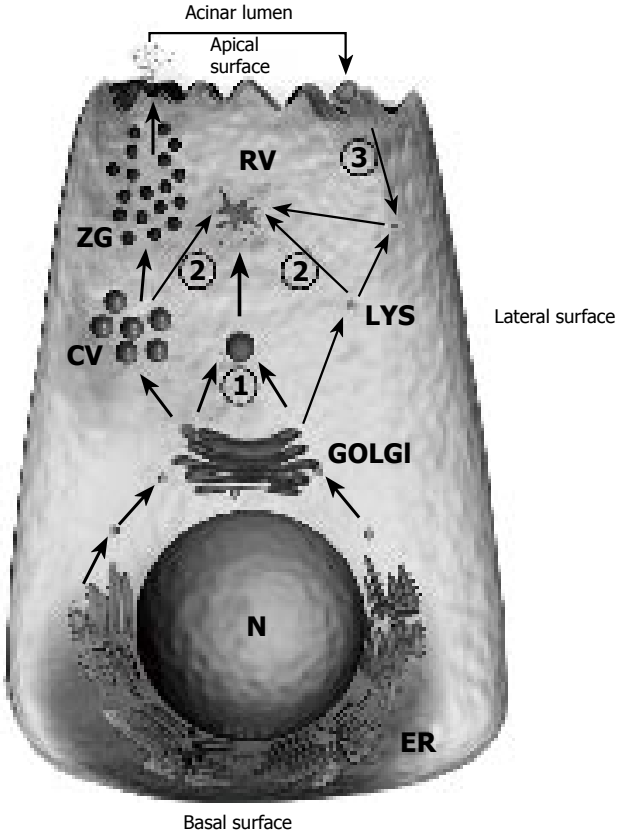

Figure 1 Acinar cell demonstrating mechanisms of co-localization. Arrows represent physiological and pathological pathways. Co-localization can occur as a result of (1) missorting of lysosomal hydrolases from digestive zymogens in the Golgi, (2) fusion of zymogen granules with lysosomes and (3) reuptake into the lysosomal compartment of secreted digestive zymogens. Note the ruptured co-localization vacuole (RV) and release of activated digestive enzymes inside the cell. (ER = endoplasmic reticulum, N = nucleus, LYS = lysosomes, CV = condensing vacuoles and ZG = zymogen granules).

examination of the evidence which suggests that the co-localization phenomenon is not simply a reaction to injury or a protective response but that it is, in fact, an early and critical event which leads to zymogen activation and acinar cell injury in pancreatitis. Ideally, studies addressing these issues would be performed using clinically derived material and patients with acute pancreatitis but, unfortunately, those studies are not possible since patients with acute pancreatitis are only identified after the very early phases of the disease have been completed and access to the pancreas of patients with early acute pancreatitis is generally not possible. Thus, by necessity, studies testing the co-localization hypothesis have all been performed using various models of acute pancreatitis in experimental animals.

Validation of the co-localization hypothesis would require fulfillment of the following 6 postulates: (1) The co-localization phenomenon should be observed in all experimental models of acute pancreatitis. (2) Lysosomal hydrolases should be capable of activating digestive enzyme zymogens such as trypsinogen. (3) During the evolution of pancreatitis, the co-localization phenomenon should occur prior to zymogen activation and cell injury. (4) The co-localization phenomenon and digestive zymogen activation should occur within the same intracellular compartment. (5) Digestive zymogen activation should be dependent upon the presence of lysosomal hydrolase activity. (6) Prevention of co-localization should interfere with zymogen activation but prevention of zymogen activation should not interfere with co-localization.

The evidence supporting fulfillment of these 6 postulates will now be summarized.

The co-localization phenomenon occurs in all models of experimental pancreatitis

As noted above, early studies performed using the diet-induced and the secretagogue (caerulein)- induced models of experimental pancreatitis and employing the techniques of both subcellular fractionation and immunolocalization, indicate that the co-localization phenomenon occurs in both of those models[16-20,9]. Subsequent studies using the rabbit model of duct obstruction[27], the closed duodenal loop model[28], and the taurocholate model of retrograde duct injection[29], indicate that the co-localization phenomenon occurs in each of those models as well. In each of these models, co-localization is an early event which can be demonstrated to occur prior to the appearance of cell injury/necrosis.

The mechanism(s) responsible for the co-localization phenomenon in each of these models appears to be different (Figure 1). In the diet-induced model, it reflects fusion of zymogen granules with lysosomes (i.e. crinophagy) while, in the secretagogue-model, it reflects both crinophagy and defective sorting of lysosomal hydrolases away from secretory proteins because of a defect in processing lysosomal hydrolases prior to their passage through the medial Golgi compartment. In the opossum model, co-localization occurs because previously secreted digestive zymogens are taken up by acinar cells via endocytosis and then transported to the endosome/lysosome compartment.

Lysosomal hydrolases such as cathepsin B can activate trypsinogen

Studies independently performed and reported by at least 3 groups of investigators have shown that cathepsin B can activate trypsinogen under in-vitro conditions[21,22,24]. The most recent of those studies evaluated the pH profile of this activation process and found that a considerable degree of activation could occur at pH 7.0[24].

The co-localization phenomenon occurs prior to acinar cell injury/necrosis

If the co-localization phenomenon is a reaction to cell injury, it should occur after changes indicative of that injury are already demonstrable while, if it is the cause of cell injury, it should occur prior to the appearance of that injury. Time-dependence studies using the secretagogue-induced model have been performed to evaluate this issue[30]. In these studies, pancreatic tissue was homogenized and subcellular fractionated to obtain zymogen granule-enriched and lysosome-enriched fractions at selected times after the start of caerulein administration. Redistribution of cathepsin B from the lysosome-enriched to the zymogen granule-enriched fraction was noted within

15 minutes after the start of caerulein administration and, at roughly this same time, evidence of trypsinogen activation (i.e. increased trypsin activity and increased pancreatic levels of trypsinogen activation peptide) was also first detected. In contrast, hyperamylasemia, pancreatic edema and acinar cell injury/necrosis were only detected at later times. Thus, these studies indicate that the co-localization phenomenon occurs prior to, and not after, cell injury in pancreatitis.

Co-localization and zymogen activation occur within the same intracellular compartment during pancreatitis

Studies addressing this issue have been performed using antibodies directed against the lysosomal hydrolase cathepsin B and antibodies directed against the peptide released during the process of trypsinogen activation (i.e. trypsinogen activation peptide, TAP). During the early stages of secretagogue-induced pancreatitis, both types of antibodies were found localized in the same cytoplasmic vacuoles[31,9,20]. Similar observations have been made in studies using antibodies directed against lysosomal membrane proteins[32]. In both cases, the site of co-localization appeared to be in the area of the *trans*-Golgi network. Taken together, these immunolocalization studies support the conclusion that co-localization and activation occur within the same intracellular compartment.

Zymogen activation and pancreatitis severity are dependent upon cathepsin B activity

This issue has been addressed in two ways. In one, cathepsin B inhibition was achieved using the cell permeant and highly specific cathepsin B inhibitor CA-074me. The effect of this inhibitor in two dissimilar models of pancreatitis (secretagogue-induced and taurocholate-induced) was evaluated[33] and, in both models, inhibition of cathepsin B was found to prevent trypsinogen activation. That inhibition was also found to reduce the severity of pancreatitis. The second approach to this issue involved the use of genetically modified mice that did not express cathepsin B and, in those mice, both trypsinogen activation and the severity of pancreatitis were found to be reduced[34]. These observations support the conclusion that cathepsin B (either alone or, perhaps with other lysosomal hydrolases) plays a critical role in trypsinogen activation during pancreatitis and that, in pancreatitis, trypsinogen activation is closely related to the severity of the disease.

Prevention of the co-localization phenomenon interferes with zymogen activation but inhibition of zymogen activation does not prevent co-localization

The co-localization of digestive zymogens with lysosomal hydrolases in both the secretagogue-induced and the taurocholate-induced models of pancreatitis is dependent upon the activity of phosphoinositide-3-kinase (PI3K) and co-localization can be prevented in those models by administration of the PI3K inhibitors wortmannin and LY49002. In a recently reported study, administration of those PI3K inhibitors was found to reduce trypsinogen activation[35]. These findings indicate that prevention of co-localization, by administration of PI3K inhibitors, can reduce zymogen activation in these models of pancreatitis.

As noted above, CA-074me is a potent in-vivo cathepsin B inhibitor and administration of CA-074me to animals can prevent trypsinogen activation during pancreatitis. To further evaluate the possible causative role of co-localization in zymogen activation, mice subjected to secretagogue-induced pancreatitis were given CA-074me and the effect of inhibiting trypsinogen activation (via inhibition of cathepsin B) on the co-localization phenomenon was evaluated (Van Acker *et al*, submitted for publication). Under these conditions, co-localization of lysosomal hydrolases with digestive enzymes was still observed even though zymogen activation had been prevented. Taken together, these findings are consistent with the conclusion that zymogen activation is dependent upon the co-localization phenomenon and they are inconsistent with the theory that zymogen activation leads to the co-localization phenomenon.

SUMMARY

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment.

This review has been focused on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. Indeed, there is considerable evidence that, under appropriate conditions lysosomal hydrolases such as cathepsin B are capable of activating digestive enzyme zymogens such as trypsinogen. Normally, that activation does not occur because, under physiological conditions, newly synthesized lysosomal hydrolases are segregated from digestive enzyme zymogens as the two types of enzymes traffic through acinar cells. However, during pancreatitis, that segregation is perturbed and lysosomal hydrolases become co-localized with digestive enzyme zymogens within cytoplasmic vacuoles. Evidence supporting the co-localization hypothesis as an explanation for intracellular zymogen activation include the following: (a) the co-localization phenomenon is observed in virtually all of the experimental models of pancreatitis that have been studied; (b) lysosomal enzymes such as cathepsin B can activate digestive zymogens such as trypsinogen and trypsin can activate the other zymogens; (c) the co-localization phenomenon occurs prior to the appearance of cell injury/necrosis during pancreatitis; (d) co-localization and zymogen activation occur within the

same intracellular compartment; (e) zymogen activation is dependent upon the presence of lysosomal enzyme (i.e. cathepsin B) activity; and finally, (f) preventing the co-localization phenomenon prevents zymogen activation during pancreatitis but preventing zymogen activation does not, necessarily, prevent the co-localization phenomenon. Taken together, these findings support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

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REVIEW

Novel susceptibility genes in inflammatory bowel disease

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Abstract

The inflammatory bowel disease, Crohn's disease and ulcerative colitis, are polygenic disorders with important environmental interactions. To date, the most widely adopted approach to identifying susceptibility genes in complex diseases has involved genome wide linkage studies followed by studies of positional candidate genes in loci of interest. This review encompasses data from studies into novel candidate genes implicated in the pathogenesis of inflammatory bowel disease. Novel techniques to identify candidate genes-genome wide association studies, yeast-two hybrid screening, microarray gene expression studies and proteomic profiling, are also reviewed and their potential role in unravelling the pathogenesis of inflammatory bowel disease are discussed.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Yeast- two hybrid; Genomics

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INTRODUCTION

Following the identification of the NOD2/CARD15 gene in 2001[1,2], the impetus has grown to identify novel genetic determinants of susceptibility and phenotype in the inflammatory bowel diseases, Crohn's disease and ulcerative colitis. The inflammatory bowel disease are now considered to be non-Mendelian polygenic disorders with important environmental interactions[3], and as such the accepted approach to identifying candidate genes in complex disease genetics has been adopted, namely genome wide scanning followed by studies of positional candidate genes in subchromosomal regions of association (loci).

A number of candidate genes and susceptibility loci have been discussed elsewhere in this issue, so in this review we first summarise recent data with regards to genome wide scanning and candidate gene studies, which may lead to gene identification. Novel techniques to identify candidate genes - genome wide association studies, yeast- two hybrid screening, microarray gene expression studies and proteomic profiling, are subsequently discussed.

GENE FINDING APPROACHES

Genome wide scan approach

Genome-wide linkage analysis using highly polymorphic microsatellite markers identified during the course of the Human Genome Project has led to success in identifying genetic determinants both in single gene disorders and in complex genetic diseases. To date, successful genome scans have typically involved several hundred microsatellite markers and a large number of multiply affected inflammatory bowel disease families (typically sibling pairs), with the aim of examining whether the degree of sharing variant alleles between affected individuals exceeds that as expected by chance alone[4].

To date, fourteen genome wide scans have been carried out in patients with inflammatory bowel disease. The most widely accepted guidelines for assessing the results are those defined in 1995 by Lander and Kruglyak who proposed criteria for reporting areas of linkage[5], with areas of 'suggestive linkage' having LOD scores of 2.2 and above and P values of less than 7×10^{-4}, areas of 'significant linkage' having LOD scores above 3.6 and P values of less than 2×10^{-5}, and areas of 'highly significant linkage' having LOD scores of 5.4 and above and P values of less than 3×10^{-7}. Areas of' confirmed linkage defined as areas of significant linkage have been replicated in an independent cohort, with a nominal P value of less than 0.01. Using these criteria, loci with confirmed linkage have been identified on chromosomes 1[6], 3 (IBD9)[7,8], 5 (IBD5)[9-11], 6 (IBD3; HLA)[10,12], 12 (IBD2)[13], 14 (IBD4)[9,14], 16 (IBD1)[13,15] and 19 (IBD6)[10] (Figure 1).

These confirmed regions of linkage need to be further narrowed by fine mapping of these areas, as each spans a large genomic region. The classical positional cloning approach is used by Hugot and colleagues to identify NOD2/CARD15 as the gene confers the critical mutations in IBD1 on chromosome 16[1]. Alternative strategies for fine mapping these regions or providing a short-cut to the critical genes are discussed in the following.

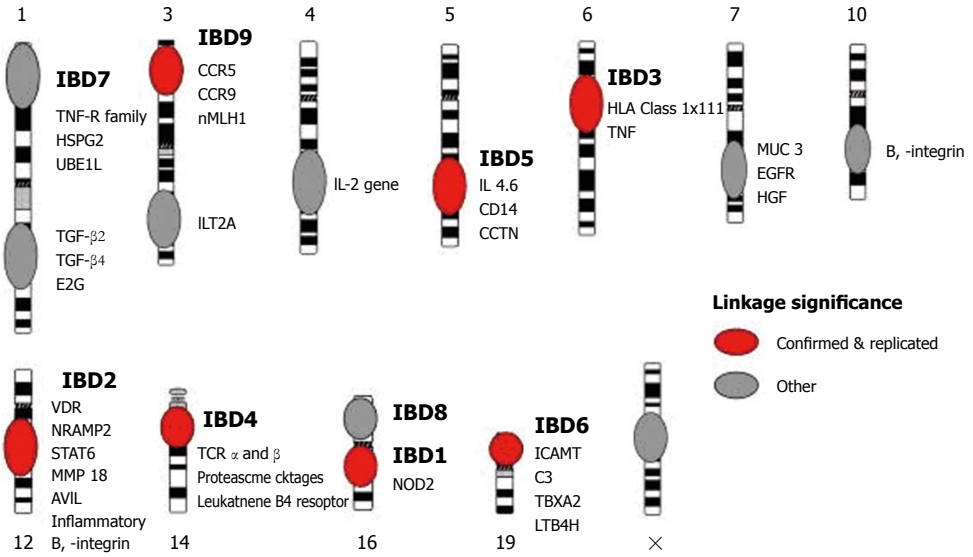

Figure 1 Confirmed and suggested linkage intervals from genome wide scans in inflammatory bowel diseases with the potential candidate genes coloured in red.

Significance levels defined by Lander and Kruglyak [95].

Positional candidate gene approach

The positional candidate gene approach to identifying association involves the use of data concerning the chromosomal location of the candidate gene as well as functional or expression data. In inflammatory bowel disease genes involved in the regulation of the innate immune system, mucosal integrity and cell-cell interactions are all clearly plausible candidate genes. By comparing the allelic frequencies of variants in these genes between patients with IBD and matched controls (case control analysis), or by investigating intra-familial association/ linkage, genes containing critical disease causing mutations may be identified.

Using this approach, Ogura and colleagues[2] identified the frameshift mutation in the NOD2/CARD15 gene (Leu1007fsinsC). Stoll and colleagues[16] also identified the DLG5 (Drosophila Discs Large Homolog 5) gene located on chromosome 10q23 by candidate gene approach allied to its position as being associated with inflammatory bowel disease in the German population[16].

However, whilst these approaches have had notable success, especially with the identification of the NOD2/CARD15 gene within the IBD1 locus, there have been difficulties in identifying candidate genes within other loci, notably the IBD5 locus as discussed elsewhere. Due to the tight linkage disequilibrium across the IBD5 locus and the wealth of candidate genes in the region[17], it has been impossible to identify with certainty the causative mutations in this region. Although Peltekova and colleagues[18] have suggested that the OCTN1/2 genes contain the causative mutations, considerably larger cohorts of inflammatory bowel disease patients (around 3000 patients) are needed to confirm these findings using genetic studies alone and functional and expression data can help delineate this region[19].

NOVEL GENES AND LOCI CURRENTLY UNDER INVESTIGATION

Genes involved in initiation and regulation of the innate immune system

The function of the immune system is to be able to recognise the vast number of antigens that are present in our environment, to mount an appropriate response to invasive pathogens and also to limit damage during inflammation and immune response to these pathogens. Dysregulation of the immune system can lead to immune-mediated diseases such as inflammatory bowel disease. Following the discovery of NOD2/CARD15, attention has focussed on the innate immune system[20]. From an evolutionary prospective, the innate immune response is more ancient than the adaptive immune response present in nearly all taxa[21]. As a further measure of the importance of the innate immune response in plants, genes involved in plant disease resistance occupy >1% of the genome of primitive species, such as *Rockcress*[22].

Toll like receptors (TLRs) are transmembrane proteins which play a pivotal role in mediating the innate immune response to viral, bacterial and fungal pathogens[23]. TLRs were initially identified in *Drosophila* and they are widely conserved across animal species[24]. In mammals TLRs are integral membrane glycoproteins which recognise conserved products unique to microbial metabolism and signal via a number of downstream signalling molecules (MyD88, Il-1R-associated kinases, TGF-β, and TNF- receptor associated factor 6)[25]. To date 11 members of the TLR family have been identified in mammals[24].

TLR4

TLR4 functions as a sensor for lipopolysaccharide (LPS), part of the cell membrane of Gram negative bacteria[26], and as such has been considered a strong functional candidate gene in the pathogenesis of inflammatory bowel disease as well as in other immunoinflammatory diseases. Variant alleles of the TLR4 Asp299Gly polymorphism have been associated with decreased bronchial responsiveness to LPS in humans and reduced activation in transfection experiments[27]. The Asp299Gly polymorphism has been associated with Crohn's disease and ulcerative colitis both in a cohort of Belgian patients[28] and in cohorts from Greece[29] (Table 1).

An association was observed between ulcerative colitis

Table 1 Number of patients studied and Toll-like receptor 4 Asp299Gly allele frequency (as a percentage of each group) in healthy controls, ulcerative colitis and Crohn's disease patients

	Healthy controls (%)	Ulcerative colitis (%)	Crohn's disease (%)	*P* value for controls versus Crohn's disease (NS: not significant)
Franchimont *et al*, Belgium (28)	139 (5.0)	163 (10.0)	334 (11.0)	0.007
Arnott *et al*, Scotland (32)	189 (8.8)	246 (6.8)	234 (10.3)	NS
Torok *et al*, Germany (30)	145 (4.0)	98 (9.0)	102 (7.0)	NS
Gazouli *et al*, Greece (29)	100 (3)	85 (3.5)	120 (7.9)	0.026
Brand *et al*, Germany (31)	199 (7.5)		204 (14.2)	0.038
Oostenbrug *et al*, Netherlands (33)	296 (4.6)	179 (5.9)	393 (6.7)	NS

These data from 6 European case control studies looking at the allelic frequencies of the Asp299Gly polymorphism of the TLR4 gene illustrate the contrasting results from these studies, and furthermore suggest a degree of genetic heterogeneity between the different populations.

and the TLR4 Thr399Ile polymorphism but not the Asp299Gly variant in one German cohort[30]. However, in a further cohort of German patients Asp299Gly variants were associated with Crohn's disease[31]. No association between inflammatory bowel disease and the TLR4 Asp299Gly polymorphism has been observed in cohorts of patients from Scotland[32]. In the light of these discordant data-sets, it is difficult to ascertain whether these two polymorphisms represent the critical disease causing mutations in the TLR4 gene[33]. Recent data from two independent mouse models have shown that treatment with TLR4 antagonists may prevent the development of colitis, further suggesting that this innate immune receptor may be central to the pathogenesis of inflammatory bowel disease[34].

TLR5

TLR5 functions by recognising flagellin, a common bacterial antigen that is present in enteric bacteria[35, 36]. Strong serological response to flagellin in multiple animal models of colitis has been observed by Lodes and colleagues[37], who demonstrated that colitis can be induced by transferring flagellin-specific T cells to immunodeficient animals. Further evidence implicating TLR5 polymorphisms in the pathogenesis of inflammatory bowel disease suggests that carriage of a dominant negative TLR5 polymorphism (TLR5-stop) appears to protect against Crohn's disease and results in a significant reduction in flagellin-specific circulating concentrations of IgA and IgG[38]. These data linking a genetic defect in TLR5 to an alteration in the acquired immune response are intriguing and of great pertinence as recent studies report synergism between NOD2/CARD15 and TLR5 signalling[39].

NOD-LLR protein family

NOD (nucleotide- binding oligomerization domain) - LLR (leucine rich region) proteins are part of the CATERPILLER (CARD, transcription enhancer, R [purine]- binding, pyrin, lots of purine repeats) family, and these proteins are key regulators of innate immunity and apoptosis in mammals and plants[40]. NOD- LLR proteins play a central role in recognising pathogens on the cell surface and in the cytosol, and a family of greater than twenty human proteins possessing a NOD domain has been identified[41]. NOD-LLR proteins are comprised of three distinct functional domains: the amino terminal effector domain involved in signalling, the centrally located NOD domain and the LLR ligand-sensing domain[42]. Three of the human NOD-LLR proteins have effector domains, namely caspase-recruitment domains (CARD): NOD1/CARD4, NOD2/CARD15 and Apaf-1.[40]

With the established role of NOD2/CARD15 in the pathogenesis of Crohn's disease, attention has focused on NOD1/CARD4 identified by two groups in 1999[43,44]. NOD1/CARD4 has a similar structure to NOD2/CARD15 with it having only one CARD domain, a central NOD domain and a LLR[45]. NOD1 detects intracellular diaminopimelic acid, a tripeptide motif found in many Gram negative bacteria and unique to Gram negative proteoglycans[46,47]. NOD1/CARD4 plays a role in colonic epithelial defence against the intracellular pathogens *E. coli* and *Shigella flexeneri*[48,49]. Its effector domain is associated with RipK2(a CARD-containing interleukin-1 beta-converting enzyme-associated kinase), thus mediating NFκB activation[43,44,50].

Linkage to the area that encompasses the NOD1/CARD4 gene, chromosome 7p14, was first found on a genome wide scan of the UK population in 1996[13]. This initial finding is replicated in two further genome wide scans involving patients in North America (USA and Canada)[6,10]. In a pan-European study involving 381 inflammatory bowel disease families from France, Sweden, Belgium, Spain, Denmark, Italy and Ireland (235 CD and 58 UC), no association between susceptibility to IBD and the E266K variant which lies within the CARD domain of NOD1/CARD4 was observed[51].

However, a further recent UK study of 556 IBD trios (294 CD and 252 UC) a significant association was observed between a complex insertion deletion allele (CCCCCCAC/ CCCCCCCCC) of NOD1/CARD4 at nucleotides 30, 258, 950 (Ensembl build 35) which is partially identified by rs6958571 and is located within intron 9, and predisposition to inflammatory bowel disease ($P = 0.002$)

and, also to ulcerative colitis ($P=0.01$)[52]. By creating a sliding two marker haplotype using this insertion deletion allele and the rs2907748 (T→ C) variant at nucleotides 30,246,263, the association was strengthened with *P* values of 3×10^{-6} for inflammatory bowel disease, 7×10^{-4} for Crohn's disease and 3×10^{-5} for ulcerative colitis. The deletion allele was significantly associated with an early age of onset of IBD (<25 years) and there was no evidence of an epistatic effect when NOD1/CARD4, NOD2/CARD15 and IBD5 are investigated.

Interestingly allelic variation of the same insertion deletion polymorphism (CCCCCCAC/ CCCCCCCCC) at nucleotides 30, 258, 950, which appear to alter the protein binding of NOD1/CARD4, is also associated with susceptibility to childhood onset of asthma and elevated serum IgE levels[53]. Further genetic and functional data with regards to NOD1/CARD4 are required to discover whether these variants play a role in the pathogenesis of inflammatory bowel disease in racially diverse populations.

CCL20

CC chemokine ligand (CCL) 20 is responsible for the chemoattraction of immature dendritic cells (DC) expressing the CCR6 receptor in the epithelium and Peyer's patches in the bowel mucosa[54]. The ligand- receptor pair of CCL20 and CCR6 also play a role in the chemoattraction of effector/memory T-cells and B-cells under homeostatic and inflammatory conditions, in diseases including cancer and rheumatoid arthritis[55]. Microarray and real-time PCR analysis of endoscopic colonic biopsies of patients with IBD have revealed increased levels of CCL20 mRNA in histologically inflamed biopsies when compared to non-inflamed IBD biopsies and inflamed non-IBD colonic biopsies[56,57].

Using microarray analysis of peripheral blood mononuclear cells in 8 patients with ulcerative colitis and 8 healthy controls, Choi and colleagues showed that the CCL20 gene is upregulated in ulcerative colitis samples[58]. Five SNPs in the promoter region of CCL20 are identified and analysed in a case control study of 118 ulcerative colitis patients and 300 healthy controls in the Korean population. Three of the CCL20 variants are significantly associated with ulcerative colitis ($P<0.0038$) and further replication data in ethnically diverse cohorts of patients with IBD are required.

Other loci

The identification of other genetic variants in loci identified by genome wide scan continues to yield other potential determinants of inflammatory bowel disease. These genes may play a role that is limited to specific populations and disease phenotypes. However, their identification can unravel the pathogenesis of inflammatory bowel disease and may reveal new therapeutic targets.

Other loci of current interest include the IBD2 locus on chromosome 12 first described in the UK[13] and subsequent analysis suggests that this locus is most strongly associated with colonic disease[59]. In the Flemish population the IBD4 region on chromosome 14 appears to be an important determinant of Crohn's disease[60] and this association has been replicated in the USA[14] and by the International IBD genetics consortium[61]. Fine mapping of the IBD2 and the IBD4 loci are required to establish which genes in these regions contain critical disease causing mutations.

NOVEL TECHNIQUES TO IDENTIFY CANDIDATE GENES

Genome wide association studies

With the advent of the HapMap project[62] and plans to include information on around 300 million genotypes, genome-wide association studies have been proposed as a powerful tool in identifying common variants that contribute to complex genetic traits[63]. Genome wide association studies compare the frequency of alleles and genotypes between cases and controls on a genome-wide scale, thus creating a comprehensive unbiased method to identify candidate genes. Advances in technology lowering the price of genotyping on a large scale and genome wide linkage disequilibrium mapping using data from the HapMap project have made this method available. However, significant concerns remain about the power of these studies and it has been proposed that levels of significance of $P<5\times10^{-8}$ are required to constitute significance in genome wide association studies[64].

Using this approach Tamiya and colleagues[65] placed 27 039 microsatellite markers across the human genome at 100kb intervals in 470 patients with rheumatoid arthritis and 470 controls. Forty seven candidate regions were identified and the previously implicated major histocompatibility complex gene HLA- DRB1 in the chromosomal region 6p21.3 was shown to be associated with rheumatoid arthritis. In the UK, the Wellcome Trust has provided initial funding for a phase I study including 1 000 patients with Crohn's disease and 3 000 controls, 675 000 SNPs will be genotyped to cover the entire genome and results are expected in 12 months.

Yeast two- hybrid screening

The yeast two-hybrid assay is an elegant means of investigating protein-protein interactions, which have become increasingly important in our understanding of biological systems and pathways. The yeast two- hybrid model can also be used to characterize interactions already known to occur, thus helping delineate the protein domains responsible for interaction and the environmental conditions involved[66].

The yeast two-hybrid assay is performed in the budding yeast, *S cerevisiae* using two fusion proteins: the target protein of interest, known as 'bait' is fused to a DNA binding domain attached to its N-terminus. The second protein, the 'prey' is fused to an activation domain. If the bait protein interacts with the prey, these bring the binding domain and the activation domain of transcriptional activator together, which in turn switches on the expression of the reporter genes. The reporter genes are constructed to allow growth of the yeast in a selective medium when the interaction occurs. When investigating 'protein- protein' interactions, a single bait protein is used to search for interaction with a library of proteins fused to the activation domain. The choice of library is determined

by the tissue of interest, e.g. intestinal cell library for inflammatory bowel disease[67].

Schizophrenia is a disease of polygenic genetic susceptibility where the yeast two hybrid systems have been successfully used to identify candidate genes. The disrupted-in- schizophrenia 1 (DISC1) gene was identified in 2 000[68] and confirmed in other cohorts,[69,70] was used as bait. DISC1 encodes a novel protein of unknown function and full-length human DISC1 protein was used to screen human adult and foetal brain libraries for interacting proteins, using the yeast two-hybrid system[71]. Twenty-one proteins from a variety of locations have been identified implicating DISC1 in several aspects of central nervous system signalling and confirming data from other yeast two- hybrid scans using DISC1 as bait[72,73]. From these data the authors are able to identify a number of potential DISC1 interactions and to speculate that DISC1 may be at the centre of an extensive protein interaction network.

Yeast two- hybrid as an investigative tool in inflammatory bowel disease

Barnich and colleagues[74] used NOD2/CARD15 gene as 'bait' to screen a bone marrow library and identified GRIM19, a protein with homology to the NADPH dehydrogenase which interacts with endogenous NOD2[74]. GRIM19 is required for NFκB activation following NOD2-mediated recognition of bacterial muramyl dipeptide and the authors hypothesised that GRIM19 is a key component of the CARD15/NOD2 signalling pathway which currently remains under detailed investigation[75-77]. In our own protein yeast two- hybrid studies a series of 12 candidate genes which interact with CARD15/NOD2 (*Nimmo, Satsangi,* unpublished data) have been identified.

GENOMICS

Microarray expression studies

Gene expression technology using microarray allows a comprehensive picture of gene expression at the tissue and cellular level, thus helping understand the underlying physiological and pathological processes. Microarray technology has developed from spotted nylon array technology used to identify genomic inserts in bacterial colonies by hybridisation with preidentified cDNAs[78]. In the seminal microarray experiment in 1995, a two-colour fluorescent pattern of differential gene expression is generated when comparing 48 genes in the root and the shoot of *Arabidopsis*[79].

Since 1995 there has been a rapid increase in the number of papers published using microarray technology from 7 in 1995-1996 to 139 in 1999 and to 3 000 in 2003[80]. The initial optimism set out by Mark Schena, one of the authors of the *Arabidopsis* microarray experiment suggesting that all human diseases can be studied by microarray technology, With the ultimate goal of this work to develop effective treatments and cures for every human disease by 2 050 has not yet fully been born out by the published data. However, a number of exciting and novel observations have been generated.

Microarray design

RNA isolated from the tissue sample or cells can be used to generate cRNA or cDNA[78]. The probe set is then designed with transcripts targeting the genes of interest and care needs to be taken to prevent cross- hybridization. It is worth noting at this point that the probe refers to the reporter sequence placed at a particular position on the array which interrogates the sample and not the other way around. The probes are hybridised on the chip with the target unknown cRNA or cDNA. Following this process which can take several hours, the unbound target is washed off and the arrays are fluorescently labelled, so that they can be analyzed by confocal laser scanning. Expression of the entire human genome can be analyzed on one chip and complex computational and statistical techniques have been developed to analyze expression data because of the large amount of data microarrays[81].

Clinical data generated by microarray

Probably the most provocative and clinically relevant data generated by microarray have been in the field of cancer research. An example of the potential of microarray has demonstrated by Alizadeh and colleagues[82]. By comparing expression analysis on a 'lymphochip' panel of 3186 genes between patients with diffuse large B- cell lymphoma (DLBCL) prior to treatment, the investigators are able to group these patients into two discrete groups: germinal centre DLBCL and activated DLBCL. When the clinical progress of these two groups of patients were examined, the germinal cell DLBCL had a higher five year survival rate than the activated DLBCL (75% $n=25$ versus 16% $n=37$ respectively, $P \leqslant 0.01$). Gene expression profiles have also been successfully used to predict prognosis in 295 patients with breast cancer[83]. However, a meta- analysis of 84 studies found that DNA microarrays have a variable performance in measuring prognosis in a number of different cancers[84].

Microarray experiments in subjects with inflammatory bowel disease

Microarray has been used to compare synovial tissue obtained from patients with severe rheumatoid arthritis and macroscopically affected bowel of patients undergoing surgery for Crohn's disease[85]. A number of inflammatory genes were commonly expressed in diseased tissues. In 2000 Dieckgraefe and colleagues[86] published a study using microarray technology to compare patients with ulcerative colitis undergoing colectomies for disease refractory to medical management and a control group. Six thousand five hundred genes were analysed and the results confirmed an increase in a number of genes previously implicated in the pathogenesis of ulcerative colitis (IL-1, IL-1 RA and IL8) and suggested that multiple members of the chemokine subfamily may play a role in disease pathogenesis.

In a further microarray experiment using surgically resected inflammatory bowel disease tissue, 7 070 genes were examined and 170 genes were differentially expressed in ulcerative colitis and Crohn's disease with almost an equal number up regulated and down regulated[87]. Twenty percent of the differentially regulated genes were common to both forms of inflammatory bowel disease and when the locations of these genes were mapped several were found to lie within IBD2 locus on chromosome 12.

More recently Langman and colleagues[88] used microarray to analyze biopsies taken from patients with Crohn's disease, ulcerative colitis and control patients, 22 283 genes were analysed. They found that genes involved in cellular detoxification and biotransformation (pregnane X receptor and MDR1) are significantly down regulated in the colon of patients with ulcerative colitis.

Endoscopic investigation of inflammatory bowel disease with the ability to take pinch mucosal biopsies has allowed investigators to take microarray tissue from a larger range of patients including those with less severe disease compared to patients requiring rescectional surgery. In a study of 24 patients with ulcerative colitis, Okahara and colleagues[89] investigated the difference in gene expression between endoscopic biopsies taken from inflamed and non inflamed areas using a 1300 gene microarray, and found that migration inhibitory factor- related protein 14 (MRP14), growth-related oncogene gamma (GROγ) and serum amyloid A1 (SAA1) were upregulated whereas TIMP1 and PDZ and LIM domain 1 (elfin) were down regulated in the inflamed biopsies when compared to the non- inflamed biopsies.

In a study involving endoscopic biopsies of patients with Crohn's disease, ulcerative colitis and controls, Costello and colleagues[90] found that 500 and 272 transcripts are differentially regulated in CD and UC, respectively. Candidate genes are confirmed by real-time PCR and immunohistochemistry, and a number of genes involved in immune regulation were identified.

Further microarray studies in inflammatory bowel disease

Microarray has been useful in studies thus far and continues to be a powerful tool in investigation of the pathogenesis of inflammatory bowel disease. Further studies need to be designed in order to allow the collection of accurate clinical data describing the phenotype and activity of inflammatory bowel disease at the time of sample collection as these data are critical in analysis and interpretation of the results. The confounding problems associated with the heterogeneous mixture of cells, which is inevitable in the analysis of entire biopsy samples, can be reduced by using laser capture micro-dissection, in order to analyze only the cells of interest. By obtaining painstakingly accurate clinical information at the time of biopsy collection and narrowing down cell heterogeneity, the amount of background 'noise' that has hampered previous microarray studies can be reduced[91]. Minimum standards of information reported on microarray data have been proposed with the aim of establishing a standard for recording micoarray-based gene expression data[92], and replication of results with real-time PCR helps to validate these studies.

PROTEOMICS

Proteomic analysis is another relatively new investigative tool that has not been used to any large extent in the field of inflammatory bowel disease. Proteomics refers to the study of the total protein content in cells and the products of genes, so as to identify differences between normal and diseased tissue[93]. This is achieved by combining the techniques of protein electrophoresis and mass spectrometry. Expression profiles of proteomes may be generated from samples of serum or secreted fluid, and may be able to differentiate disease progression, response to therapy and to identify novel therapeutic targets.

Preliminary data have been generated in our unit by comparing serum of patients with severe ulcerative colitis who responded to corticosteroid therapy and matched patients who were resistant to and failed corticosteroid therapy[94]. Proteomic profiles of corticosteroid resistant and responsive groups are significantly different at 19 protein biomarkers: 12 proteins were up-regulated and 7 proteins were down-regulated in the corticosteroid resistant group. These results suggest that protein profiling may be useful in predicting patient response to corticosteroid therapy and identification of these proteins is currently underway.

CONCLUSION

The application of novel technologies has catalyzed the search for novel determinants in inflammatory bowel disease. Proof of the principle for genome wide studies is provided by the NOD2/CARD15 discovery and investigation of genes at other loci is underway. Rigorous attention to statistical design and phenotypic classification of disease is of paramount importance in gene discovery.

With the advent of new powerful investigative tools such as microarray and proteomic analysis, large amounts of data can be generated from small studies in inflammatory bowel disease. Among the clinical challenges in harnessing the power of these new investigative tools will be to accurately define clinical phenotype in patients studied, given the heterogeneity inevitable in these studies.

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REVIEW

Chemotherapy as a component of multimodal therapy for gastric carcinoma

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Abstract

Prognosis of locally advanced gastric cancer remains poor, and several multimodality strategies involving surgery, chemotherapy, and radiation have been tested in clinical trials. Phase III trial testing the benefit of postoperative adjuvant chemotherapy over treatment with surgery alone have revealed little impact on survival, with the exception of some small trials in Western nations. A large trial from the United States exploring postoperative chemoradiation was the first major success in this category. Results from Japanese trials suggest that moderate chemotherapy with oral fluoropyrimidines may be effective against less-advanced (T2-stage) cancer, although another confirmative trial is needed to prove this point. Investigators have recently turned to neoadjuvant chemotherapy, and some promising results have been reported from phase II trials using active drug combinations. In 2005, a large phase III trial testing pre- and postoperative chemotherapy has proven its survival benefit for resectable gastric cancer. Since the rate of pathologic complete response is considered to affect treatment results of this strategy, neoadjuvant chemoradiation that further increases the incidence of pathologic complete response could be a breakthrough, and phase III studies testing this strategy may be warranted in the near future.

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INTRODUCTION

Despite its declining incidence in Western Europe[1] and the United States[2], gastric carcinoma remains the second most common cause of cancer death worldwide with over 600 000 deaths per year[3]. The curative treatment of gastric carcinoma remains primarily surgical. Although developments in surgery have been slowed in the West by the large percentage of patients presenting at advanced stages[4] radical gastrectomy with extended lymphadenectomy[5,6] has been performed in Japan and other East Asian countries[7] as well as at specialized centers in the West[8-12] and is now recognized as a reasonably safe procedure in experienced hands[13,14]. However, the survival benefit of extended lymphadenectomy is yet to be proven in a large-scale randomized trial[15,16], and the prognosis of patients with locally advanced gastric cancer remains dismal even after potentially curative resection. Consequently, multimodal treatment strategies involving surgery, chemotherapy, and radiation have been explored to improve on the survival of the patients with resectable advanced cancer. The current review focuses on the development and states of the art of chemotherapy given as a component of such multimodal treatments. It is not within the scope of this review to describe in detail the chemotherapeutic regimens given concurrently with radiation.

Postoperative adjuvant chemotherapy

One straightforward strategy against resectable advanced gastric cancer is to do the best that can be done by surgery and then supplement it with chemotherapy to eliminate micrometastases that may have developed before the surgery or viable cells that may have been disseminated during the surgical procedure. Based on the fractional cell kill hypothesis [17], it would be expected that the highest tolerable drug doses given at the shortest possible interval after surgery would maximize the rate of cell kill[18, 19]. The authors have shown through an in vivo model of peritoneal carcinomatosis that either oral S-1 (1M tegafur-0.4M gimestat-1M otastat potassium)[20] or intraperitoneal paclitaxel[21] can control viable intraperitoneal cancer cells at an early stage of the metastatic process, although the therapeutic effect proved limited once they have developed into gross metastases. This strategy has long been a standard of care in other cancer types including colorectal cancer, reflecting the results of several randomized phase III trials. In the case of gastric carcinoma, however, the results of phase III trials testing this strategy have been inconsistent. An early success was reported from Japan in the 1970s, where a treatment by twice weekly intravenous administrations of

0.08 mg/kg mitomycin for 5 weeks reportedly improved the 5-year survival of curatively resected Stages I~IV gastric carcinoma by 13.5%[22]. This was the result of a large-scale nationwide trial in which 714 patients had been enrolled. Survival analysis, however, showed that the number of patients receiving chemotherapy was 242, whereas the number treated by surgery alone was 283. Details of a large number of patients who had been excluded from the survival analysis have not been reported, and the reliability of the study is questionable from the current point of view. Nevertheless, the result was taken seriously at the time, and several trials in the decade to follow explored the benefits of new combinations, mostly with oral fluoropyrimidines, as well as of new routes of delivery versus intravenous mitomycin, the gold standard. It was only in late 1980s after a long dispute that the Gastric Cancer Surgery Study Group in the Japan Clinical Oncology Group (JCOG) declared that treatment with surgery alone should once again be considered as the standard of care for curatively resected gastric cancer. Since that time, several new phase III trials have been launched in Japan with surgery alone as a standard arm. However, these new generation studies have repeatedly produced negative results. The failure to prove a survival benefit may be attributed to inadequate eligibility criteria that allowed the inclusion of early-stage cancers[23], faulty study design[24], and selection of ineffective antineoplastic agents[25]. The first JCOG study to be reported was a phase III study testing the impact of intravenous mitomycin (1.4 mg/m^2) and fluorouracil (166.7 mg/m^2) twice weekly for 3 week followed by oral UFT for 18 months[23]. From subset analyses of this negative study that enrolled 579 patients, Nakajima suggested that T1 stage cancer should be excluded from future trials, whereas T2 stage cancer could be a promising target for postoperative adjuvant chemotherapy. However, eligibility criteria of the next JCOG trial testing intravenous mitomycin 1.33 mg/m^2, fluorouracil 166.7 mg/m^2, and cytosine arabinoside 13.3 mg/m^2 given twice weekly for 3 wk followed by oral fluorouracil 134 mg/m^2 daily for the next 18 mo were based on surgical rather than histopathologic findings, and this again resulted in the inclusion of several T1 stage cancers. Consequently, the excellent survival achieved by surgery alone left little room for improvement by adjuvant chemotherapy, and the study, designed unfortunately to detect a large difference of 15% in 5-year survival, wasdestined to be under-powered[24]. Another JCOG phase III trial testing the survival benefit of a combination chemotherapy with intraperitoneal CDDP (70 mg/m^2) on d1, intravenous CDDP at 70 mg/m^2 on d14, and continuous 5FU at 700 mg/m^2 on d14~16 followed by one year of oral UFT (267 mg/m^2) over surgery alone for T3~T4 stage cancer had been powered to detect a difference of 12% in 5-year survival[25]. Survival curves in this study that were found to be almost identical suggest that this trial failed because of an inadequate regimen rather than a flaw in the statistical considerations, since intraperitoneal CDDP had already proven ineffective as was reported in 1994 by a much smaller Austrian study[26].

After the aforementioned JCOG trials, a trial testing oral UFT (350 g/m^2) to be continued for 16 months was conducted for pathological T2/N1 and T2/N2 stage cancer (NSAS-GC). Although there were several new participants in the study in addition to the members of JCOG (33 institutions in all), the patient accrual was poor with only 199 patients participating in 4 years, whereas 488 were needed to detect an 8.8% difference in 5-year survival. The trial was eventually discontinued in order to carry out the next randomized trial testing a new and more promising drug, S-1[27], in the adjuvant setting (ACTS-GC). Nevertheless, a planned interim analysis at the median follow-up of 3.8 years revealed significant improvements in overall and relapse-free survival in the chemotherapy arm[28]. Patient characteristics had been well-balanced between the arms, and the study had been carefully conducted. However, this was considered too small a study to definitively prove the benefit of postoperative chemotherapy in gastric cancer. In the meantime, ACTS-GC, a nationwide trial comparing postoperative adjuvant chemotherapy by oral S-1 with surgery alone for Stages II and III gastric cancer, completed a planned enrollment of 1000 patients in the year 2005. It is hoped that the results of this trial will in the near future provide a decisive answer regarding the survival benefit of adjuvant chemotherapy with oral fluoropyrimidines following formal D2 dissection. Since the result of ACTS-GC could turn out to be negative after all, another confirmative phase III trial testing the benefit of UFT is currently planned by JCOG so that the enrollment may be completed before the interim analysis for the ACTS-GC study due to take place in 2008. Although the response rate of S-1 in cancer with measurable lesions was higher than that of UFT, there is currently no guarantee that S-1 is more effective than UFT in the adjuvant setting.

In the meantime, two randomized trials in the West have shown the advantage of adjuvant chemotherapy, one with mitomycin and tegafur[29], and the other with epidoxorubicin, 5FU and leucovorin[30]. However, prognosis of those patients treated with surgery alone in these trials had been so dismal that the patients enrolled for the trial might have been treated with or inadequately staged by suboptimal surgery. On the contrary, an adjuvant postoperative chemotherapy regimen consisting of EAP (etoposide, doxorubicin, cisplatin) followed by intravenous 5FU/LV had no survival benefit in a trial by the Italian Medical Oncology Group. Interestingly, the 5-year survival rate of the surgery alone group in that trial was relatively good at 44% [31]. Survival benefit was not proven in other modern adjuvant trials exploring the FAM (5-FU, doxorubicin, mitomycin) regimen[32] or 5FU/LV and cisplatin[33], though meta-analyses of the Western trials demonstrated some potential of this strategy[34-38]. A meta-analysis of the three Japanese randomized trials with serosa-negative cancer as a target[23, 24, 27] also suggested a survival advantage[39]. Thus, attempts to confirm a definitive survival advantage of postoperative adjuvant chemotherapy should be continued.

There is a significant difference between the Japanese and Western principles for the selection of chemotherapeutic regimens to be used in the postoperative adjuvant setting. In the West, any regimen found to be active in the treatment for unresectable/metastatic cancer could also be regarded as a candidate to be tested in the adjuvant setting. For instance, the EAP regimen tested in the Italian Trials in the Medical Oncology group study[33] had caused

concern due to its severe toxicity[40]. A Swiss study currently exploring the impact in both a neoadjuvant and adjuvant setting of a combination of 5-FU, cisplatin and docetaxel which has revealed a superior response rate compared with 5FU and cisplatin among advanced gastric cancers [41] but has also been recognized as highly toxic. The Gruppo Onocologico Italia Merideionale (GOIM) recently conducted a trial to explore the efficacy and tolerability of the addition of epirubicin to a combination of etoposide, leucovorin and 5FU (ELF), and found this regimen (ELFE) to be active for advanced gastric cancer[42]. Here again, the next step they took was to test this regimen in the adjuvant setting in a phase III study with surgery alone as a control arm[43]. Since most of the patients treated with potentially curative surgery still have dismal prognosis in the West, investigators there do not hesitate to introduce toxic regimens that were nevertheless found to be active against advanced cancer into trials for postoperative adjuvant chemotherapy.

The situation is different in Japan where regimens used in adjuvant settings tend to be relatively mild. This is because a larger proportion of patients to be treated with postoperative adjuvant chemotherapy will survive by surgery alone, so that treatment-related toxicities and impairment of patient's quality of life are less likely to be tolerated. For these reasons, adjuvant chemotherapies in Japan have centered around oral fluoropyrimidine-based regimens with mild toxicities. The aforementioned regimen of intravenous mitomycin, fluorouracil, and cytosine arabinoside followed by oral fluorouracil, for example, induced Grade III leucopenia in 1.6% and Grade III gastrointestinal symptoms in 0% of patients treated in the adjuvant setting[24]. A highly regarded combination of oral S-1 (80 mg/m^2 for 21 d with 14 d of rest) and cisplatin (60 mg/m^2 on d8) that achieved a response rate of >70%[44], for instance, had been frequently used in Japan for unresectable/metastatic cancer, but has not been considered as a candidate for postoperative adjuvant chemotherapy. Use of mild regimens demonstrating moderate reponse rates are regarded by some investigators as old-fashioned[45], but as described earlier, the possibility that these regimens may still prove useful in pT2 stage cancers treated with adequate surgery cannot be currently denied.

Finally, a positive result in a trial testing adjuvant chemoradiation needs to be mentioned. In the United States, an Intergroup Trial (SWOG-9008/INT0116) was launched in 1991 to test the effect of combined radiation and fluorinated pyrimidine in the adjuvant setting[46]. The treatment consisted of five consecutive days of bolus 5FU (425 mg/m^2) /LV (20 mg/m^2) before and after 45 Gy of radiation given for 5 weeks, with intravenous 5FU (400 mg/m^2) /LV (20 mg/m^2) on the first four and last three days of radiotherapy. The trial enrolled 556 patients, and the median overall survival in the surgery-only group was 27 mo as compared with 36 mo in the chemoradiotherapy group, showing a significant survival benefit for chemoradiation. Since radiation was delivered to the gastric bed and regional lymph nodes, the object of chemoradiation would seem to have been to combat any locoregional residual disease, and it was indeed for this pattern of recurrence that a significant decrease in the incidence was observed among the treatment group. The extent of lymph node dissection being D0 in 54% of the patients enrolled, some skepticism arose as to whether chemoradiotherapy might have effectively compensated for the suboptimal surgery in terms of local control[47, 48]. These observations point to the importance of quality control in surgery even when multimodality treatments are being discussed[26]. Although postoperative chemoradiation has not been seriously explored in Japan where investigators believe that local control can be achieved through extended lymphadenectomy, this may be a useful option in countries where systematic lymphadenectomy has not become a standard practice.

Future perspective regarding postoperative adjuvant chemotherapy

A breakthrough in clinical trials testing postoperative adjuvant chemotherapy may be achieved through a customized approach in which the candidates for adjuvant chemotherapy are more meticulously selected. Detection of minimal residual disease may be one of the options, since chemotherapy given to a patient with no residual disease would only be needlessly harmful. This detection can be done through immunostaining or polymerase chain reaction of protein, gene mutation, or mRNA expression that may be present in cancer cells while absent in non-cancer cells that may be included in the samples[49]. Although the prognostic value of micrometastasis detected in the lymph node through immunostaining remains controversial[50, 51], detection of free cancer cells in peritoneal washing samples by a conventional cytologic examination is a strong prognostic factor predicting the risk for peritoneal carcinomatosis[52]. Enhanced detection through reverse-transcriptase polymerase chain reaction was found to be even more potent as a prognostic factor[53-55].

Identification of patients at risk will have little value unless effective drugs are available. Adequate selection of anticancer drugs could be achieved through *in vitro* chemosensitivity testing in which the rates of growth in relation to a control of viable cancer cells from the surgical specimens are tested in culture media containing various antineoplastic drugs. A retrospective study has shown that patients treated with postoperative adjuvant chemotherapy using a certain drug had a better outcome when the result of *in vitro* chemosensitivity testing for that drug had been positive[56]. Prospective studies to confirm this phenomenon are currently underway by several study groups in Japan. However, a randomized trial comparing patients whose treatments are selected based on chemosensitivity testing and those who were treated with empirical treatments is still needed to definitively assess the benefit of this costly and time-consuming procedure[57,58].

Neoadjuvant chemotherapy for resectable gastric cancer

Due to the consistently poor outcome of patients treated with a strategy of surgery followed by chemotherapy, preoperative chemotherapy has for several years attracted the attention of investigators in the West[59]. This is considered effective for patients in advanced T and N stages, and may result in downstaging of the tumors and consequently improving the curative resection rate. Since the best response is likely to be achieved within a few months from

the initiation of therapy, 2 to 3 courses of preoperative chemotherapy are usually performed in most clinical trials. Naturally, a high response rate is required for the chemotherapeutic regimens to be used in this setting in order to achieve downstaging. Chemotherapeutic regimens with high response rates usually are associated with greater toxicity and may not be feasible in the postoperative adjuvant setting, since chemotherapy performed immediately after gastric surgery is often marred by surgery-related gastrointestinal symptoms. Preoperative delivery of chemotherapy would be much less problematic, and this underscores the major appeal of neoadjuvant therapy[60]. Another theoretical benefit of neoadjuvant chemotherapy concerns micrometastases that are undetected at the initiation of treatment. Patients with locally advanced cancer are more or less likely to harbor distant micrometastases which could remain untreated for several weeks when the surgery-first strategy is selected.

It appears so far that survival rates are excellent among patients who respond to neoadjuvant chemotherapy[60]. However, whether a group of patients as a whole benefits from this strategy needs to be carefully elucidated, and several phase III trials are ongoing after producing promising phase II results[61-63]. Of these, a MAGIC trial exploring pre- and postoperative ECF regimens (a combination of epirubicin, CDDP, and continuous infusion of 5FU) has enrolled patients as planned and has proved the significant benefit of chemotherapy (improvement of 13% in a 5-year survival rate)[64]. Although three cycles each of ECF were to be performed before and after surgery, compliance with chemotherapy was unexpectedly lower after surgery (only 42% of patients completed postoperative chemotherapy), so that the result of MAGIC trial is considered mainly to reflect the effect of preoperative ECF. Tumor diameter, depth of invasion, and nodal status of the surgical specimens were significantly decreased or improved in the chemotherapy group, indicating that downstaging actually took place in a significant proportion of patients. Despite the toxicity of neoadjuvant chemotherapy, the mortality and morbidity in surgery following chemotherapy seems to have been comparable to what it would have been without preoperative chemotherapy.

In Japan, two phase II studies await survival analysis after enrollment as planned: one is exploring a combination of CPT-11 and CDDP[65] performed against gastric cancer with bulky nodal metastasis, and the other is testing CDDP and oral S-1[44] against linitis plastica type (type 4) and large type 3 cancers. A phase III trial comparing neoadjuvant chemotherapy by CDDP and S-1 with surgery alone for types 3 and 4 cancers will soon be launched. The concept of chemotherapy in the neoadjuvant setting is similar between Japan and Western countries in that regimens with high response rates have usually been selected. It has been proven in other types of cancer that only neoadjuvant chemotherapy using a regimen with a high pCR (pathological complete response) rate can really affect survival[66]. A high incidence of PR (partial response) was reported in a phase II trial of chemotherapy with CDDP and S-1, but CR was seldom observed as a result of treatment with this combination[44]. Thus, there is a theoretical concern that the expected survival benefit may not be obtained by a neoadjuvant chemotherapy relying on this regimen, although the consequences of neoadjuvant chemotherapy followed by gastrectomy plus extended lymphadenectomy with sufficient nodal clearance remain of interest.

The pCR rate can be enhanced by the addition of radiation, which has been shown to have a beneficial impact on the surgical outcome in esophageal and rectal cancer. Anticancer drugs in this case are in part considered as radiosensitizers, and 5FU, cisplatin, and paclitaxel have been used alone and in combination[67-69]. A pCR was achieved in 20% of patients who received two cycles of continuous 5FU, paclitaxel, and cisplatin followed by 45 Gy radiotherapy with concurrent 5FU and paclitaxel[70]. Although chemotherapy performed concurrently with radiation may be somewhat inadequate against potential micrometastases that may exist outside the field of radiation, a three- step strategy with intense induction chemotherapy prior to the preoperative chemoradiation and surgery may overcome this weakness. The validity of this strategy needs to be proven by a well-designed phase III trial.

CONCLUSIONS

Since the prognosis of locally advanced gastric cancer remains poor, several multimodality strategies involving surgery, chemotherapy, and radiation have been tested in clinical trials. Postoperative adjuvant chemotherapy with highly active regimens generally had disappointingly little impact on survival for advanced cancer, although there is a hard evidence that locoregional control could be improved by postoperative chemoradiation where systemic removal of the regional lymph nodes has not been conducted.

Moderate chemotherapy with oral fluoropyrimidines may be effective against T2 N+ stage cancer, although another confirmative trial is needed to prove this point. For more advanced disease, investigators have turned to neoadjuvant chemotherapy in the hope that downstaging might facilitate an R0 resection and that distant micrometastasis could be eliminated. A large randomized study testing pre- and postoperative ECF has confirmed the survival benefit of this strategy, and several phase III trials exploring other promising regimens are ongoing. The outlook is even brighter with neoadjvant chemoradiation that further increases the incidence of pathologic CR, and phase III studies testing this strategy are warranted.

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ESOPHAGEAL CANCER

Expression of midkine and its clinical significance in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of midkine in esophageal squamous cell carcinoma (ESCC) and analyze its relationship with clinicopathological features.

METHODS: RT-PCR and immunocytochemical staining were used to detect the expression of midkine mRNA and protein in EC109 cells, respectively. Then the expression of midkine in 66 cases of ESCC samples were detected by immunohistochemistry using monoclonal antibodies against human midkine.

RESULTS: Midkine was expressed in EC109 cell by RT-PCR and immunocytochemistry. The immunoreactivity was detected in 56.1% (37/66) of the ESCC samples. The expression of midkine was found in cytoplasm of tumor cells. Notably, the intensity of midkine was stronger at the area abundant in vessels and the invading border of the tumors. Midkine was more intensely expressed in well differentiated tumors (76.9%) than in moderately and poorly differentiated tumors (43.1% and 41.2%, respectively) ($P < 0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or survival in ESCC.

CONCLUSION: Midkine is overexpressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine is correlated with tumor cell differentiation in ESCC. The more poorly tumor cells differentiate, the weaker midkine expresses.

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Key words: Midkine; Esophageal squamous cell carcinoma; Expression

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INTRODUCTION

Midkine is a novel heparin-binding growth factor, originally identified as the product of a retinoic acid-responsive gene. Midkine and pleiotrophin (PTN; also called HB-GAM) are the only members of a family distinct from other heparin-binding growth factor families[1,2]. In the last few years, midkine has been found to be overexpressed in various human malignant tumors, such as esophageal[3-5], gastric[4,5], colorectal[4-6], liver[4,7,8], lung[9], thyroid[10], urinary bladder[11] and prostate carcinomas[12], as well as neuroblastomas[13] and astrocytomas[14,15]. Especially in neuroblasmas[13] and bladder carcinomas[11], the level of midkine expression correlates negatively with the patients' prognosis. Furthermore, it has also been reported that midkine is extensively expressed in the early stages of carcinogenesis[6,12]. All of these studies suggested that midkine may play an important role in carcinogenesis, development and metastasis of tumors, and that it could serve as a novel tumor marker.

In the present study, we investigated midkine expression in esophageal squamous cell carcinoma (ESCC). First, we detected midkine mRNA and midkine protein expression in EC109 cells (a cell line of well differentiated human esophageal squamous cell carcinoma) by means of RT-PCR and immunochemical staining, respectively. Then we examined midkine expression in 66 cases of ESCC tissues by immunohistochemical staining using monoclonal antibody against human midkine, and analyzed the correlation between midkine expression and clinicopathological features.

MATERIALS AND METHODS

Cell lines

EC109 cells, a well differentiated human ESCC cell line, established by National Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College

in 1976, and generously supplied by Department of Immunology, the School of Oncology, Peking University, were maintained in RPMI1640 with 100 mL/L heat-inactivated fetal bovine serum in humidified 50 mL/L CO_2 atmosphere at 37 ℃.

RT-PCR

Total RNA was extracted from EC109 cells with a single method using the Trizol reagent according to the manufacturer's protocol. Complementary DNA (cDNA) of EC109 cells was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase. PCR was performed in a total of 20 μL reaction mixture, comprising Taq DNA polymerase, dNTPs, buffer, 10 μmol/L of each gene-specific forward and backward primers and 2 μL of cDNA. β-actin mRNA levels were used as internal controls. The sequences of PCR primers are as follows: for midkine, 5' AAAGAATTCGAGATGCAGCAC CGAGG 3' (forward) and 5' AAACTCGAGCCAG GCTTGGCGTCTAGTC3' (backward); for β-actin, 5' AACTGGGACGACATGGAGAA 3' (forward) and 5' GGTAGTCAGTCAGGTCCC3' (backward). Expected RT-PCR product sizes were 477 bp for midkine and 318 bp for β-actin. PCR conditions consisted of an initial denaturation step for 5 min at 94 ℃, followed by 35 cycles of amplification (denaturation for 30 s at 94 ℃, annealing for 30 s at 62.5 ℃, and extension for 30 s at 72 ℃) and a final extension for 10 min at 72 ℃. Then 7 μL of PCR product was electrophoresed on 15 g/L agarose gels to visualize cDNA products.

Immunocytochemical staining of EC109 cells

Dako EnVision system was used for imm-unocytochemical staining of midkine. EC109 cells were fixed with acetone/methanol (1∶1, V/V). Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. After washed with PBS, the slides were blocked with 50 mL/L horse serum for 1 h at 37 ℃, followed by incubation with mouse anti-human midkine antibody overnight at 4 ℃. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 ℃ and then further washed for 3 times with PBS. After that the sections were reacted with DAB for 5 to 7 min to allow visualization. Finally, the slides were counterstained with hematoxylin, dehydrated, and evaluated under a light microscope.

Patients

Sixty-six specimens of ESCC were obtained from patients who underwent surgery at Beijing Cancer Hospital from Aug. 1998 to Dec. 2000. None of the patients received chemo- or radiotherapy prior to resection. These patients included 56 males and 10 females, aged from 38 to 76 years with a median age of 57.5 years. All the cases had been pathologically proven to be ESCC, including 26 well, 23 moderately and 17 poorly differentiated tumors; and 35 metastatic lymph node specimens.

Immunohistochemical staining of tumor tissues

Mouse monoclonal antibody against human midkine was prepared by our laboratory[16]. Secondary antibody of EnVision kit was purchased from Dako Inc. The sections were stained using standard EnVision, peroxidase method. Four-micrometer-thick tissue sections were dewaxed with xylene and rehydrated with graded ethanol, then briefly immersed in water. Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. Then heat-mediated antigen retrieval was performed by heating the sections (immersed in 0.01 mol/L citrate buffer, pH 6.0) in a microwave oven for 10 min. After washed with PBS, the slides were blocked with 50 mL/L milk to prevent nonspecific binding for 1 h at 37 ℃, followed by incubation with mouse anti-human midkine antibody overnight at 4 ℃. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 ℃ and then further washed for 3 times with PBS. Finally, the sections were reacted with DAB for 5 to 7 min to allow visualization, and counterstained with hematoxylin, dehydrated, and evaluated under a light microscope. The sections known to be midkine positive were stained under the same conditions as above and served as positive controls. For negative controls, sections were processed as above but the primary antibody was replaced by PBS. Anti-midkine immunoreactivity was confined primarily to the cytoplasm. One hundred cells from 5 randomly selected representative fields (× 400) of each section were counted. It was consid-ered positive when all immunoreactivity levels were more than 10% with anti-midkine antibody.

Statistical analysis

The χ^2 test was used to examine the differences of midkine expression between groups, and Cox regression was employed to analyze the correlation between midkine expression and patients' post-operation survival time by SPSS 11.0 software. $P<0.05$ was considered as statistically significant.

RESULTS

Midkine expression in EC109 cells

Midkine mRNA transcription was detected in EC109 cells by means of RT-PCR (Figure 1). After amplification of RT-PCR, we observed that there were two strands located in the gaps from 400 bp to 500 bp and from 300 bp to 400 bp, respectively. The sizes of RT-PCR product indicated by strands were conformed with expected RT-PCR product sizes of 477 bp for midkine and 318 bp for β-actin. The positive signals of midkine protein immunocytochemical staining were located in cytoplasm. Expression of midkine protein was observed in EC109 cells (Figure 2).

Expression of midkine protein in ESCC tissues

Midkine expression was confined primarily to the cytoplasm, shown as brown granules (Figure 3A). Midkine was expressed in 37 of 66 ESCCs (56.1%), while it was present at a very low level in just a few normal esophageal epithelia adjacent to tumor tissues. Midkine positive tumor cells were distributed in foci, and the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors (Figure 3B). In addition, Midkine ex-

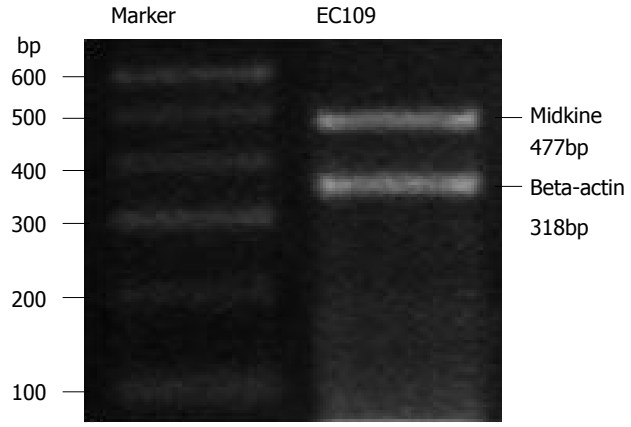

Figure 1 Amplification of midkine mRNA in EC109 cells by RT-PCR.

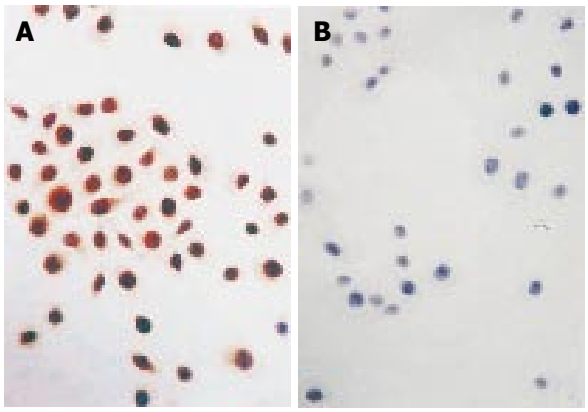

Figure 2 Midkine expression in EC109 cells (Immunoperoxidase method ×400). **A**: Positive in EC109 cells; **B**: Negative control.

pression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal elements.

Correlation between midkine protein expression and clinicopathological features

Midkine was expressed in 20 of 26 well differentiated ESCCs (76.9%), more than that in moderately (10/23, 43.1%) and poorly (7/17, 41.2%) differentiated tumors ($P<0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or post-operation survival time in ESCC ($P>0.05$, Table 1).

DISCUSSION

Many studies have shown that growth factors not only promote tissue proliferation but also induce malignant transformation, and they play important roles in the development of neoplasm[17]. Over-expression of growth factors has been found in many human tumors. Midkine, a novel heparin-binding growth factor, is strongly expressed in mid-gestational period. While in normal adult tissues, its expression is highly restricted. It is normally highly expressed in small bowel epithelium, moderately expressed in the thyroid and lowly expressed in lung, stomach, colon and kidney[18]. In the last few years, Midkine has been found

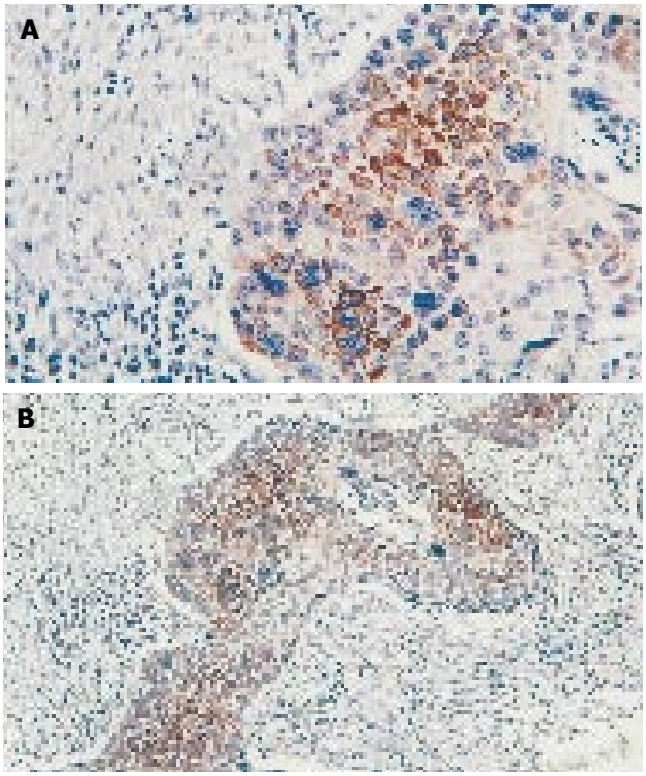

Figure 3 Midkine expression in ESCC (Immunoperoxidase method). **A**: In cytoplasm (×400); **B**: Stronger at the area abundant in vessels (×200).

to be over-expressed in various human malignant tumors, such as esophageal[3-5], gastric[4,5], colorectal[4-6], liver[4,7,8], lung[9], thyroid[10], urinary bladder[11] and prostate carcinomas[12], as well as neuroblastomas[13] and astro-cytomas[14,15]. Especially in neuroblasmas[13] and bladder carcinomas[11], the level of midkine expression correlates negatively with the patients' prognosis. It has also been reported that midkine is extensively expressed in the early stages of carcinogenesis[6,12]. Further studies showed that midkine had several cancer-related activities. Midkine could transform NIH3T3 cells[19] and enhanced the plasminogen activator/plasmin levels in bovine endothelial cells[20]. It also promotes cell growth[21,22], cell survival[23] and migration of various cells such as neutrophils and macrophages[24]. These biological activities of midkine supported the possible involvement of midkine in carcinogenesis and tumor advancement.

To further investigate the role of midkine in carcinogenesis and tumor advancement, we examined midkine expression in ESCC. First, we found that midkine was expressed in EC109 cell by means of RT-PCR and immunocytochemistry. Further, we examined midkine expression in 66 ESCC samples using immunohistochemistry. Our data showed that midkine was over-expressed in ESCC with the positive rate of 56.1% (37/66), while it was present at a very low level in just a few normal esophageal epithelium adjacent to tumor tissues. And the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors. Midkine expression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal tissues. These characteristics of distribution were confirmed in Kato's study[10], suggesting

Table 1 Intensity of midkine expression in relation to clinicopathological variables in esophageal squamous cell carcinoma

Variable		*n*	Positive *n*	Negative *n*	Positive rate (%)	χ^2 value	*P* value
Gender	Male	56	31	25	55.4	0.074	0.785
	Female	10	6	4	60.0		
Age (yr)	60	35	15	20	57.1	0.035	0.851
	⩾60	31	17	14	54.8		
Differentiation[a]	Well	26	20	6	76.9	7.906	0.019
	Modertate	23	10	13	43.5		
	Poor	17	7	10	41.2		
TNM Classification	I~II	15	16	7	69.6	2.669	0.102
	III~IV	13	21	22	48.8		
Lymph node metastasis	Positive	35	18	17	51.4	0.651	0.420
	Negative	31	19	12	61.3		

[a]$P<0.05$ as compared between well differentiated and moderately or poorly differentiated tumors.

that midkine may play a role in tumor angiogenesis and invasion. It has been demonstrated that midkine could promote the endothelial cells growth and it is a novel molecular mediator in tumor angiogenesis[25].

Our study also showed that the expression of midkine was correlated with tumor cell differentiation in ESCC. Midkine was more intensively expressed in well differentiated tumors (76.9%) than in moderately (43.1%) and poorly (41.2%) differentiated tumors ($P<0.05$). The phenomenon that the more poorly tumor cells differentiated, the weaker midkine was expressed was also observed in other studies on midkine expression in esophageal cancer and vulvar tumors[2,26].

In summary, our study showed that midkine was overexpressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine was correlated with tumor cell differentiation in ESCC: the more poorly tumor cells differentiated, the weaker midkine was expressed. Since midkine is a secretory protein, midkine level in blood can be detected when it over-expresses in tumors. It has been reported that an elevated serum midkine level can be detected in many malignant tumors, and serum midkine level decreases after the removal of tumors[27, 28]. In addition, the elevated midkine level even can be detected in urine[29]. These studies suggest that midkine could serve as a tumor marker which can be conveniently detected.

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Relationship between somatostatin receptor subtype expression and clinicopathology, Ki-67, Bcl-2 and p53 in colorectal cancer

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Abstract

AIM: To study the SSTR1, 2, 3, 4, 5 expression and their relationships with clinico-pathological factors, cell proliferation, Bcl-2 and p53 expression in colorectal cancer cells.

METHODS: Immunohistochemical staining of five SSTR subtypes, Ki-67, Bcl-2 and p53 was performed by the standard streptavidin-peroxidase (SP) technique for the paraffin sections of 127 colorectal cancers. and expression of five SSTR subtypes in 40 specimens of normal colorectal mucosae was detected with the same method.

RESULTS: Positive staining for five SSTR subtypes was observed in colorectal cancer cells and normal colorectal mucosae. SSTR1 was the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% *vs* 18.9%, $P < 0.05$); the expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated ones ($P < 0.05$), the SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, $P < 0.05$). In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased ($P < 0.05$); the correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes'stages ($P > 0.05$), but the frequency of SSTR1 expression increased with Dukes' stage, while SSTR3 and SSTR5 expression decreased with Dukes' stage. Moreover, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis. The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression ($P < 0.05$). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3, 5 was significantly lower than that with negative expression ($P < 0.05$). There was no correlation between five SSTR subtypes and p53 expression.

CONCLUSION: The most predominant SSTR subtype is SSTR1, and the second is SSTR2 or SSTR5. Five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells.

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Key words: Somatostatin receptor subtype; Cell proliferation; Apoptosis; p53; Colorectal cancer; Immunohistochemistry

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INTRODUCTION

Somatostatin analogue (SSTA) may inhibit the growth of various tumor cells by direct interaction with specific somatostatin receptors (SSTR) on tumor cells. However, conflicting clinical results were obtained in patients with colorectal cancers treated with SSTA[1]. Many authors think the rationale for the clinical efficacy of SSTA in the management of colorectal cancer may be related to the direct inhibitory effect of SSTA, consequently, with the expression of SSTR subtypes on tumor cells[2,3]. To date, five SSTR subtypes, SSTR1-SSTR5, have been cloned in human tissues, however, few researches on their expres-

cance were reported, and the results were inconsistent[3-6]. In this study, we used an immunohistochemical method to detect the expression of five SSTR subtypes protein in colorectal cancer cells and to explore correlation between each subtype expression and clinicopathological factors, cell proliferation or apoptosis. It will provide the basis for the treatment of colorectal cancer with SSTA.

MATERIALS AND METHODS

Patients and samples

A total of 127 cases of colorectal adenocarcinoma were involved in this study. The patients with colorectal carcinomas, who underwent surgical resection at the Second Hospital Affiliated to Fujian Medical University (Quanzhou, China) from January 1, 2003 to June 30, 2004, had received neither chemotherapy nor radiation therapy before surgery. There were 73 men and 54 women, and their mean age was 58.5 (SD, 13.9; range, 24-84) years. specimens were obtained from right colon (31), left colon (27), rectum (69). According to the Chinese Dukes' Staging criteria, 15 cases were stage A, 31 cases were stage B, 46 cases were stage C and 14 cases were stage D. Among 127 patients, 102 were moderately to well differentiated and 25 poorly differentiated adenocarcinomas. Meantime, 40 specimens of morphologically normal colorectal mucosae were examined from the same patients at a minimal distance of 10 cm from the diseased area.

All specimens were routinely fixed in neutral-buffered formalin and embedded in paraffin. Serial 4 μm sections were cut from archived paraffin blocks and subjected to immunohistochemical study. In each case, standard hematoxylin-eosin staining was employed for routine histological examination.

Reagents

Goat anti-human SSTR2, SSTR3, SSTR4 and SSTR5 polyclonal antibody were purchased from Santa Cruz Biotechnology Co. (California, U.S.A.). Rabbit anti-human SSTR1 polyclonal antibody, mouse anti-human Ki-67 monoclonal antibody (MBI.1), mouse anti-human Bcl-2 monoclonal antibody (100/D5), mouse anti-human p53 monoclonal antibody (DO-7), SP staining kit and DAB kit were supplied by Maixin-Bio Co., Fuzhou, China.

Methods

The immunohistochemical staining for five SSTR subtypes, Ki-67, Bcl-2 and p53 expression in colorectal cancer cells and five SSTR subtypes expression in normal colorectal mucosae were carried out by the standard streptavidin-peroxidase (SP) technique. Briefly, after deparaffinization and rehydration, the antigen retrieval of the sections was achieved by incubation in 0.01mol/L citric acid buffer (pH 6.0) and boiled for 1 min in a pressure cooker, and then cooled and washed in tap water. The sections were incubated with hydrogen peroxide for 10 min and washed in PBS. Nonspecific reactions were blocked by incubating the sections in a solution containing normal serum. The sections were incubated with a primary antibody (anti-SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Ki-67, Bcl-2 or p53 antibodies) overnight at 4℃. The working dilution of anti-SSTR2, SSTR3, SSTR4, SSTR5 antibodies was 1:400. Rinsed with PBS, then the sections were incubated for 10 min at room temperature with biotinylated secondary antibody. After washing, streptavidin biotin complex conjugated to horse-radish peroxidase was applied for 10 min at room temperature. Again after three rinses with PBS, the sections were incubated with diaminobenzidine substrate, then rinsed with distilled water and counterstained with hematoxylin, finally dehydrated and cover slipped. The sections were prepared for microscopic observation. The known positive colorectal cancer tissues were used as positive control. As negative control, PBS was used to replace primary antibody.

Scoring criteria for SSTR subtypes expression

Intensity and percentage of positive cells were used to evaluate each tissue section. The mean percentage of positive tumor cells to normal epithelial cells in at least five areas at 400 magnification was determined and assigned to one of five categories: 1) 0, < 5%; 2) 1, 5% to 24%; 3) 2, 25% to 49%; 4) 3, 50% to 74%; and 5) 4, ≥ 75%. The intensity of SSTR subtype immunostaining was scored as 0 (achromatic), 1(light yellow), 2(yellow), and 3(brown). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores less than 1 were defined as negative; all others were defined as positive.

Evaluation of staining for p53 and Bcl-2

For p53 or Bcl-2 expression, cases with ≤ 10% positively-stained tumor cells were defined as negative; otherwise, the definition was positive.

Determination of the Ki-67 proliferative index

At least 5 high-power fields were chosen randomly in each section, and 500 cells were counted for each field. The Ki-67 proliferative index was defined as the number of Ki-67-positive nuclei divided by the total number of colorectal cancer cells counted and was expressed as a percentage.

Statistical analysis

The statistical software package SPSS 11.5 was used. Clinicopathological variables associated with SSTR subtypes expression as well as the correlation between SSTR subtypes and p53 or Bcl-2 expression were analyzed by either the χ^2 test or Fisher's exact test. The correlation between SSTR subtypes and proliferative index was analyzed by t test. $P < 0.05$ was considered significant.

RESULTS

Expression of five SSTR subtypes

Positive-staining for all SSTR subtypes was identified in the cytoplasm and membrane of colorectal cancer cells and normal colorectal mucosa with brown-yellow granules (Figures 1-5). The positive rates of SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5 expression were respectively 64.6% (52/127), 36.2% (46/127), 18.9% (24/127), 18.9% (24/127), 38.6% (49/127) in colorectal cancer and 52.5% (21/40), 40% (16/40), 30% (12/40), 2.5% (1/40), 32.5% (13/40) in normal colorectal mucosa (Table 1). SSTR1 was

Table 1 SSTR expression in colorectal cancer

Tissue	*n*	Expression *n* (%)				
		SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Normal mucosa	40	21(52.5)	16(40.0)	12(30.0)	1(2.5)	13(32.5)
Colorectal cancer	127	52(64.6)	46(36.2)	24(18.9)	24(18.9)[a]	49(38.6)

[a]$P<0.05$ *vs* normal mucosa.

the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% *vs* 18.9%, $P < 0.05$), and the positive rates of other subtypes expression were not different between colorectal cancer and normal colorectal mucosa ($P > 0.05$).

Correlation between SSTR expression and clinicopathological factors

The expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated colorectal adenocarcinoma ($P < 0.05$)(Table 2). The SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, $P < 0.05$). The correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes' stages ($P > 0.05$), but the frequency of SSTR1 expression increased with Dukes' stage, while SSTR3 and SSTR5 decreased with Dukes' stage. In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased ($P < 0.05$); however, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis.

Correlation between SSTR expression and proliferative index and Bcl-2 or p53

The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression ($P < 0.05$, Table 3). A positive cytoplasmic immunoreactivity for Bcl-2 was detected in 67 of 127 cases (52.8%). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3 ,5 were significantly lower than that with negative expression ($P < 0.05$, Table 3). In contrast, nuclear accumulation of p53 was demonstrated in 81 of 127 cases (63.8%). There was no correlation between five SSTR subtypes and p53 expression.

DISCUSSION

Five SSTR subtypes belong to G proteins family, and different SSTR subtypes can be expressed in various patterns in different normal tissues or tumors. Evaluation of five SSTR subtypes expression in tumors may help understand carcinogenesis, and progression of tumors, as well as diagnosis and treatment. There are some controversies on the dominant SSTR subtype and the correlation between five SSTR subtypes expression and clinicopathological factors in colorectal cancer cells. Buscail *et al* found that SSTR2 expression was lost in colorectal cancer[3]. However, some subsequent studies demonstrated that there was no difference between SSTR subtypes expression between normal colorectal mucosae and colorectal cancer[5-7]. Also some results indicated the most frequent subtype was SSTR2 or SSTR5 in colorectal cancer[3,6,8]. The expression of SSTR2 or SSTR5 was significantly lower in Dukes' C and D stages of colorectal cancer than that in Dukes' A and B stages. The decrease of SSTR2 or SSTR5 expression may be related to tumor local invasion and metastasis, which may provide a growth advantage in colorectal cancer. It is possible that SSTR2 or SSTR5 acts as a tumour suppressor[3,6]. But recent research showed there was no correlation of SSTR mRNA expression with Dukes' stages[7].

In this study, only the frequency of SSTR4 expression in colorectal cancer cells was higher than that in the colorectal mucosae. SSTR1 was expressed predominantly in colorectal cancer cells, followed by SSTR5 and SSTR2. There was no correlation between five subtypes expression and Dukes' stages. The differences of our results from previous findings may be accounted for by different research methods. SSTR subtypes can express not only in colorectal cancer cells but also in other constituents of cancer tissue, such as vessels[9]. Thus, the PCR method tends to overestimate the real contribution of the various messengers for receptors owing to the outstandingly sensitive technique, and mRNA levels do not necessarily reflect the presence of the SSTR protein in certain cancers. To detect protein expression of SSTR by receptor autoradiography can not completely reflect protein levels of five SSTR subtypes, because there are major differences in binding affinity of SSTA to various SSTR subtypes[1]. In addition, the discrepancy may be relevant to different reagents and the small sample size of our study. Cascade reaction induced by activation of SSTR2 and SSTR5 can inhibit proliferation of tumor cells. In our study, the expression of SSTR2, SSTR4 and SSTR5 reduced in poorly differentiated colorectal cancer, and the SSTR3 and SSTR5 expression showed a tendency to reduction with Dukes' stages. The loss of these receptors can weaken inhibition on the proliferation of tumor cells, and may provide a growth advantage in colorectal cancer. It suggests SSTR2, 3, 4, 5 may be related to the development of colorectal cancer.

This study also demonstrated that the Ki-67 proliferative index in colorectal cancer cells with positive expression of SSTR2 or SSTR3 was significantly lower than that with negative expression of SSTR2 or SSTR3. This further proved that somatostatin *in vivo* can directly inhibit colorectal cancer cell proliferation by activating SSTR2 and SSTR3. The transplantable models of pancreatic primary tumor and hepatic metastases were established in hamsters which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, and the following effects were induced: growth of tumors with SSTR2 positive expression was delayed and the Ki-67 proliferative index was decreased significantly[10,11]. Activation of SSTR2 and

Table 2 Correlation between SSTR expression and clinicopathology in colorectal cancer *n* (%)

Clinico-pathological factor		*n*	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Sex	Male	73	47(64.4)	26(35.6)	13(17.8)	13(17.8)	28(38.4)
	Female	54	35(64.8)	20(37.0)	11(20.4)	11(20.4)	21(38.9)
Age (yr)	< 60	66	45(68.2)	20(30.3)	12(18.2)	12(18.2)	24(36.4)
	⩾ 60	61	37(60.7)	26(42.6)	12(19.7)	12(19.7)	25(41.0)
Tumor site	Right colon	31	18(58.1)	11(35.5)	7(22.6)	6(19.4)	11(35.5)
	Left colon	27	17(63.0)	12(44.4)	5(18.5)	6(22.2)	13(48.1)
	Rectum	69	47(68.1)	23(33.3)	12(17.4)	12(17.4)	25(36.2)
Macroscopic type	Elevated	40	27(67.5)	17(42.5)	10(25.0)	9(22.5)	17(42.5)
	Ulcerative	59	37(62.7)	13(22.0)[a]	8(13.6)	8(13.6)	21(35.6)
	Infiltrative	28	18(64.3)	16(57.1)	6(21.4)	7(25.0)	11(39.3)
Differentiation	Moderate to well	102	70(68.6)	43(42.2)[a]	22(21.6)	24(23.5)[a]	46(45.1)[a]
	Poor	25	12(48.0)	3(12.0)	2(8.0)	0(0.0)	3(12.0)
Tumor depth	Muscularis	24	14(58.3)	11(45.8)	5(20.8)	3(12.5)	9(37.5)
	Serosa	103	68(66.0)	35(34.0)	19(18.4)	21(20.4)	40(38.8)
Lymph	(+)	72	52(72.2)[a]	24(33.3)	12(16.7)	15(20.8)	26(36.1)
node metastasis	(-)	55	30(54.5)	22(40.0)	12(21.8)	9(16.4)	23(41.8)
Distant metastasis	(+)	20	15(75.0)	6(30.0)	3(15.0)	2(10.0)	7(35.0)
	(-)	107	67(62.6)	40(37.4)	21(19.6)	22(20.6)	42(39.3)
Dukes′ stage	A	13	7(53.8)	5(38.5)	4(30.8)	2(15.4)	6(46.2)
	B	40	22(55.0)	16(40.0)	8(20.0)	7(17.5)	16(40.0)
	C	54	38(70.4)	19(35.2)	9(16.7)	13(24.1)	20(37.0)
	D	20	15(75.0)	6(30.0)	3(15.0)	2(10.0)	7(35.0)

[a]$P < 0.05$.

Table 3 Correlation between SSTR expression and proliferative index, Bcl-2 or p53 expression

		n	Ki67 proliferation index mean ± SD	Expression of Bcl-2 positive (%)	Expression of p53 positive (%)
SSTR1	(+)	82	34.22 ± 24.33	37(45.1)	53(64.6)
	(-)	45	40.21 ± 22.97	30(66.7)[a]	28(62.2)
SSTR2	(+)	46	28.65 ± 18.80	15(32.6)	33(71.7)
	(-)	81	40.71 ± 25.50[a]	52(64.2)[a]	48(51.3)
SSTR3	(+)	24	25.72 ± 17.88	5(20.8)	17(70.8)
	(-)	103	38.82 ± 24.55[a]	62(60.2)[a]	64(62.1)
SSTR4	(+)	24	28.02 ± 20.12	9(37.5)	16(66.7)
	(-)	103	38.29 ± 24.42	58(56.3)	65(63.1)
SSTR5	(+)	49	32.20 ± 21.92	17(34.7)	34(69.4)
	(-)	78	38.95 ± 24.91	50(64.1)[a]	47(60.3)

[a]$P < 0.05$.

SSTR3 can inhibit the proliferation of cancer cell through the following mechanisms: SSTR2 and SSTR3 can inhibit adenylate cyclase activity, which results in reduction of intracellular cAMP concentration; they also can stimulate tyrosine phosphatase activity, modulate the mitogen activated protein kinases (MAPK) and upregulate the expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ so that cell cycle arrest is induced; the effects on K^+ and Ca^{2+} channels lead to increased intracellular K^+ concentration and reduced intracellular Ca^{2+} concentration[12-15]. This study further verified that five SSTR subtypes play different roles in development of colorectal cancer. SSTR2 and SSTR3 can obviously inhibit the cell proliferation of colorectal cancer and have an important effect on the progression of colorectal cancer.

The activation of some SSTR subtypes not only directly inhibits the proliferation of tumor cells, but also is relevant to apoptosis. Bcl-2 is an apoptosis suppressor gene and important parameter of apoptosis. In this study, Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, SSTR2, SSTR3 or SSTR5 significantly decreased, indicating the four SSTR subtypes can promote the apoptosis of tumor cells and affect the progression of colorectal cancer by down-regulating the Bcl-2 expression or counteracting the anti-apoptosis effect of Bcl-2. They promote apoptosis via different mechanisms: Apoptosis is mediated by SSTR1 via a block in the G1/S progression in the cell cycle[16]. SSTR2 activation can promote apoptosis through the following pathways, it sensitizes cancer cells to apoptosis induced by tumor necrosis factor, induces caspase-8 activation cascade, stimulates mitochondrial cytochrome c released into the cytosol and downregulates the Bcl-2 expression[17]. In the mouse models of pancreatic primary tumor and hepatic metastases, which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, it has been demonstrated that activation of caspase-3 and apoptotic index increased, expression of anti-apoptosis protein Bcl-2 decreased in tumor cells expressing SSTR2[10,18]. Apoptosis can be signaled by SSTR3 which can activate phosphoprotein phosphates and lead to cellular acidification and activation of acidic endonuclease[19].

In conclusion, five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells. The expression of SSTR2, SSTR4 and SSTR5 is correlated with malignant degree of colorectal cancer cells. Though SSTR1 is the predominant SSTR subtype, its ex-

pression is correlated with lymph node metastasis. SSTA, which has a high affinity to SSTR2, SSTR3 and/or SSTR5 should be a treatment choice for patients with colorectal carcinomas.

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BASIC RESEARCH

Adeno-associated virus-mediated heme oxygenase-1 gene transfer suppresses the progression of micronodular cirrhosis in rats

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Abstract

AIM: To test the hypothesis that enhancement of the activity of heme oxygenase can interfere with processes of fibrogenesis associated with recurrent liver injury, we investigated the therapeutic potential of over-expression of heme oxygense-1 in a CCl_4-induced micronodular cirrhosis model.

METHODS: Recombinant adeno-associated viruses carrying rat HO-1 or GFP gene were generated. 1×10^{12} vg of adeno-associated viruses were administered through portal injection at the time of the induction of liver fibrosis.

RESULTS: Conditioning the rat liver with over-expression of HO-1 by rAAV/HO-1 significantly increased the HO enzymatic activities in a stable manner. The development of micronodular cirrhosis was significantly inhibited in rAAV/HO-1-transduced animals as compared to controls. Portal hypertension was markedly diminished in rAAV/HO-1-transduced animals as compared to controls, whereas there are no significant changes in systolic blood pressure. This finding was accompanied with improved liver biochemistry, less infiltrating macrophages and less activated hepatic stellate cells (HSCs) in rAAV/HO-1-transduced livers.

CONCLUSIONS: Enhancement of HO activity in the livers suppresses the development of cirrhosis.

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Key Words: Cirrhosis; Portal hypertension; Heme oxygenase; Gene therapy; Adeno-associated virus

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INTRODUCTION

Heme oxygenase (HO) is a rate-limiting enzyme that cleaves pro-oxidant heme into equimolar amounts of carbon monoxide (CO), biliverdin/bilirubin, and free ferrous iron [1,2]. Up to date, three isoforms of HO have been identified. Among of them, HO-1 and HO-2 are expressed in livers. HO-2 expresses constitutively in hepatocyte and functions as an important enzyme in catalyzing the endogenous and exogenous heme. In contrast, HO-1 is an inducible form of HO in livers [3]. Although expression of HO-1 in the liver is primarily restricted to a subpopulation of Kupffer cells [3,4], the protein is expressed in both parenchymal and particularly non-parenchymal liver cells, and therefore results in higher enzymatic activities of HO under stress conditions [4]. The cytoprotective effects of enhanced HO activities in liver might be through the mechanisms associated with its catalytic products. All of its three catalytic products can contribute the protective effects of HO [5-7].

HO-1 was induced in the livers with cirrhosis [8]. It was thought to be an adoptive response to oxidative stresses, inflammatory insults of persistent or recurrent liver injury as well as the responsiveness to the increase of intrasinusoidal resistance. HO-1 was mainly expressed in Kupffer cells, sinusoidal endothelial cells, stellate cells and in a part of hepatocyte in livers with cirrhosis [8,9]. HO-1 was also expressed in vascular system that might contribute to the development of hemodynamic changes in the rats with cirrhosis [9]. In addition, higher levels of CO production might be associated with the development of hepatopulmonary syndrome in patients with cirrhosis [10]. Thus, it becomes controversy whether the expression of HO-1 or enhancement of HO activities contribute to the pathophysiological changes in the development of cirrhosis, or whether HO-1 functions as homeostatic molecule in controlling of disease progression. To elucidate the role of

HO-1 in the context of recurrent hepatocellular injury and the development of cirrhosis, here we take the advantage of adeno-associated viral vector to increase the overall HO enzymatic activity of the liver in a stable manner and to investigate the effects of HO-1 in the disease progression in carbon tetrachloride (CCl_4)-induced cirrhosis model.

MATERIALS AND METHODS

Generation of recombinant adeno-associated viral vectors (rAAVs)

rAAVs were produced and purified as previously described [11]. In brief, a full-length HO-1 gene originally cloned from the spleen of a LEW rat or the reporter gene GFP was inserted into the vector plasmid to construct pSNAV1/GFP or pSNAV1/HO-1. rAAV/GFP or rAAV/HO-1 was generated in BHK-21 cells (American Type Culture Collection, Manassas, VA) by transfection of vector plasmid and subsequent rescue rAAVs (serotype 2) by co-infection with recombinant HSV1-rc/ΔUL2, which is essential for viron packaging. A large scale of rAAV was purified as described [12].

Animal model and gene delivery protocol

Inbred LEW rats (230-250 g) were purchased from the Animal Institute of Medical School Hannover, Germany and were maintained in the Laboratory Animal Unit of the University of Hong Kong. The study was approved by the Committee on the Use of Live Animals for Teaching and Research, the University of Hong Kong. Micronodular liver cirrhosis in LEW rats was induced using a protocol described previously [13]. In brief, phentobarbital sodium (35 mg/L) was given one week before the first dose of CCl_4 in order to increase the sensitivity of the rat liver to CCl_4 and was present in drinking water in the whole period of induction. A total of 9 doses of CCl_4 (0.2 mL/kg per wk) were given to the rats intragastrically. rAAVs (1×10^{12}v.g.) was given through the portal vein of rats at the time pentobarbital was given in drinking water and 7 d before the first dose of CCl_4.

Liver biochemistry, histology, immunohistochemistry and ELISA

Serum samples were collected at the end point of experiments. The activities of alanine aminotransferase (ALT) and total bilirubin in the plasma were measured in the Department of Clinical Biochemistry, the University of Hong Kong. Liver samples were snap frozen and stored at -75 ℃ until further applications. Five micrometers of frozen sections were used for hematoxylin and eosin staining, Masson's trichrome staining, and immunohistochemistry. The area of fibrotic tissues in the cross section of livers was measured by the computer software (MetaMorph imaging system, Universal Imaging Corporation, PA) after Masson's trichrome, collagen 1α and fibronectin staining. All measurements were done in a double-blind manner with at least 20 areas/liver (fibrotic area, original 200×) in the 5 livers/group. Mouse anti-rat ED1 (infiltrating macrophage), anti-ED2 (tissue residential macrophage), anti-HO-1 (OSA-111) monoclonal antibodies and polyclonal anti-TGF-β1, anti-desmin, anti-collagen 1α, and anti-fibronectin antibodies (Serotec Ltd., Oxford, UK; Stressgene, Victoria, British Columbia, Canada; Chemicon, Temecula, CA; Santa Cruz, CA) were applied in this study for immunohistochemistry using the standard horseradish peroxidase protocol. Serum macrophage migration inhibitory factor (MIF) level was detected by ELISA according to the instruction of manufacturer (Chemicon).

Measurement of portal and systolic blood pressure

Animals were anesthesized with ketamine (100 mg/kg, ip) and Rompun (0.2 mg/kg, ip). Portal pressure and systolic blood pressure were measured by directly introducing 24G Angiocath® (BD Biosciences, San Jose, CA) into the portal vein or abdominal aorta and connecting with a saline filled strain gauge transducer. The signals were monitored by Colin BP-408 Mark III (Japan) and were set to zero before the measurement.

HO enzymatic activity

The HO enzymatic activity was measured by the production of microsomal bilirubin in the liver. Frozen samples of livers were homogenized in ice-cold sucrose and Tri-HCl buffer. Microsomal pellet was obtained after centrifugation and was then re-suspended in $MgCl_2$-potassium phosphate buffer. Sample protein was further incubated with the reaction mixture containing rat liver cytosol, hemin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37 ℃. The generated bilirubin was measured using spectrophotometry. The level of bilirubin production was demonstrated by the ratio of sample/normal liver.

RNase protection assay

The mRNA level of target genes was determined by RNase protection assay according to the instruction of manufacturer (RiboQuant kit, BD Biosciences Pharmingen, San Diego, CA). In brief, total RNA of transduced and non-transduced liver samples were extracted and purified using Rneasy kit (Qiagen, Hilden, Germany). Three micrograms of RNA/samples were hybridized with complimentary [^{32}P]UTP labeled riboprobes overnight. The probes were digested with Rnase and were loaded on a denatured polyacrylamide gel. The radioactive signals were then detected by exposure to X-ray film (BioMax, Kodak, Rochester, NY) and quantified by phosphorimaging (Strom, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data were demonstrated as mean ± SEM. One-way ANOVA was used to compare the difference of means between the experimental groups with the Bonferroni's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

rAAV-mediated stable HO enzymatic activity in rat liver

To enhance HO activity in a stable manner, we administered the rAAV carrying rat HO-1 cDNA to the liver through portal injection. After the injection, a large num-

ber of HO-1 positive non-parenchymal cells were found in both rAAV/GFP and rAAV/HO-1-transduced livers on d 7 (17.7 ± 0.5 cells/mm^2 versus 10.8 ± 0.7 cells/mm^2, n = 3). However, the number of positive cells decreased dramatically to a level similar to normal livers in rAAV/GFP transduced liver on day 30, whereas there was a significantly larger number of HO-1-positive non-parenchymal cells in rAAV/HO-1-transduced livers (3.9 ± 0.8 cells/mm^2 *vs* 5.8 ± 1.0 cells/mm^2, n = 3, P < 0.05; Figures 1A and 1B). No HO-1 positive hepatocyte was found in both rAAV/GFP and rAAV/HO-1 treatment groups on d 7. The expression of HO-1 in hepatocyte was first detected sporadically on d 14 after portal injection of rAAV/HO-1, whereas there was no HO-1 positive hepatocyte in rAAV/GFP-transduced livers (data not shown). More HO-1 positive hepatocytes could be detected in rAAV/HO-1-transduced livers on d 30 (2.2 ± 0.3 cells/mm^2, n = 3, P < 0.05), whereas they remained undetectable in rAAV/GFP-transduced livers (Figures 1A and 1B). To evaluate the biological activity of transduced HO-1 in the livers, we determined the enzymatic activity of HO by measuring the generated bilirubin of microsomal protein isolated from the livers. There was a significant increase of HO activity of the rAAV-transduced livers on d 7 (the earliest time point that we detected). Both rAAV/GFP and rAAV/HO-1 transduced livers showed increased amount of generated bilirubin in isolated microsomal proteins. Impressively, the amount of generated bilirubin (0.9 fold over the basal level of normal rat liver) remained elevated in the rAAV/HO-1 treatment group and sustained for over 3 mo of observation time. In contrast, the amount of generated bilirubin decreased to the level of normal rats in the rAAV/GFP treatment group on d 14. (Figure 1C). This suggests the specificity of rAAV/HO-1 in the induction of stable HO activity in rat liver.

Enhancement of HO activity suppressed the development of cirrhosis in rats

Intragastrically administration of CCl_4 to adult LEW rats for 9 wk resulted in the formation of micronodular cirrhosis. To further evaluate the effects of elevated HO activity on the development of cirrhosis, we examined various parameters relating to cirrhosis after over-expression of HO-1 in rat liver by rAAV gene transfer (Figure 2A). Histological examination showed massive amount of fibrotic tissues accumulated in the portal tract areas of non-transduced or rAAV/GFP-transduced livers and led to the formation of micronodular cirrhosis accompanied with portal hypertension and lower systolic blood pressure in the majority of rats. rAAV/HO-1 gene transfer markedly diminished the amount of accumulated fibrotic tissues as shown in Masson's trichrome (MT) staining and immunohistochemistry. No micronodular cirrhosis was formed and only minimal fibrotic tissues could be found in the portal tract areas of rAAV/HO-1-transduced livers (Figure 2B). Analysis of fibrotic areas using the computer software showed that there was significantly less fibrotic element deposition in the rAAV/HO-1-transduced liver (7.9% ± 0.9% by MT staining, 3.1% ± 2.6% by collagen 1α

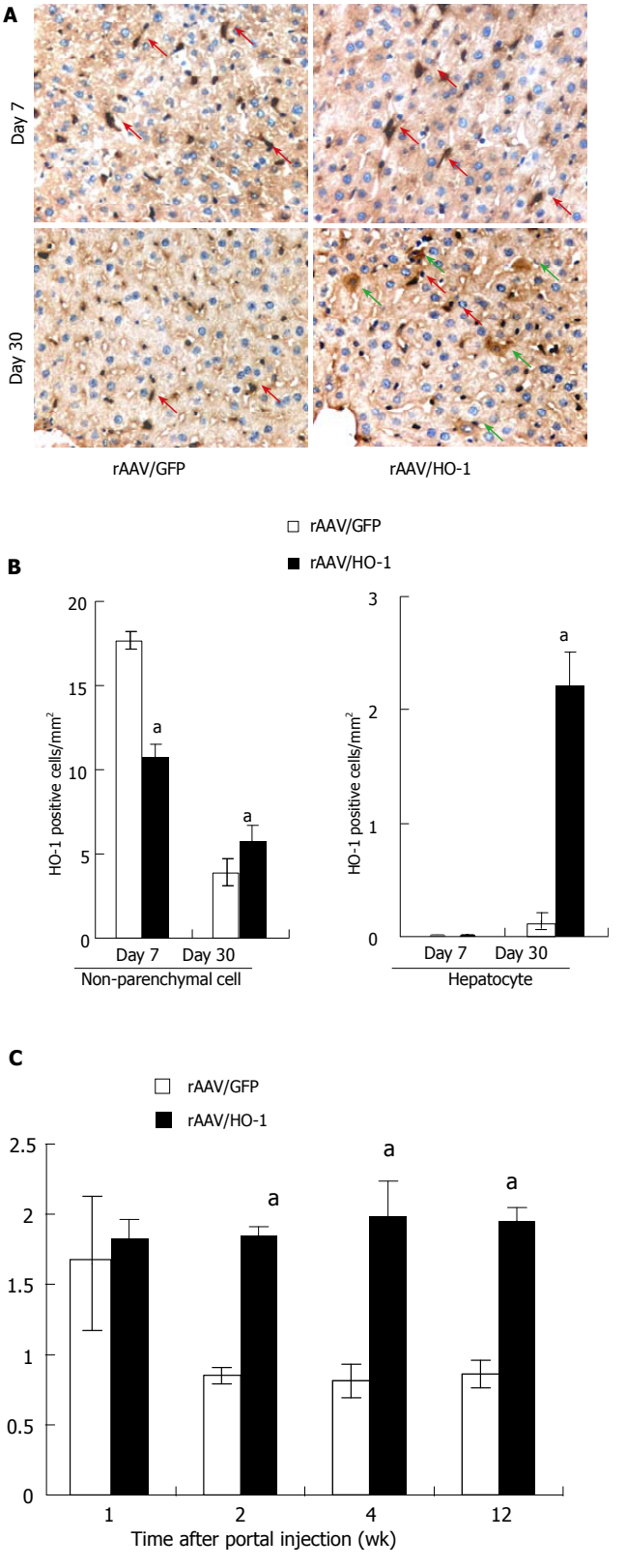


Figure 1 **A:** The expression patterns of HO-1 in the livers after portal injection of rAAV/HO-1 in LEW rats. Red arrow indicated the HO-1-positive non-parenchymal cells; green arrow indicated the hepatocyte; **B:** The quantification of the HO-1 positive cells in the liver after administration of rAAV/HO-1. Data were presented as number of HO-1 positive cells per mm^2; n = 3-5, mean ± SE, ^aP<0.05; **C:** The HO enzymatic activity of rAAV/GFP or rAAV/HO-1 transduced livers. The data were demonstrated as the amount of generated bilirubin of microsomal protein from liver tissues and were shown as the fold changes over the normal rat liver, mean ± SE, n = 3, ^aP < 0.05.

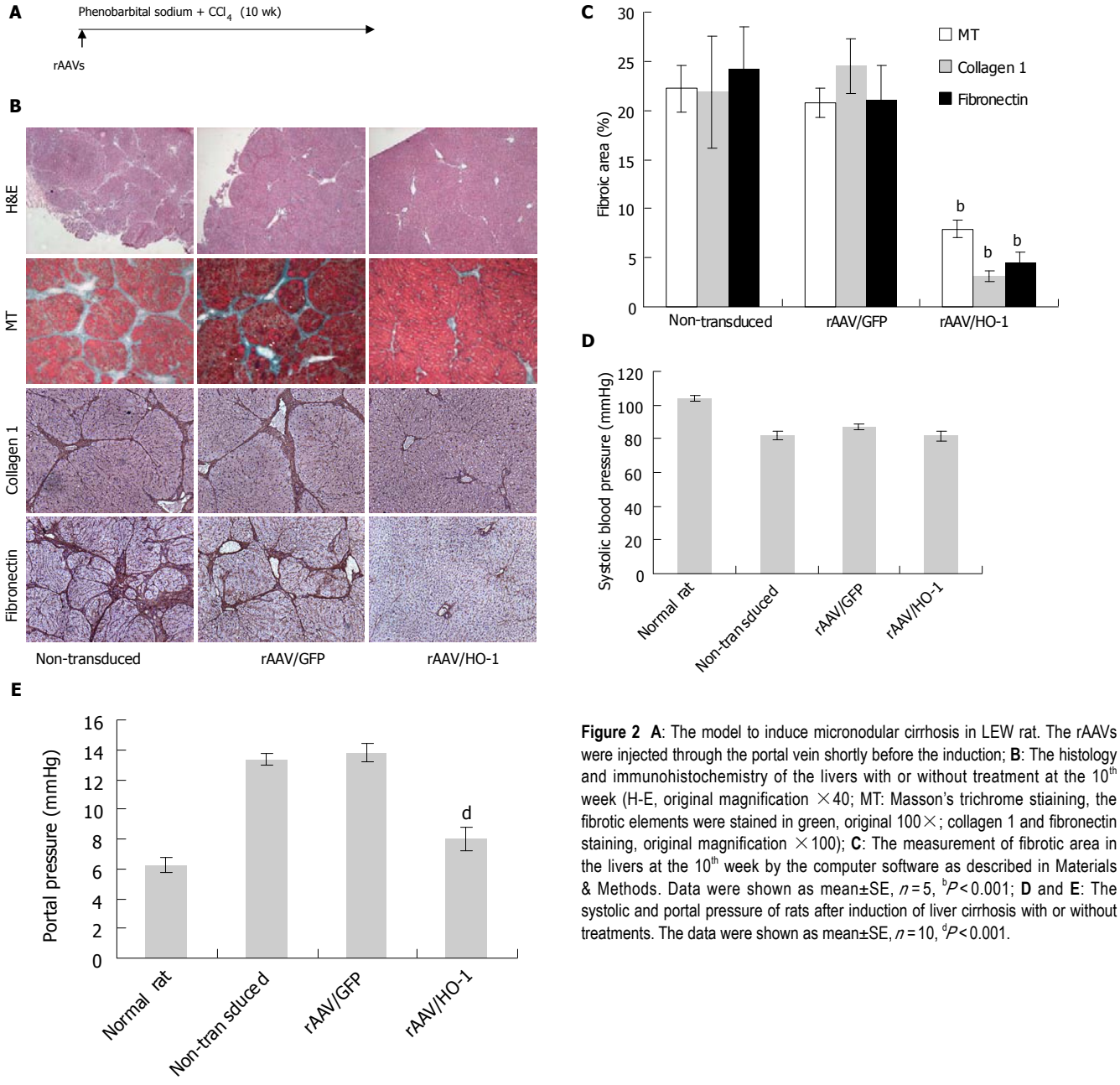
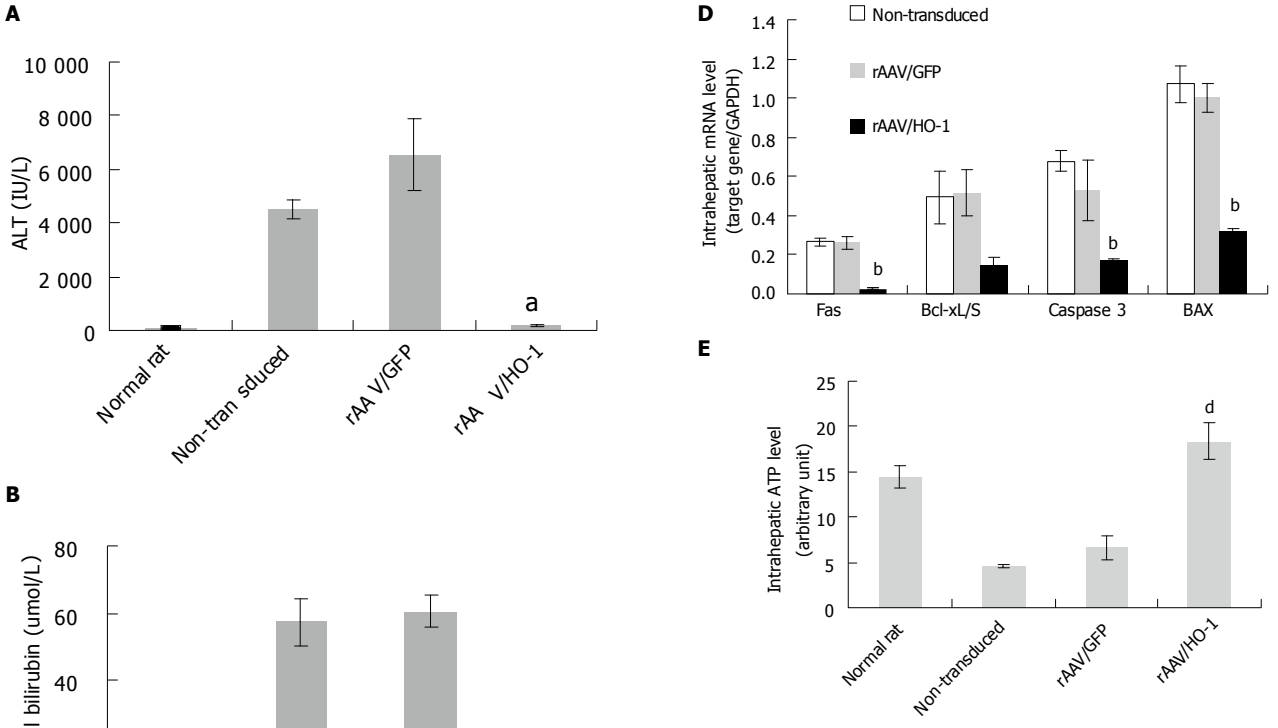


Figure 2 **A**: The model to induce micronodular cirrhosis in LEW rat. The rAAVs were injected through the portal vein shortly before the induction; **B**: The histology and immunohistochemistry of the livers with or without treatment at the 10th week (H-E, original magnification ×40; MT: Masson's trichrome stiaining, the fibrotic elements were stained in green, original 100×; collagen 1 and fibronectin staining, original magnification ×100); **C**: The measurement of fibrotic area in the livers at the 10th week by the computer software as described in Materials & Methods. Data were shown as mean±SE, $n=5$, $^bP<0.001$; **D** and **E**: The systolic and portal pressure of rats after induction of liver cirrhosis with or without treatments. The data were shown as mean±SE, $n=10$, $^dP<0.001$.

staining, 4.4%±1.2% by fibronectin staining, $P<0.001$). In contrast, in the livers of non-treatment or rAAV/GFP treated groups, there was a higher level of fibrotic element deposition (22.2%±5.8% or 20.8%±3.8% by MT staining, 21.9%±5.7% or 24.5%±2.8% by collagen 1α staining, 24.1%±4.4% or 21.1%±3.6% by fibronectin staining, Figure 2C). In addition, the beneficial effects of HO-1 on the development of liver cirrhosis could be reflected by the reduction of portal hypertension (8.0±0.8 mmHg versus 13.3±0.4 mmHg in the non-treatment group and 13.8±0.6 mmHg in the rAAV/GFP treatment group, $P<0.001$), whereas there was no significant difference at the systolic blood pressure (81.7±0.3 mmHg) in comparison to non-treatment (81.8±0.6 mmHg) or vector controls (87.0±0.2 mmHg, $P>0.31$, Figures 2D and 2E).

Stable HO activity protected against CCl_4-mediated recurrent liver injury

Repeated administration of CCl_4 to adult rats led to chronic liver injury with elevated plasma level of total bilirubin (no treatment group: 57.25±6.95 μmol/L; rAAV/GFP group: 60.5±4.87 μmol/L) and ALT (non-treatment group: 4 522±334 IU/L; rAAV/GFP group: 6 552±1 363 IU/L, $n=5$). Increasing HO activity in the liver by rAAV/HO-1 significantly improved the liver function of rats under long-term CCl_4 toxicity, the amount of total bilirubin and ALT decreased dramatically in the rAAV/HO-1-treated rats in comparison to non-treatment or rAAV/GFP controls (total bilirubin: 6.20±3.49 μmol/L; ALT: 199.6±80.5 IU/L, $n=5$, $P<0.005$, Figures 3A and 3B). To further examine the protective effects of over-expressing HO-1 in the livers, we determined the transcript level of apoptotic genes and energy exchanges of livers under long-term CCl_4 toxicity. Rnase protection assay showed the transcript level of Fas, caspase 3, and BAX genes significantly diminished in the rAAV/HO-1-transduced livers ($n=3$, $P<0.05$,



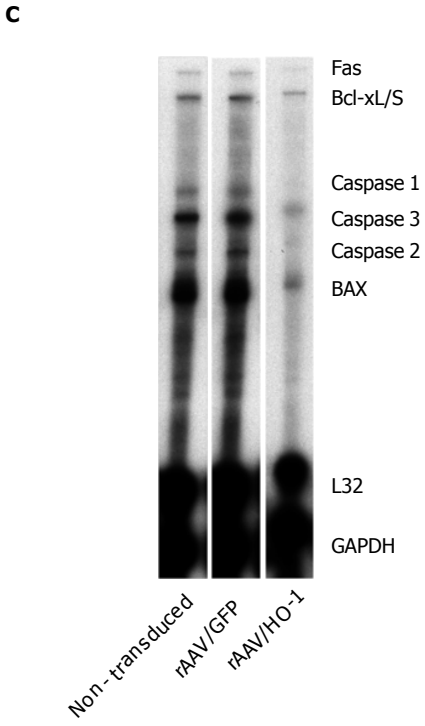


Figure 3 **A** and **B**: The liver function of rats with or with treatment under long-term CCl_4 toxicity was presented by the determination of plasma ALT and total bilirubin level. The data was shown as mean±SEM, n = 5-10, ^aP<0.001; **C** and **D**: The representative picture of the expression patterns of apoptotic genes in the livers at the 10th week. The mRNA level of genes was detected by RNase protection assay. The quantification of the expression level was measured by phosphoimager as described in Materials & Methods. The data were shown as mean ± SE, n = 3-5, ^bP<0.05; **E:** The ATP level of the livers of rats with or without treatments at the 10th wk, n = 5-10, ^dP<0.001.

Figures 3C and 3D), whereas these genes were strongly expressed in the non-transduced or rAAV/GFP-transduced livers. The beneficial effects of HO-1 on CCl_4-mediated chronic liver injury were also shown by the improvement of liver energy exchanges. Over-expression of HO-1 by rAAV significantly improved the intrahepatic ATP level in rAAV/HO-1transduced liver in comparison with non-treatment or rAAV/GFP-transduced livers of rats under long-term CCl_4 toxicity (n = 3-5, P < 0.05; Figure 3E).

Stable HO activity suppresses the pro-inflammatory and pro-fibrogenic responses in CCl_4-treated rats

To characterize the effects of HO-1 on the chronic inflammatory response followed by liver injury, we examined the histopathological parameters of liver tissues and transcript or protein level of pro-inflammatory cytokine-MIF. A large amount of ED1-positive (infiltrating) macrophages accumulated in the portal tract areas in the non-transduced or rAAV/GFP-transduced livers, whereas only a few ED1-positive macrophages were found in rAAV/HO-1-transduced livers (Figure 4A). In contrast, there was no significant change in the number of ED2-positive (tissue-residential) macrophages in the livers of CCl_4-treated rats. In consistent with the immunohistochemical stainings, detection of intrahepatic mRNA level by Rnase protection assay and serum levels of MIF by ELISA method showed significant reduction of the expression level of MIF in rAAV/HO-1-treated rats in comparison to non-treatment or rAAV/GFP controls (n = 3-5, P < 0.001, Figures 4B and 4C).

To examine the effects of rAAV/HO-1 on the fibrogenic process after liver injury, we next examined the

expression patterns of pro-fibrogenic cytokine-TGF-β1, which plays an important role in the activation and transition of HSCs to myofibroblast-like cells after liver injury. Immunohistochemistry showed that TGF-β1 was highly expressed in the portal tract areas in the livers of long-term CCl_4-treated rats. In contrast, only a minimal number of cells expressed TGF-β1 in rAAV/HO-1-transduced livers of rats under the same CCl_4 protocol (Figure 4A). Parallel to the immunohistochemical stainings, RNase protection assay also showed significant reduction in the transcript level of TGF-β1 in rAAV/HO-1-transduced livers (n = 3-5, P < 0.001, Figure 4B). This finding was associated with the decreased number of activated HSCs (desmin-positive) in rAAV/HO-1-transduced livers (Figure 4A).

DISCUSSION

There is a wide range of factors can result in hepatocellular injury. However, the primary response leading to subsequent pro-inflammatory and pro-fibrogenic responses in liver is extraordinary similar [14]. Analysis of clinical parameters based on the treatment of primary liver disease showed encouraging results in the decrease of the severity of liver fibrosis/cirrhosis [15,16]. However, none of anti-fibrotic treatment has been shown clinically effective. A number of therapeutic approaches targeting on anti-oxidant, anti-inflammatory response, suppression of HSC activation, induction of HSC apoptosis or increase of the degradation of extracellular matrix have been shown effective in the attenuation of the severity of liver fibrosis/cirrhosis in various animal models [14]. Most of them, however, cannot easily translate to the clinical setting, because the majority of approaches focus on the single step of disease progression and the clinical situation is far more complex than that in the experimental setting. Thus, it is still urged to find out a therapeutic target that has multiple effects on disease progression and can be applied potentially in the clinical setting.

HO-1-mediated anti-apoptotic and anti-inflammatory activities are among the most attractive mechanisms in cellular protection [17]. In fact, HO-1 only expresses in a subpopulation of Kupffer cells in normal liver, whereas a small amount of HO-2 is constitutively being expressed in hepatocyte [3,4]. None of them expresses in sinusoidal endothelial cells and hepatic stellate cells [3]. However, a significant increase in HO-1 expression was observed on the whole organ level under stress conditions [4]. In the liver with acute injury, HO-1 was expressed in the majority of Kupffer cells and infiltrating macrophages, which might function as a feedback loop to control the macrophage activation [18-20]. Although the whole HO activity increases dramatically, the need for the liver to overcome the insults of cellular injury seems to be relatively insufficient. Increasing HO activity by introduction of exogenous HO-1 by rAAV/HO-1 in our model that significantly improved the long-term outcomes of CCl_4-induced liver cirrhosis could be based on (1) the anti-apoptotic effects of HO-1 on hepatocyte; (2) the anti-inflammatory effects of HO-1 on the control of the cellular response of hepatocyte in the production of MIF under stress and/or injury condi-

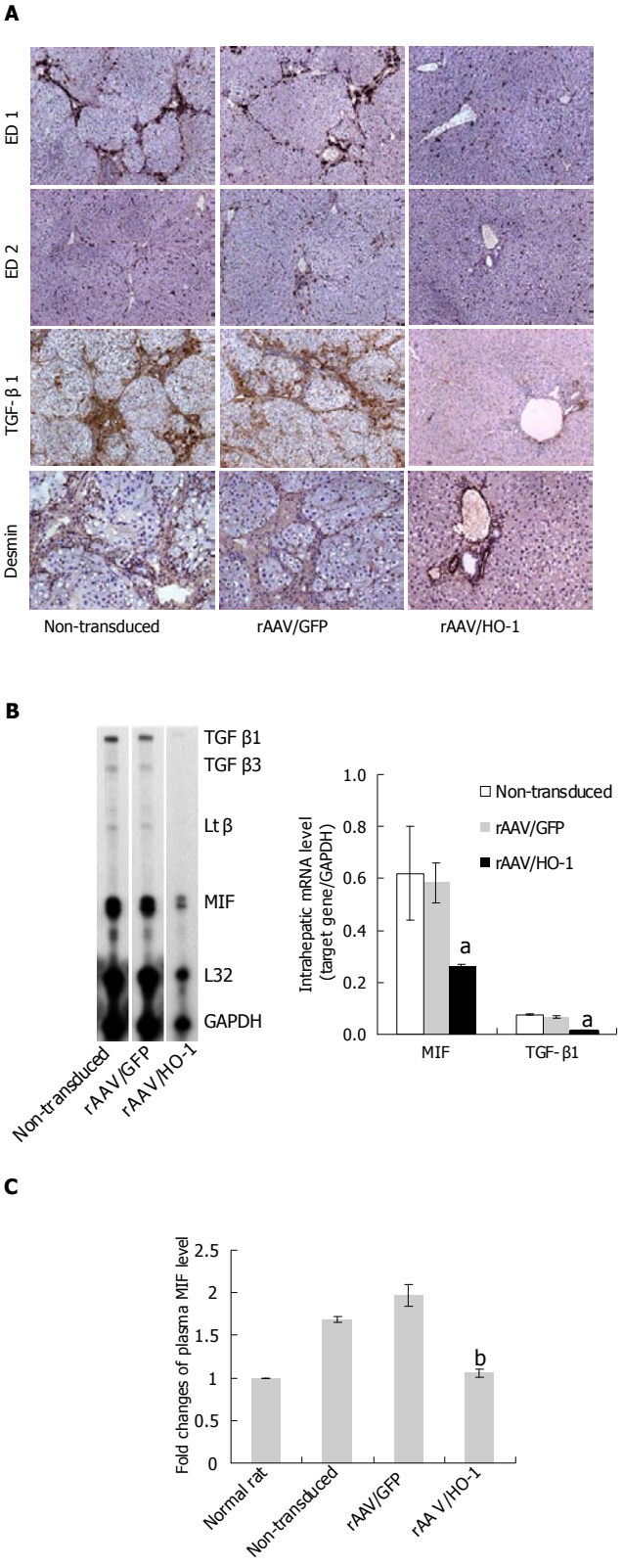


Figure 4 **A:** The representative pictures of pro-inflammatory and pro-fibrogenic responses in the livers of rats with or without treatment at the 10th week. Infiltrating macrophage (ED1), tissue residential macrophages (ED2), TGF-β1, and activated hepatic stellate cells (desmin) were detected by immunohistochemistry; original magnification, ×100; **B:** The profile of pro-inflammatory and profibrogenic cytokine expression. The level of mRNA was detected by RNase protection assay and was quantified by phosphoimager, n = 3-5, ^aP<0.05; **C**: The plasma level of macrophage migration inhibitory factor (MIF) was detected by ELISA. The data were shown as fold changes of over the level in normal rat, n = 3-5, ^bP < 0.001.

tions and the suppression of the macrophage activation; and (3) the anti-fibrogenic effects of HO-1 on the suppression of the collagen synthesis and/or proliferation of activated HSCs.

Improvement of liver function under long-term CCl_4 toxicity by rAAV/HO-1 may reflect the fact that the expression of HO-1 in the hepatocyte was able to prevent liver damage, which was supported by the down-regulation of pro-apoptotic genes and enhancement of liver ATP level in our model. In acute liver injury and ischemia/reperfusion injury of transplanted liver, recent data suggested that the resistance of HO-1 expressing cells to the pro-apoptotic stimuli might be directly through its enzymatic product CO and/or indirectly through the induction of the Fe^{2+}-sequestering protein ferritin. Exposure of CO to the primary hepatocyte could prevent the tumor necrosis factor-α-mediated and anti-CD95-mediated apoptotic events through the down-regulation of caspase-3 activity [21], whereas the induction of ferritin suppressed serum-deprived or oxidative stress-mediated hepatocyte apoptosis by modulation of intracellular Fe^{2+} level and inhibition of Fe^{2+}-mediated conversion of hydrogen peroxide into OH^- and OH through the Fenton reaction [7,22]. In addition to anti-apoptotic effects, HO-1 could also suppress the production of pro-inflammatory cytokine-MIF production in a human primary hepatocyte culture (Tsui TY, et al. unpublished data). This may suggest that HO-1 can function as homeostatic molecule in the prevention of apoptotic event and the control of cellular response.

HO-1 can be induced in various cell types including HSC through direct or indirect mechanisms. Our data are consistent with a recent finding in primary human HSC culture showing that the induction of HO-1 expression in HSC suppressed the transcript level of pro-collagen 1α and serum-mediated HSC proliferation [23]. This may suggest that HO-1 can serve as a negative regulator in the control of HSC activation and proliferation. Thus, less accumulation of fibrotic elements and prevention of the development of portal hypertension in rAAV/HO-1-transduced animals might reflect the outcomes of cellular protection, anti-inflammatory and anti-fibrogenic responses. Although further investigation is needed to clarify the mechanisms in more details, increased HO activity in liver and systemic effects of the products of HO enzymatic activity can be the key in the control of the development of cirrhosis and portal hypertension in our model.

In conclusion, our data demonstrated that enhancement the HO activity in a stable manner can suppresses the pathophysiological changes of cirrhosis. In addition, rAAV-mediated gene transfer may represent an attractive approach in controlling the development of cirrhosis. However, further studies should be carried out in order to answer the question of whether the rAAV approach is suitable for the hepatitis virus-mediated chronic liver injury and the subsequent outcome.

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BASIC RESEARCH

Rosuvastatin reduces rat intestinal ischemia-reperfusion injury associated with the preservation of endothelial nitric oxide synthase protein

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Abstract

AIM: To investigate the protective effect of rosuvastatin on ischemia-reperfusion (I-R)-induced small intestinal injury and inflammation in rats, and to determine the effect of this agent on the expression of endothelial nitric oxide synthase (eNOS) protein.

METHODS: Intestinal damage was induced in male Sprague-Dawley rats by clamping both the superior mesenteric artery and the celiac trunk for 30 min, followed by reperfusion for 60 min. Rosuvastatin dissolved in physiological saline was administered intraperitoneally 60 min before ischemia. The severity of the intestinal mucosal injury and inflammation were evaluated by several biochemical markers, as well as by histological findings. The protein levels of eNOS were determined by Western blot.

RESULTS: The levels of both intraluminal hemoglobin and protein, as indices of mucosal damage, were significantly increased in the I-R group compared with those in the sham-operated group. These increases, however, were significantly inhibited by treatment with rosuvastatin in a dose-dependent manner. The protective effects of rosuvastatin were also confirmed by histological findings. Exposure of the small intestine to I-R resulted in mucosal inflammation characterized by significant increases in thiobarbituric acid-reactive substances, tissue-associated myeloperoxidase activity, and the mucosal contents of rat cytokine-induced neutrophil chemoattractant-1 (CINC-1) and tumor necrosis factor-α (TNF-α). These increases in inflammatory parameters after I-R were significantly inhibited by pretreatment with rosuvastatin at a dose of 10 mg/kg. Furthermore, mRNA expression of CINC-1 and TNF-α was increased after I-R, and this increase was also inhibited by rosuvastatin. The mucosal protein levels of eNOS decreased during I-R, but were preserved in rats treated with rosuvastatin.

CONCLUSION: Rosuvastatin inhibits rat intestinal injury and inflammation induced by I-R, and its protection is associated with the preservation of eNOS protein.

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Key words: Rosuvastatin; Rat; Intestine; Ischemia-reperfusion injury; Endothelium; Nitric oxide synthase

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INTRODUCTION

Intestinal ischemia-reperfusion (I-R) injury is a grave condition resulting from acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, hemorrhagic, traumatic or septic shock, or severe burns[1,2]. Recent reports have demonstrated that neutrophil infiltration in the intestinal mucosa via neutrophil-endothelial cell interactions plays a significant role in the pathogenesis of I-R-induced intestinal injury because activated neutrophils generate tissue damaging products such as reactive oxygen species, protease, collagenase, and a ferrous iron-ferritin complex [3-5].

Leukocyte accumulation is a complex phenomenon that also involves endothelium-based adhesion molecules as well as leukocyte chemotaxis factors such as interleukin-8 (IL-8). Intercellular adhesion molecules (ICAMs) are normally expressed at a low basal level, but their expression can be enhanced by various inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α). A variety of cytokines, including TNF-α, interferon-γ and IL-1β, are released from post-ischemic tissues.

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used in the treatment of hyperlipidemia and coronary artery disease [6]. Recently accumulating evidence suggests that statins have anti-inflammatory and endothelial cell protective actions that are independent of their cholesterol-lowering effect [7]. Statins can increase the expression of endothelial nitric oxide synthase (eNOS) by blocking Rho geranylgeranylation [8], and they can also block the lymphocyte function-associated antigen-1 (LFA-1)-ICAM-1 interaction by binding the L-site [9]. Rosuvastatin, a new HMG-CoA reductase inhibitor, has exhibited a more potent affinity for the active site of HMG-CoA reductase than other statins. In addition, the cytoprotective action of rosuvastatin against ischemic injury has been clearly documented [10-14]. Ikeda *et al* [10], for example, demonstrated that rosuvastatin reduces neutrophil-induced cardiac contractile dysfunction in the isolated ischemic reperfused rat heart. However, to date, the effects of rosuvastatin on intestinal I-R injury have not yet been investigated. In this study, we show that rosuvastatin dramatically attenuates I-R-induced intestinal injury and inflammation. Moreover, rosuvastatin was found to reverse the decrease in eNOS expression after I-R in intestinal mucosa.

MATERIALS AND METHODS

Chemicals

All chemicals were prepared immediately prior to use. Rosuvastatin was a gift from AstraZeneca UK, Ltd. (London, UK). Thiobarbituric acid (TBA) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako Pure Chemical Indust. (Osaka, Japan), and 1,1,3,3-tetramethoxypropane was obtained from Tokyo Kasei (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for rat TNF-α and cytokine-induced neutrophil chemoattractant-1 (CINC-1) were obtained from BioSource International (Camarillo, CA) and Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan), respectively. All other chemicals used were of reagent grade.

Preparation of rats for intestinal I-R

Male Sprague-Dawley rats weighing 180-200 g were obtained from Keari Co., Ltd. (Osaka, Japan). The animals were housed at 22℃ in a controlled environment with 12 h of artificial light per day. They were allowed access to rat chow and water ad libitum. The rats were anesthetized with urethane (1 mg/kg, ip). After a midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. Intestinal ischemia was induced by clamping both the superior mesenteric artery and the celiac trunk for 30 min, resulting in a total occlusion of these arteries, following our previously reported method [15, 16]. After this period of occlusion, the clamps were removed. The animals were randomized into groups receiving different concentrations of rosuvastatin or physiological saline alone by intraperitoneal injection 1 h before ischemia. The maintenance of the animals and the experimental procedures performed on them were carried out in accordance with National Institutes of Health (NIH) guidelines for the use of experimental animals. All procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Assessment of intestinal mucosal injury induced by I-R

The animals were killed after reperfusion, and the intestine was removed and submitted for examination. After making a 30-cm proximal intestinal loop, saline (10 mL) was injected into the loop. The contents were aliquoted after centrifugation. Intestinal injury after reperfusion was evaluated by examining the luminal contents of hemoglobin and protein, and by histological examination. Intestinal bleeding was quantified indirectly as the hemoglobin concentration in luminal lavage fluid using a kit and following the manufacturer's protocol (Wako). Luminal protein levels were also determined using a kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). For histological evaluation, formalin-fixed tissues were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist who was not informed of the experimental conditions for any given specimen.

Measurements of TBA-reactive substances and MPO activity

The concentrations of TBA-reactive substances were measured in the intestinal mucosa using the method described by Ohkawa *et al* [17] as an index of lipid peroxidation. The animals were killed by exsanguination from the abdominal aorta after the experiments, and their small intestines were removed. The intestinal mucosa was then scraped off using two glass slides, and homogenized with 1.5 mL of 10 mmol/L potassium phosphate buffer (pH 7.8) containing 30 mmol/L KCl in a Teflon Potter-Elvehjem homogenizer. The level of TBA-reactive substances in the mucosal homogenates was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as the standard. The total protein in the tissue homogenates was measured using a kit, according to the manufacturer's protocol (Bio-Rad).

Tissue-associated myeloperoxidase (MPO) activity was determined using a modification of the method described by Grisham *et al* [18] as an index of neutrophil accumulation. Briefly, two milliliters of mucosal homogenates were centrifuged at 20 000 g for 15 min at 4℃ to pellet the insoluble cellular debris. The pellet was then rehomogenized in an equivalent volume of 0.05-M potassium phosphate buffer (pH 5.4), containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20 000 g for 15 min at 4℃ and the supernatants were saved. The MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at

655 nm and 25 ℃.

Determination of mucosal content of CINC-1 and TNF-α

The concentrations of the inflammatory cytokines TNF-α and CINC-1 in the supernatant of mucosal homogenates were determined by rat TNF-α and CINC-1-specific ELISA kits according to the manufacturer's instructions.

RT-PCR

The mRNA expression of intestinal TNF-α and CINC-1 was determined by reverse transciprion-polymerase chain reaction the (RT-PCR). Samples of intestinal tissue for mRNA isolation were removed from the intestine. The total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method using Isogen (Nippon Gene, Tokyo, Japan) and the concentration of RNA was determined by absorbance at 260 nm in relation to that at 280 nm. RNA was stored at –70 ℃ until it was used for the RT-PCR. Amplification was carried out in a 50-μL mixture containing 2 μL of the RT product, 0.6 μmol/L of both the sense and antisense primers, 0.4 mmol/L dNTP mix, and 0.5 μL Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan). The reaction was performed as follows: 35 cycles of amplication (denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s, and extension at 72 ℃ for 60 s), followed by a final extension step of 7 min at 72 ℃. The primers had the following sequences: for TNF-α, sense 5'-ATGAGCACAGAAAGCATGATC-3', and antisense 5'-TACAGGCTTGTCACTCGAATT-3'; for CINC-1, sense 5'-CTGTGCTGGCCACCAGCCGC-3', and antisense 5'-ACAGTCCTTGGAACTTCTCTG-3'; and for ß-actin, sense 5'-ATCGTGGGCCGCCCTAGGCA-3', and antisense 5'-TGGCCTTAGGGTTCAGAGGGG-3'. The PCR products were separated electrophoretically in a 25 g/L agarose gel and stained by ethidium bromide.

Western blotting

Intestinal cells were thawed on ice and homogenized at 4 ℃ in a solution of 50 mmol/L Tris–HCl (pH 7.6), 300 mmol/L NaCl, 0.5 g/L Triton X-100, 10 g/L aprotinin, 10 g/L leptin, 1 mmol/L phenylmethysulfonyl fluoride (PMSF), 1.8 g/L iodoacetamide, 50 mmol/L NaF, and 1 mmol/L DTT in order to extract the total cell protein. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (Bio-Rad). Membranes were probed with specific antibodies against eNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and immune complexes were visualized by Western blotting with a commercial kit (ECL, Amersham, Buckinghamshire, England), following the manufacturer's recommendations.

Statistics

All results are presented as mean ± SE. The data were compared by two-way analysis of variance (ANOVA), and differences were considered to be significant if the *P* value was less than 0.05 based on Scheffe's multiple comparison test. All analyses were performed using the Stat View 5.0-J program (Abacus Concepts, Inc., Berkeley, CA, USA) on a Macintosh computer.

RESULTS

Rosuvastatin inhibits intestinal reperfusion injury

We found that 60 min of reperfusion after 30 min of ischemia resulted in increases in luminal protein and hemoglobin concentrations (Figure 1). In contrast, pre-treatment with rosuvastatin 1 h before the ischemia was found to inhibit these increases in a dose-dependent manner (Figure 1). Neither vehicle alone nor rosuvastatin alone affected the luminal protein or hemoglobin concentrations (data not shown). Therefore, in the following experiments, we used rosuvastatin at a dose of 10 mg/kg and examined its pharmacological action. In rats treated with I-R, multiple erosions and bleeding developed in the small intestine; however, pretreatment with rosuvastatin at a dose of 10 mg/kg 1 h before the ischemia inhibited both the intestinal erosions and the bleeding. The protective effect of rosuvastatin was further confirmed histologically. Representative hematoxylin and eosin-stained sections of the ileum from sham, I-R, and I-R + rosuvastatin-treated animals are depicted in Figure 2. While the ileum of sham animals exhibited normal mucosal architecture with intact villi, I-R resulted in large areas of epithelial crypt loss, predominantly neutrophilic infiltrate throughout the mucosa, erosion, and mucosal bleeding. In contrast, pretreatment with rosuvastatin resulted in smaller erosions with few neutrophils. Rosuvastatin alone did not produce any macroscopic or microscopic lesions in the rat intestine.

Effect of rosuvastatin on TBA-reactive substances

The extent of lipid peroxidation was determined by measuring TBA-reactive substances in the small intestine. No significant differences were found in the intestinal TBA-reactive substance levels between vehicle- and rosuvastatin-treated rats in the sham-operated group. Sixty min of reperfusion caused a significant increase in TBA-reactive substances in the control rats. This increase in TBA-reactive substances in the intestinal mucosa was significantly inhibited by 10 mg/kg of rosuvastatin (Figure 3A).

Effect of rosuvastatin on MPO activity

A hallmark of intestinal reperfusion injury is the accumulation of neutrophils in the injured tissue. Therefore, we next evaluated neutrophil accumulation based on tissue-associated MPO activity. There were no differences in intestinal MPO activities between vehicle- and rosuvastatin-treated rats in the sham-operated group, while the MPO activity in the intestinal mucosa markedly increased from a basal activity of 0.23 ± 0.07 nkat/g protein to 5.20 ± 1.65 nkat/g protein after I-R in the control group. The increase in MPO activity in the intestinal mucosa after reperfusion was significantly inhibited by treatment with rosuvastatin (Figure 3B).

Effect of rosuvastatin on mRNA expression and mucosal protein contents of CINC-1

In order to determine whether or not treatment with rosuvastatin can modulate the inflammatory response through the regulation of cytokine production, we analyzed intestinal mucosal levels of CINC-1 and TNF-α. The intestinal concentrations of TNF-α and CINC-1 increased signifi-

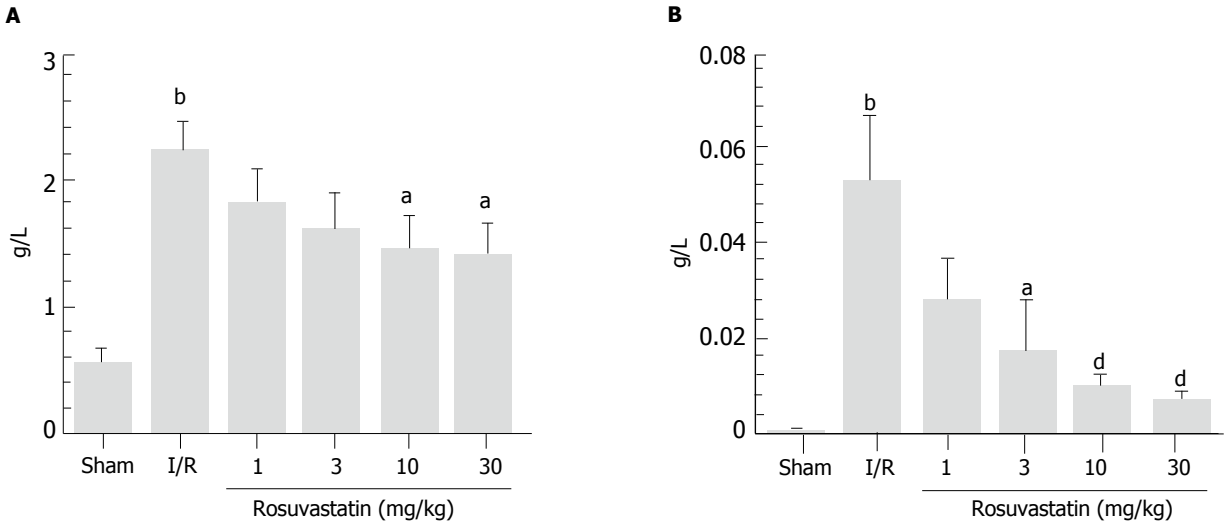

Figure 1 Dose-response of rosuvastatin on acute intestinal I-R injury in rats (mean ±SE). **A:** Intraluminal protein; **B:** Hemoglobin. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$, $^{d}P<0.01$ *vs* Vehicle + I-R.

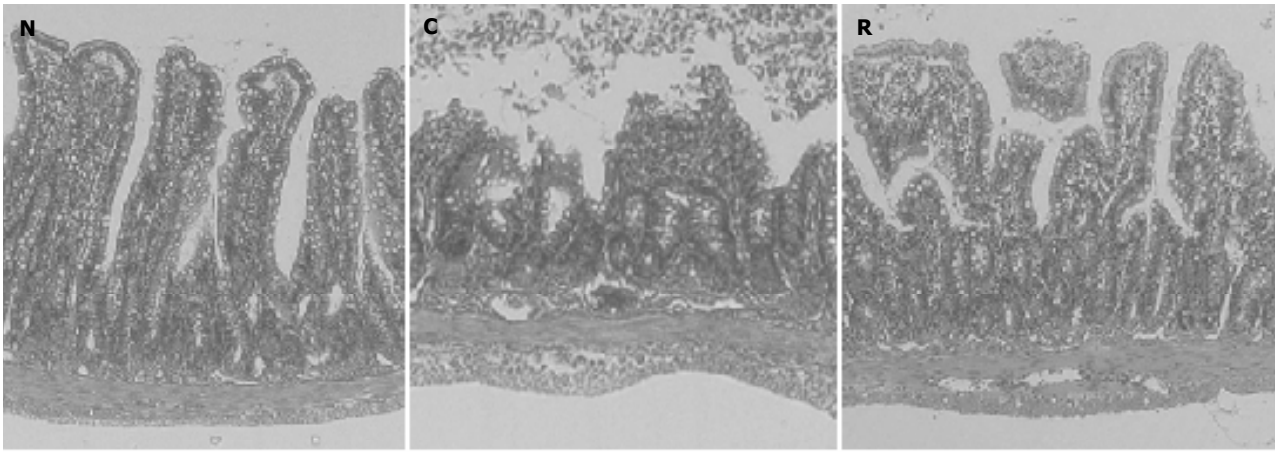

Figure 2 Histological appearance of the small intestine in rats (HE ×20). **N:** Sham. **C**: I-R. **R**: I-R plus rosuvastatin.

cantly in rats treated with I-R, and these increases were significantly inhibited by treatment with rosuvastatin at a dose of 10 mg/kg (Figures 4A, 4B). To further confirm the inhibitory effect of rosuvastatin on CINC-1 and TNF-α production, we analyzed the intestinal expression of CINC-1 and TNF-α using RT-PCR yielding 495 and 225 base pair products to identify CINC-1 and TNF-α gene expression, respectively. As shown in Figure 5, we found the expression of these genes in rats treated with sham-operation to be negligible or faint. In contrast, transcription was readily enhanced in I-R-treated rats. Treatment with rosuvastatin suppressed the level of mRNA expression for each gene.

Effect of rosuvastatin on eNOS protein expression

Since previous studies have shown that statins exerts beneficial effects on the endothelium through an increase in NO production from eNOS [19, 20], we investigated whether eNOS expression could be affected by I-R with or without rosuvastatin pre-treatment. Figure 6 was a representative of at least 3 experiments performed on different experimental days. The expression levels of eNOS protein were decreased during the I-R. However, pretreatment with rosuvastatin significantly reversed the decrease in eNOS protein. Rosuvastatin alone did not affect intestinal eNOS protein levels in sham-operated rats.

DISCUSSION

The present study clearly demonstrates that rosuvastatin, a new HMG-CoA reductase inhibitor, has a protective effect against reperfusion-induced intestinal injury and inflammation in rats. In this study, intestinal injury was assessed by a variety of methods including luminal protein/hemoglobin concentrations and histological evaluation. The results of each assessment showed that rosuvastatin treatment significantly inhibited intestinal injury. In addition, we demonstrated that, in I-R-induced intestinal inflammation, the expression of pro-inflammatory cytokines (CINC-1 and TNF-α) was enhanced in association with neutrophil accumulation, as determined by MPO activity in the homog-

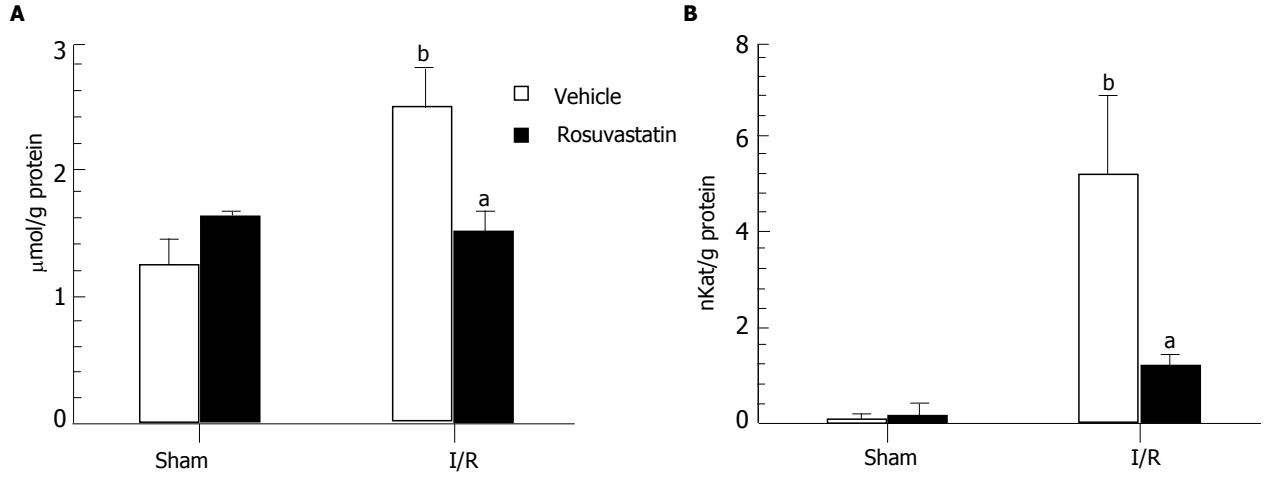

Figure 3 Effects of rosuvastatin on lipid peroxide concentrations and neutrophil accumulation in the intestinal mucosa of rats treated with I-R (mean±SE). **A:** TBA-reactive substance; **B:** Myeloperoxidase activity. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$ *vs* Vehicle + I-R.

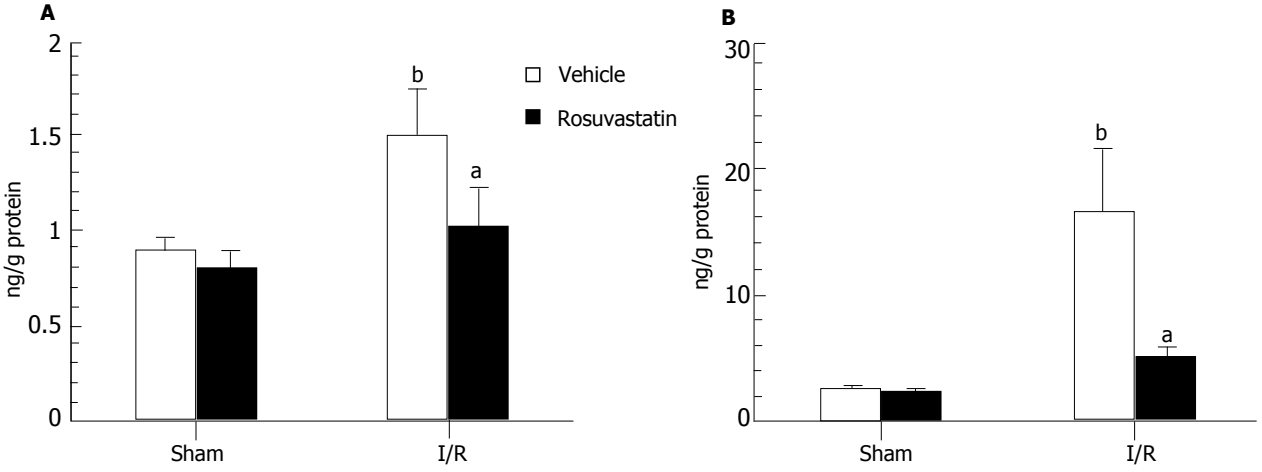

Figure 4 Effects of rosuvastatin on the intestinal CINC-1 and TNF-α after I-R in rats (mean±SE). **A:** CINC-1; **B:** TNF-α. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$ *vs* Vehicle + I-R.

enate of the small intestine. Rosuvastatin treatment inhibited both the up-regulation of pro-inflammatory cytokine expression and the neutrophil infiltration, which prevents tissue injury. These results suggest that rosuvastatin has a protective action against I-R-induced intestinal mucosal injury as well as an anti-inflammatory action against reperfusion-induced inflammation.

Oxygen-derived free radicals produced during I-R are known to cause peroxidation of polyunsaturated fatty acids in cell membranes. The resulting increase in lipid peroxides can lead to changes in membrane fluidity and permeability, and finally to cell lysis. In the present study, TBA-reactive substances in intestinal mucosa, which are an index of lipid peroxidation, were significantly increased in I-R-treated rats compared to sham-operated rats, and rosuvastatin treatment significantly inhibited the increase in these substances. However, neither rosuvastatin nor other statins have been reported to have antioxidant activities *in vitro*, nor has the inhibition of lipid peroxidation been previously reported. Our recent study also showed that rosuvastatin had no effect on scavenging hydroxyl and superoxide radicals determined by electron spin resonance-spin trapping assay (data not shown). Therefore, the inhibitory effect of rosuvastatin on I-R-induced lipid peroxidation in rat intestinal mucosa may result from its anti-inflammatory activity, rather than from its direct antioxidant activity.

Recent reports hypothesize that neutrophil-mediated inflammation is involved in the pathogenesis of I-R-induced intestinal injury. This hypothesis has been supported by several studies; neutrophil depletion by intraperitoneal injection of anti-neutrophil serum has been found to significantly attenuate intestinal mucosal injury elicited by I-R [21, 22]; in addition, immunoneutralization of the CD11/CD18 adherence complex on neutrophils and CINC-1 is known to attenuate intestinal reperfusion injury [23, 24]. The present study showed that MPO activity, an index of tissue-associated neutrophil accumulation, increases in the intestinal mucosa after I-R, and that this increase is significantly inhibited by treatment with rosuvastatin. The inhibition of neutrophil-endothelial interaction by statins has been demonstrated in a number of previous reports. Honjo *et al* [25] have shown that both cerivastatin and pravastatin significantly reduce the number of rolling/accumulated leukocytes in the retinal veins after I-R. Naidu *et al* [26] have

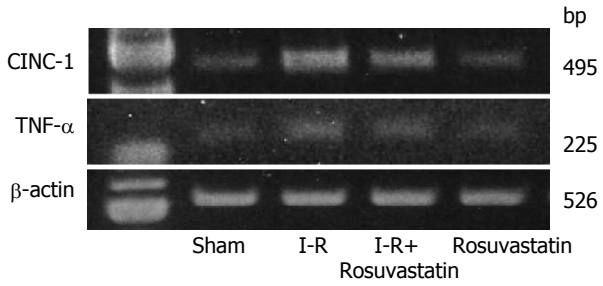

Figure 5 Effects of rosuvastatin on the expression of CINC-1 and TNF-α mRNA in the intestinal mucosa after I-R in rats.

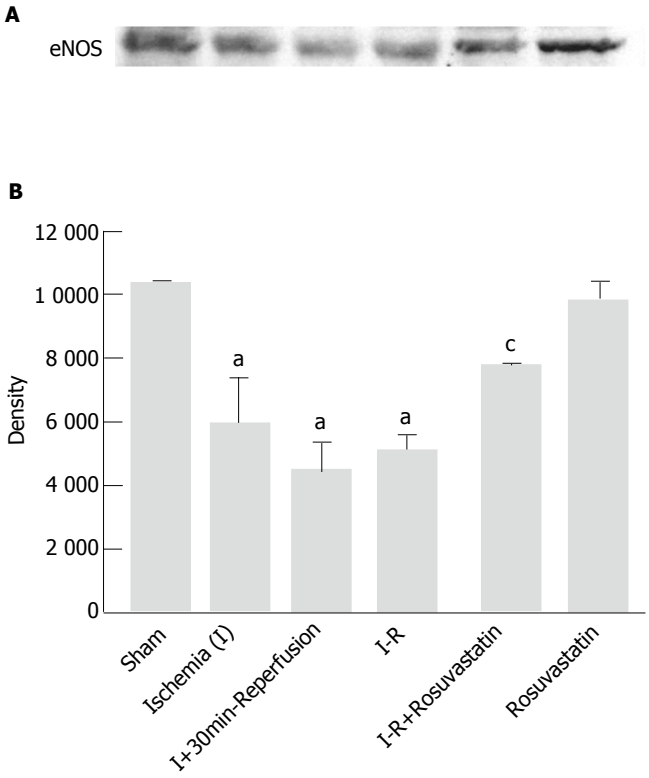

Figure 6 Effect of rosuvastatin on eNOS protein expression during I-R. $^{a}P<0.05$ *vs* Sham, $^{c}P<0.05$ *vs* Vehicle + I-R.

also demonstrated that pretreatment with simvastatin inhibits increases in tissue MPO content in lungs treated with I-R. In addition, the present study showed that the expression of the pro-inflammatory cytokines CINC-1 and TNF-α markedly enhanced in the intestine after I-R, and that these increases are also significantly inhibited by rosuvastatin. The inhibitory effect of statins against mRNA expression for inflammatory genes was also confirmed by a recent study using DNA microarray technology [27], in which it was demonstrated that the statins atorvastatin and pitavastatin directly affect the expression levels of genes involved in inflammation, including interleukin-8 (similar to rat CINC-1), in cultured human umbilical vein endothelial cell. These results, together with the present data, indicate that the inhibition of neutrophil accumulation as well as the inhibition of cytokine production by rosuvastatin may be one of the protective factors decreasing I-R-elicited intestinal mucosal injury.

The beneficial effects of statins on acute inflammation are abolished when it is administered together with an NOS inhibitor [20] or when it was administered to eNOS knockout mice [19], indicating that an enhanced release of NO from eNOS may be involved in the action of statins. Statins are known to up-regulate eNOS expression and activate this enzyme in the systemic vasculature by blocking Rho geranylgeranylation [8, 28] as well as by activating the PI-3 kinase/Akt pathway [20]. As shown in the present study, the intestinal levels of eNOS protein gradually decreased during 30-min ischemia and 60-min reperfusion, indicating that reperfusion may be associated with a posttranscriptional reduction in the expression of the eNOS enzyme. In addition, rosuvastatin-treated animals demonstrated preserved expression of eNOS protein compared to the I-R-treated group, although this agent did not affect the level of eNOS protein in normal rats. Therefore, the persistent expression of eNOS protein at 60 min of reperfusion in rosuvastatin-treated rats is likely to explain the reduction in tissue injury and neutrophil accumulation. In line with our observations, NO is reported to prevent leukocyte adhesion to the endothelium by repressing the up-regulation of cell adhesion molecules in the endothelial cells [29, 30].

Our results showed that I-R-induced intestinal inflammation is characterized by increased production of inflammatory mediators and decreased expression of eNOS. The blockade of these mediators and the preservation of eNOS expression by rosuvastatin were accompanied by significant suppression of intestinal inflammation *in vivo*. These data suggest that rosuvastatin may represent a novel therapeutic approach for the treatment of I-R-induced intestinal injury.

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BASIC RESEARCH

Usefulness of biopsying the major duodenal papilla to diagnose autoimmune pancreatitis: A prospective study using IgG4-immunostaining

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Abstract

AIM: To examine the histological and immunohistochemical findings of biopsy specimens taken from the major duodenal papilla of autoimmune pancreatitis (AIP) patients.

METHODS: The major duodenal papilla in the resected pancreas of 3 patients with AIP and of 5 control patients [pancreatic carcinoma (n = 3) and chronic alcoholic pancreatitis (n = 2)] was immunostained using anti-CD4-T cell, CD8-T cell and IgG4 antibodies. Forceps biopsy specimens taken from the major duodenal papilla of 2 patients with AIP and 5 control patients with suspected papillitis were prospectively taken during duodenoscopy and immunohistochemically examined.

RESULTS: Moderate or severe lymphoplasmacytic infiltration including many CD4-positive or CD8-positive T lymphocytes and IgG4-positive plasma cells ($\geqslant$10/HPF), was observed in the major duodenal papilla of all 3 patients with AIP. The same findings were also detected in the biopsy specimens taken from the major duodenal papilla of 2 patients with AIP, but in controls, there were only a few ($\leqslant$3/HPF) IgG4-positive plasma cells infiltrating the major duodenal papilla.

CONCLUSIONS: An abundant infiltration of IgG4-positive plasma cells is specifically detected in the major duodenal papilla of patients with AIP. Although this is a preliminary study, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may support the diagnosis of AIP.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of pancreatitis in which autoimmune mechanisms are involved in the pathogenesis. It is characterized by irregular narrowing of the main pancreatic duct, enlargement of the pancreas, increased levels of serum γ globulin or IgG, presence of autoantibodies, and responsiveness to steroid therapy[1,2]. Histological findings of the pancreas of AIP are also characteristic, and include dense lymphoplasmacytic infiltration with fibrosis[3]. Recently, it was reported that serum concentrations of IgG4 are significantly and specifically raised in patients with AIP[4]. However, the sensitivity of raised serum IgG4 concentrations is 67%[5]-68%[6] in some reports.

AIP occurs predominantly in elderly males and frequently presents as obstructive jaundice[7]. This presentation is also typical of pancreatic carcinoma. In typical cases of AIP that show diffuse change of the pancreas, the diagnosis can be easily made based on the combination of computed tomography (CT) and endoscopic retrograde cholangiopancreatography (ERCP) findings. However, in segmental mass-forming cases, the differentiation between AIP and pancreatic carcinoma remains difficult. The "double-duct sign", representing stricture in both the pancreatic and bile ducts, is often found in patients with pancreatic head carcinoma. This finding, however, is also frequently observed in AIP[7], because the inflammatory process compresses both the main pancreatic duct and the distal common bile duct[3]. As most patients with AIP respond to oral steroid therapy[2], an accurate diagnosis of AIP can avoid unnecessary

laparotomy or pancreatic resection. On the other hand, histopathological approach to the pancreas is sometimes difficult.

We previously reported that IgG4-positive plasma cells abundantly infiltrated various organs, as well as the pancreas of a patient with AIP, and that an abundant infiltration of IgG4-positve plasma cells was not observed in the organs of patients with pancreatic carcinoma or chronic alcoholic pancreatitis[3,8]. In order to find a useful new method to diagnose AIP on the basis of histological and immunohistochemical studies of the resected pancreas, we prospectively examined the histological and immunohistochemical findings of biopsy specimens obtained from the major duodenal papilla of AIP patients.

MATERIALS AND METHODS

The major duodenal papilla in the resected pancreas of 3 patients with AIP who underwent pancreatoduodenectomy due to suspected pancreatic carcinoma was histologically examined. The specimens were fixed in 10% formaldehyde. Serial sections were cut from paraffin-embedded tissue blocks, and immunostained using anti-CD4-T (Novocastra, Newcastle upon Tyne, UK), CD8-T (DakoCytomation, Glostrup, Denmark) cell subsets, as well as IgG4 (The Binding Site, Birmingham, UK) antibodies with avidin-biotin-peroxidase complex (ABC). The number of immunohistochemically identified cells per high power field (HPF) in each section was counted.

We recently treated 2 patients with AIP, a 73-year-old male and a 60-year-old female. Both patients had obstructive jaundice due to stenosis of the lower bile duct. Segmental narrowing of the main pancreatic duct was observed in the pancreatic head on ERCP, and focal enlargement of the pancreatic head was detected on CT. Serum IgG4 concentrations were elevated to 325 mg/dL in the male and 825 mg/dL in the female. The major duodenal papilla was normal on duodenoscopy during ERCP. Forceps biopsy specimens from the major duodenal papilla were taken during duodenoscopy. Histological and immunohistochemical studies were done on these biopsy specimens. After the biopsy was taken, steroid therapy was given to both patients, who showed marked responsiveness both morphologically and serologically. In 1 patient, rebiopsy from the major duodenal papilla was done after steroid therapy.

Histological and immunohistochemical studies were also done on control specimens. Controls consisted of the major duodenal papilla specimens of the pancreases resected by pancreatoduodenectomy for pancreatic head carcinoma ($n = 3$) and chronic alcoholic pancreatitis ($n = 2$), as well as endoscopically biopsied specimens taken from the major duodenal papilla of patients with suspected papillitis ($n = 5$).

RESULTS

Moderate or severe lymphoplasmacytic infiltration was observed in the major duodenal papilla of all 3 patients with AIP. Immunohistochemically an abundant infiltration of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (≥10/HPF) was observed in these 3 major duodenal papillae. Moderate or severe lymphoplasmacytic infiltration which included many CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (≥10/HPF) was also observed in the biopsy specimens taken from the major duodenal papilla of 2 AIP patients (Figures 1 and 2). Although infiltration of CD4- or CD8-positive T lymphocytes was detected to some extent in the major duodenal papilla of controls, there were few IgG4-positive plasma cells infiltrating the major duodenal papilla of controls (≤3/HPF). The abundant infiltration of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells disappeared in the biopsy specimen taken from the major duodenal papilla of 1 patient after steroid therapy.

Figure 1 Histology of biopsy specimen taken from the major duodenal papilla of a patient with autoimmune pancreatitis showing severe lymphoplasmacytic infiltration.

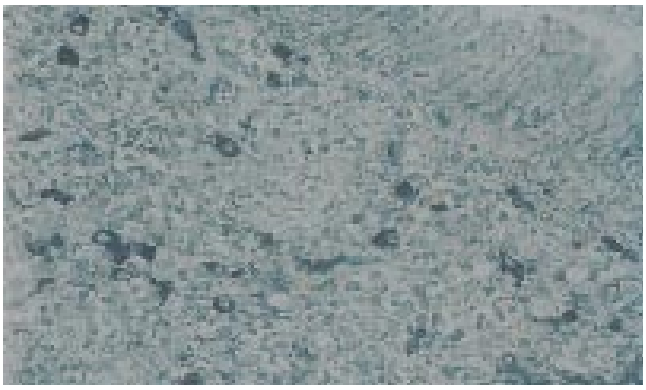

Figure 2 IgG4-immunostaining of biopsy specimen taken from the major duodenal papilla of a patient with autoimmune pancreatitis showing an abundant infiltration of IgG4-positive plasma cells (≥10/HPF).

DISCUSSION

Chronic pancreatitis and several other pancreaticobiliary diseases may be associated with inflammation of the major duodenal papilla, histologically showing infiltration of neutrophils or lymphocytes, or fibrosis[9]. In 2002, Unno *et al*[10] reported that a swollen major duodenal papilla was detected in 41% of 17 patients with AIP, and many infiltrating T lymphocytes were present in the biopsy specimens taken from the swollen papillary tissue. In 2004,

Sahin *et al*[11]reported that dense T-lymphocytic infiltration was present in the resected major duodenal papilla of 2 patients with AIP. In the present study, dense infiltration of CD4- or CD8-positive lymphocytes was also detected in the resected major duodenal papilla of 3 patients with AIP. Furthermore, an abundant infiltration of IgG4-positive plasma cells was detected in the major duodenal papilla of these patients and was not observed in the major duodenal papilla of controls.

Our previous immunohistochemical studies[3,8] of resected pancreases taken from AIP patients showed that the infiltrating inflammatory cells consisted of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells, and that an abundant infiltration IgG4-positive plasma cells in the pancreas was not detected in other diseases. In addition, an abundant infiltration of IgG4-positive plasma cells was detected in various organs of patients with AIP, including peripancreatic retroperitoneal tissue, biliary tract, salivary glands, lymph nodes, and others. We therefore proposed the existence of a novel clinicopathological entity, an IgG4-related systemic disease characterized by extensive IgG4-positive plasma cell infiltration of organs together with CD4- or CD8-positive T lymphocytes[8]. Based on this concept, the dense infiltration of IgG4-positive plasma cells along with CD4- or CD8-positive lymphocytes that are detected in the major duodenal papilla of patients with AIP seems to be induced by the same mechanism as is operative in the pancreas.

These findings led us to do a prospective immunohistochemical study using an anti-IgG4 antibody to study the biopsy specimens taken from the major duodenal papilla of AIP patients. An abundant IgG4-positive plasma cell infiltration was detected in the biopsy specimens taken from the non-swollen major duodenal papilla of 2 AIP patients, and was not detected in the biopsy specimens taken from controls. Of note, the abundant infiltration of IgG4-positive plasma cells disappeared after steroid therapy. Although the number of examined cases is small, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may be useful to support the diagnosis of AIP.

In conclusion, an abundant infiltration of IgG4-positive plasma cells was detected in the major duodenal papilla of patients with AIP. Although this is a preliminary study, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may be useful to support the diagnosis of AIP.

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BASIC RESEARCH

Effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine levels and gastric ulcer healing in rats

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Abstract

AIM: To compare the effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine levels and gastric ulcer healing.

METHODS: Male Spraque-Dawley rats ($n=48$) were divided into four groups. Group1 served as control group, group 2 as gastric ulcer group without treatment, groups 3 and 4 as gastric ulcer treatment groups with sucralfate and *Aloe vera*. The rats from each group were divided into 2 subgroups for study of leukocyte adherence, TNF-α and IL-10 levels and gastric ulcer healing on days 1 and 8 after induction of gastric ulcer by 20% acetic acid.

RESULTS: On day 1 after induction of gastric ulcer, the leukocyte adherence in postcapillary venule was significantly ($P<0.05$) increased in the ulcer groups when compared to the control group. The level of TNF-α was elevated and the level of IL-10 was reduced. In the ulcer groups treated with sucralfate and *Aloe vera*, leukocyte adherence was reduced in postcapillary venule. The level of IL-10 was elevated, but the level of TNF-α had no significant difference. On day 8, the leukocyte adherence in postcapillary venule and the level of TNF-α were still increased and the level of IL-10 was reduced in the ulcer group without treatment. The ulcer treated with sucralfate and *Aloe vera* had lower leukocyte adherence in postcapillary venule and TNF-α level. The level of IL-10 was still elevated compared to the ulcer group without treatment. Furthermore, histopathological examination of stomach on days 1 and 8 after induction of gastric ulcer showed that gastric tissue was damaged with inflammation. In the ulcer groups treated with sucralfate and *Aloe vera* on days 1 and 8, gastric inflammation was reduced, epithelial cell proliferation was enhanced and gastric glands became elongated. The ulcer sizes were also reduced compared to the ulcer group without treatment.

CONCLUSION: Administration of 20% acetic acid can induce gastric inflammation, increase leukocyte adherence in postcapillary venule and TNF-α level and reduce IL-10 level. *Aloe vera* treatment can reduce leukocyte adherence and TNF-α level, elevate IL-10 level and promote gastric ulcer healing.

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Key words: *Aloe vera*; Sucralfate; Gastric microcirculation; TNF-α; IL-10; Gastric ulcer healing

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INTRODUCTION

Gastric ulcer is produced by the imbalance between gastroduodenal mucosal defense mechanism and damaging force. Impaired mucosal defense is invoked in ulcer patients with normal levels of gastric acid and pepsin. Patients chronically using non-steroid anti inflammatory drugs (NSAIDs), including aspirin, can be pointed with some assurance at suppression of mucosal prostaglandin synthesis. Cigarette smoking impairs healing and favors recurrence, possibly by suppressing mucosal prostaglandin synthesis. Alcohol is another agent causing gastric mucosal lesion. It rapidly penetrates the gastroduodenal mucosa causing membrane damage, exfoliation of cells and erosion[1]. Corticosteroids at a high dose and repeated use promote ulceration. Personality and psychologic stress are important contribution factors as well[2].

Gastric ulceration results from the imbalance between gastrotoxic agents and protective mechanisms result in acute inflammation. Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) are the major proinflammatory cytokines, playing an important role in production of acute inflammation[3] accompanied with

neutrophil infiltration of gastric mucosa[4].

Aloe plants have been used medicinally for centuries. Among them, *Aloe barbadensis*, commonly called *Aloe vera*, is one of the most widely used healing plants in the history of mankind[5].

Two distinct preparations of Aloe plants are most used medicinally. The leaf exudate (aloe) is used as a laxative and the mucilaginous gel (*Aloe vera*) extracted from the leaf parenchyma is used as a remedy against a variety of skin disorders[6]. Aloe leaf exudate also possesses antidiabetic[7] and cardiac stimulatory activity[8].

Aloe vera is one of the few substances known to effectively decrease inflammation and promote wound healing[9,10]. *Aloe vera* gel could promote the healing of burns and other cutaneous injuries and ulcer[11, 12], thus improving wound healing in a dose-dependent manner and reducing edema and pain[9].

Aloe vera gel has been demonstrated to protect human beings[13-15] and rats[16-22] against gastric ulceration. This antiulcer activity is due to its anti-inflammatory[20], cytoprotective[16], healing[20, 23] and mucus stimulatory effects[24].

However, the effects of *Aloe vera* on gastric microcirculation, inflammatory cytokines in gastric ulcer patients have not yet been reported. Therefore, the aim of this study was to study the effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine level and gastric ulcer healing.

MATERIALS AND METHODS

Animal preparation

Male Spraque Dawley rats weighing 200-280 g purchased from the International Animal Research Center, Salaya ($n = 48$), were used in this study. Group 1 served as control group, group 2 as gastric ulcer group without treatment, groups 3 and 4 as gastric ulcer treatment groups with sucralfate (200 mg/kg/dose, twice daily) and *Aloe vera* (200 mg/kg/dose, twice daily).

The animals were fasted but allowed only water 12 hours before experiment. On the day of experiment, the animals were weighed and anesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). After tracheostomy, carotid artery and jugular vein were canulated for blood pressure measurement using polygraph and administration of fluorescent marker. The abdominal wall was incised and the stomach was extended and fixed. Then the leukocyte adherence in stomach was observed by *in vivo* microscopy.

Study of interaction between leukocytes and endothelial cells in postcapillary venule

For visualization of leukocytes, acridine orange was infused intravenously (0.5 mg/kg BW) as previously described[25]. The number of leukocyte adhesions was recorded using video recorder. Videotape of each experiment was played back and then leukocyte adherence was monitored. The leukocytes were markedly adhered to the postcapillary venule (about 15-35 μm in diameter). The location of leukocyte adherence in three areas was observed. Leukocytes were considered adherent to the vessel endothelium if they remained stationary for 30 or longer. Adherent leukocytes were expressed as the mean number of leukocyte adherences per field of view as previously described[26].

Mean number of leukocyte adherences = [the number of (area 1 + area 2 + area 3) cells/field]/3

Determination of serum cytokine levels

After the experiment, blood samples were taken by cardiac puncture, allowed to clot for 2 h at room temperature or overnight at 2-8 ℃ before centrifugation for 20 minutes at approximately 2000 r/min. Serum was separated and stored at about -80 ℃ for determining TNF α and IL-10 levels by ELISA kit (Quantikine, R&D systems).

Histological analysis

The stomach was fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) as previously described[3]. Histopathological changes and maximum length of gastric ulcer were observed under light microscope with magnification × 20. Histopathological examination was performed by pathologists.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was done using one-way analysis of variance and comparision of results between groups was made using post hoc test. $P < 0.05$ was considered statistically significant.

Ethical considerations

This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

RESULTS

Interaction between leukocytes and endothelial cells

After gastric ulcer was induced by administration of 20% acetic acid, leukocyte adherence to endothelial cells of postcapillary venules (15-35 mm in diameter.) was observed under intravital fluorescence microscopy. The number of leukocytes adhered to postcapillary venules for 30 s or longer was counted per each field of observation. The mean number of leukocyte adherences in the ulcer group without treatment (d1: 13.13 ± 1.19 cells/field; d8: 13.61 ± 1.99 cells/field) was significantly increased compared to the control group (d1: 1.69 ± 0.17 cells/field; d8: 5.53 ± 0.65 cells/field).

On days 1 and 8 after induction of gastric ulcer, the number of leukocyte adherences was significantly decreased both in the ulcer group treated with sucralfate (d1: 3.22± 0.76 cells/field; d8: 3.80 ± 0.79 cells/field) and in the ulcer group treated with *Aloe vera* (d1: 4.29 ± 0.39 cells/field; d8: 4.46 ± 0.27 cells/field) ($P < 0.05$) compared to the ulcer group without treatment (d1: 13.13 ± 1.19 cells/field; d8: 13.61 ± 1.99 cells/field). The number of leukocyte adherences in the ulcer group treated with *Aloe vera* was reduced as the ulcer group treated with sucralfate. The mean ± SE of leukocyte adherences on days 1 and 8 is shown in Table 1 and Figure 1.

Table 1 Leukocyte adherence on postcapillary venules indifferent groups (mean ± SE, $n = 6$)

Group	Mean leukocyte adherence (cells/field)	
	Day 1	Day 8
Control	1.69 ± 0.17	5.53 ± 0.65
Ulcer	13.13 ± 1.19[a]	13.61 ± 1.99[a]
Ulcer+sucralfate	3.22 ± 0.76[c]	3.80 ± 0.79[c]
Ulcer+*Aloe vera*	4.29 ± 0.39[c]	4.46 ± 0.27[c]

[a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* ulcer groups.

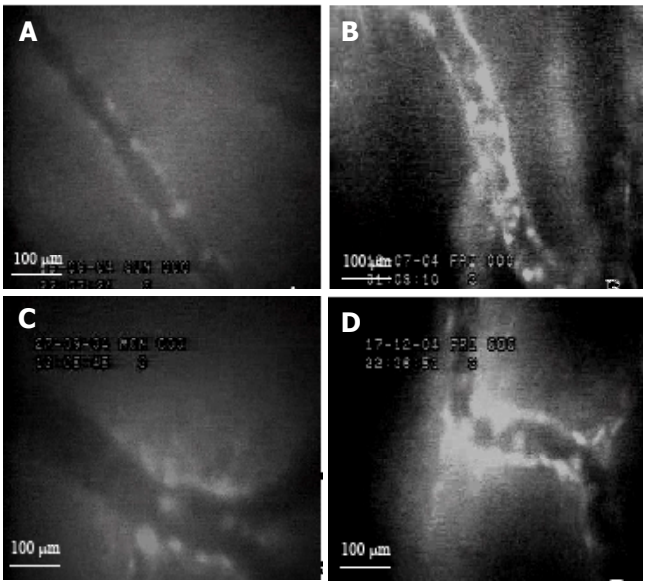

Figure 1 Intravital microscopic (´40) images of leukocyte adherence on vascular endothelium of postcapillary venules in control group (**A**), ulcer group without treatment (**B**), ulcer groups treated with sucralfate (**C**) and *Aloe vera* (**D**) on day 8.

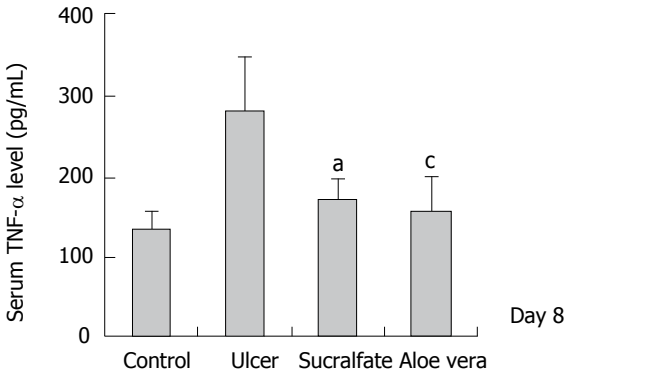

Figure 2 TNF-α level in different groups ($n = 6$) (mean ± SE), [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* ulcer groups.

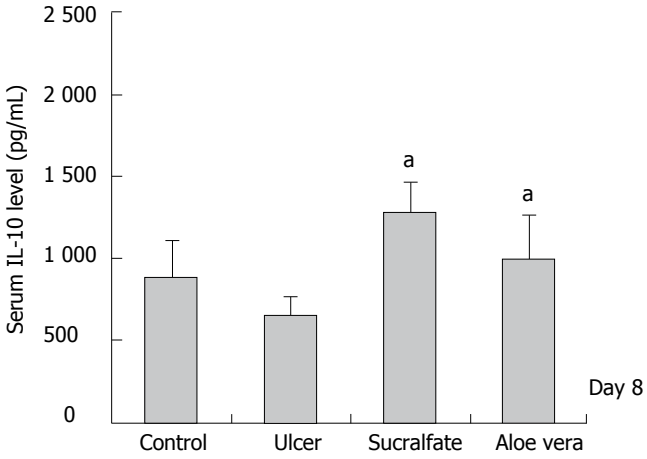

Figure 3 IL-10 level in different groups ($n = 6$) (mean±SD),[c]$P < 0.05$ *vs* ulcer groups.

Changes of TNF-α and IL-10 levels

After gastric ulcer was induced by administration of 20% acetic acid, the level of TNF-α (d_1: 151.40 ± 26.87 pg/mL; d_8: 280.44 ± 67.02 pg/mL) was significantly higher than that in the control group (d1: 12.51 ± 2.35 pg/mL; d8: 133.50 ± 20.95 pg/mL). However, the level of IL-10 after gastric ulcer was induced by administration of 20% acetic (d_1: 472.66 ± 167.75 pg/mL; d_8: 646.60 ± 118.92 pg/mL) was significantly lower than that in control group (d_1: 911.46 ± 230.81 pg/mL; d_8: 883.98 ± 227.62 pg/mL). The level of TNF-α in ulcer group treated with sucralfate (138.62 ± 47.45 pg/mL) and in ulcer group treated with *Aloe vera* (153.02 ± 26.90 pg/mL) was higher than that in control group (12.51 ± 2.35 pg/mL) on d 1. On d 8, the level of TNF-α in ulcer group treated with sucralfate (170.21 ± 23.82 pg/mL) and in ulcer group treated with *Aloe vera* (154.32 ± 43.55 pg/mL) was significantly ($P < 0.05$) lower than that in ulcer group without treatment (280.44 ± 67.02 pg/mL) and was different from that in control group (133.50 ± 20.95 pg/mL). TNF-α level was reduced in ulcer group treated with *Aloe vera* as in the ulcer group treated with sucralfate.

Furthermore, the level of IL-10 in ulcer group treated with sucralfate (d_1: 1 419.93 ± 359.81 pg/mL; d_8: 1 283.64 ± 179.72 pg/mL) and in ulcer group treated with *Aloe vera* (d_1: 1178.13 ± 159.87pg/mL; d_8: 984.02 ± 269.26 pg/mL) was higher than that in ulcer group (d_1: 472.66 ± 167.75 pg/mL; d_8: 646.60 ± 118.92 pg/mL) on days 1 and 8 after induction of gastric ulcer. The mean ± SE of TNF-α and IL-10 levels on d 1 and 8 is shown in Figures 2 and 3, respectively.

Histopathological changes

After administration of 20% acetic acid, histopathological examination revealed hemorrhage, congestion and edema in the gastric mucosa with mild to moderate leukocyte infiltration in gastric lesion. Gastric lesions were erosive and ulcerative. Congestion, edema and erosive lesion were found only in control group. Moreover, the mean maximum length of gastric ulcer in ulcer group without treatment (4.17 cm ± 0.11 cm) was significantly longer than that in control group (3.25 cm ± 0.11 cm) ($P < 0.05$). On day 8 after induction of gastric ulcer, control group and ulcer group without treatment had still mild congestion and edema, mild leukocyte infiltration and erosive lesion in gastric mucosa. The mean length of gastric ulcer in ulcer group without treatment (3.48 cm ± 0.10 cm) was larger than that in control group (3.20 cm ± 0.22 cm). On day 1, the mean maximum length of gastric ulcer in ulcer groups treated with sucralfate (3.73 cm ± 0.12 cm) and with *Aloe vera* (3.60 cm ± 0.18 cm) became shorter after treatment

Table 2 Maximum length of gastric ulcer (cm) in different groups ($n = 6$) (mean ± SE)

Group	Maximum length of gastric ulcer (cm)	
	Day 1	Day 8
Control	3.25 ± 0.11	3.20 ± 0.22
Ulcer	4.17 ± 0.11[a]	3.48 ± 0.10
Ulcer + sucralfate	3.73 ± 0.12[b]	3.33 ± 0.11
Ulcer + Aloe vera	3.60 ± 0.18[b]	3.43 ± 0.10

[a]P <0.05 *vs* control group, [b]P <0.05 *vs* ulcer groups

when compared to the ulcer group without treatment (4.17 cm ± 0.11 cm). On day 8, the mean maximum length of gastric ulcer in ulcer groups treated with sucralfate (3.33 cm ± 0.11 cm) and *Aloe vera* (3.43 cm ± 0.10 cm) was slightly reduced with no significant difference after treatment when compared to the ulcer group without treatment (3.48 cm ± 0.10 cm). The mean ± S.E of the maximum length of gastric ulcer is shown in Table 2.

DISCUSSION

In this study, after gastric ulcer was induced by administration of 20% acetic acid, gastric inflammation increased leukocyte adherence to the endothelial surface of postcapillary venules and was characterized by the migration of macrophages and PMNs in the ulcer area. The migrated macrophages then released proinflammatory cytokines such as TNF-α and interleukin-1β (IL-1β). Pro-inflammatory cytokines can up-regulate adhesion molecule expression on endothelial cells and leukocytes[27,28] and cause leukocyte recruitment[29]. Adhesion molecules on endothelial cells and leukocytes involve rolling, adhesion, and transmigration of leukocytes in gastric inflamed areas. It was reported that increment of PMNs may play an important role in the pathogenesis of NSAIDs-induced gastropathy[30]. On the other hand, NSAIDs may enhance the expression of cell adhesion molecules on the surface of endothelial cells[31]. Adhesion molecules play an important role in the recruitment of leukocytes to inflammation sites, leading to gastric mucosal injury[31, 32]. It was also reported that leukocyte adhesion and/or aggregation can occlude microcirculation, resulting in ischemic mucosal injury[31, 33]. Leukocyte infiltration in gastric mucosa can cause tissue damage leading the ulcerative lesion[34].

In this study, gastric ulcer induced by administration of 20% acetic acid had elevated TNF-α levels, demonstrating that elevated pro-inflammatory cytokine level induces interaction between leukocytes and endothelial cells. It was suggested that 20% acetic acid could stimulate macrophages to release proinflammatory cytokines. TNF-α can stimulate ICAM-1 expression on vascular endothelial cells. ICAM-1 is an adhesion molecule, which plays a pivotal role in inflammatory reaction by increasing leukocyte adhesion to endothelium and promoting transen-dothelial migration of leukocytes to inflammatory sites[3]. Moreover, it was reported that TNF-α can also stimulate expression of LFA-1 (CD11a/CD18)[35], an adhesion molecule on leukocytes. This might be the reason why of TNF-α and leukocytes-endothelium interaction is increased in the inflammatory area.

However, after gastric ulcer was induced by administration of 20% acetic acid IL-10 level was reduced on day 1, but elevated spontaneously on day 8 as compared to the control groups (Figure 3). This might be due to the fact that when the gastric mucosa was damaged by acetic acid, T and B lymphoctyes in submucosal beneath the damaged area that typically produce basal level of IL-10 were also damaged. The location of macrophages was actually beyond the damaged area, therefore, the macrophages were then survived. The survived macrophages are able to stim-ulate the release of TNF-α in response to acetic acid injury. Therefore, our findings suggest that TNF-α is synthesized more than IL-10. When inflammation occurs, IL-10 is synthesized. The increment of IL-10 level reduces gastric inflammation through its feedback inhibition of TNF-α production. Therefore, IL-10 is spontaneously elevated in chronic gastric inflammation. The elevation of IL-10 then reduces gastric tissue inflammation simultaneously.

Aloe vera and sucralfate could reduce leukocyte adherence after gastric ulcer was induced by 20% acetic acid. It has been reported that *Aloe vera* can decrease carrageenan- induced edema and neutrophil migration in rats[36]. In addition, *Aloe vera* has antiinflammatory effect on burn wounds by reducing leukocyte adhesion in rats[37, 38]. On the other hand, *Aloe vera* is able to inhibit prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane B2 production in guinea pig with burn wounds[39]. Thromboxanes and prostaglandins (PGs) can elicit platelet aggregation, leukocyte adherence and vasoconstriction, thus enhancing ischemia. Moreover, *Aloe vera* possesses bradykininase activity and also decreases inflammation[9]. Bradykinin causes increase in vascular permeability and stimulates inflammation[40]. Therefore, these effects of Aloe vera might reduce the causes of inflammatory process, the effects and leukocyte adherence after gastric ulcer is induced by 20% acetic acid.

Aloe vera and sucralfate could reduce TNF-α level on days 1 and 8 after induction of gastric ulcer (Figure 2). *Aloe vera* has cytoprotective effect on gastric mucosa by stimulating endogenous prostaglandins production[20]. Sucralfate is a cytoprotective drug which also stimulates PG production. Prostaglandins (PGs), especially prostaglandin E2 (PGE2) can protect gastric mucosa from various irritants, promote mucus production and increase mucosal blood flow[41-43]. It was reported that PGE2 plays a role in modulating TNF-α production and is also a potent inhibitor of neutrophil adherence and chemotaxis[44]. Ding *et al*[45] reported that PGE2 inhibits TNF-α release in gastric mucosa and reduces in neutrophil activation and subsequently ischemia and mucosal damage. They also showed that inhibition of TNF-α by PGE2 could result in the reduction of neutrophil CD11b/CD18 and endothelial ICAM-1 expression directly or indirectly, thus subsequently reducing neutrophil adhesion on vascular wall.

In our study, *Aloe vera* and sucralfate could elevate IL-10 level on days 1 and 8 after induction of gastric ulcer. IL-10 is an anti-inflammatory cytokine produced by various cells including monocytes/macrophages and T lymphocyte. IL-10 can inhibit cytokine synthesis by macrophages[46]. In

addition, the mild anti-inflammatory effects of IL-10 may be due to the suppression of TNF-α production[47]. Bodger *et al*[48] showed that mucosal secretion of IL-10 and TNF-α is increased in *H pylori* gastritis. IL-10 may be protective and can limit tissue damage caused by inflammation. Therefore, elevation of IL-10 can down-regulate TNF-α production in macrophage.

Aloe vera and sucralfate could reduce inflammation and promote gastric ulcer healing, which has been confirmed by histopathological examinations. *Aloe vera* and sucralfate promote epithelial cell proliferation, elongation and dilatation of oxyntic grand. *Aloe vera* and sucralfate have a cytoprotective effect on gastric mucosa by stimulating PGE_2 production. PGE_2 plays an important role in the maintenance of mucosal integrity and mucus production. It was reported that *Aloe vera* could promote burn wound healing in rats[37, 38, 49]. In addition, *Aloe vera* could induce angiogenesis *in vivo*[5], which plays an important role in wound healing. *Aloe vera* can result in reduced vasoconstriction and improve perfusion of gastric mucosal capillaries, thus promoting ulcer healing[13, 50, 51]. Furthermore, gastric acid is considered as an important aggressive factor in the stomach and is known to produce gastric injury[52]. *Aloe vera* is able to decrease gastric acid secretion and increase mucus secretion[53].

The mechanism of *Aloe vera* could be explained by its action on inflammation and ulcer healing. The results of this study suggest that *Aloe vera* could decrease leukocyte adherence and TNF-α levels in inflammatory tissue. Our findings demonstrate that *Aloe vera* could act as an antiinflammatory agent on gastric ulcer. Our findings also indicate that ulcer healing effect of *Aloe vera* is mediated by increasing IL-10, an important cytokine for wound healing process.

In conclusion, *Aloe vera* acts on inflammation and promotes ulcer healing. *Aloe vera* might be used as a therapeutic agent for gastric ulcer patients.

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BASIC RESEARCH

Construction and evaluation of anti-gastrin immunogen based on P64K protein

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Abstract

AIM: To construct two kinds of anti-gastrin immunogen based on P64K protein from *Neisseria meningitids* and to compare their immunogenic effect.

METHODS: G17P64K gene was cloned and ligated into pET28a plasmid, then transformed into BL21(DE3). After inoculation of LB medium and IPTG induction, the recombinant protein was solubly expressed at a high level. The purification of G17P64K fusion protein was similar to that of P64K. An initial step of purification consisting of 30% saturated ammonium sulfate precipitation was done. Additional fine optimizations included phenyl-sepharose, G200 Sephadex gel filtration and Q-sepharose anion exchanger chromatography. Highly purified protein was obtained and sequenced at the N-terminal amino acid residues. Polypeptide was synthesized by Fmoc solid phase chemical method and cross-linked to carrier protein P64K and DT mutant by MBS method and then the rabbit anti-gastrin 17 antibody was prepared by immunizing rabbit with cross-linked and fused protein. The titer and the activity *in vitro* of antibody were assessed.

RESULTS: G17P64K gene and the recombinant bacteria were obtained. After four steps purification, protein sample that has the purity above 90% was achieved. At the 84th day after the first immunization, the titer of antibody against cross-linked protein reached 51 200. Evaluation of the antibody *in vitro* manifested that it had a high inhibitory activity on the growth of tumor cell SW480.

CONCLUSION: The P64K-polypeptide cross-linked immunogen immunized rabbit and achieved a higher titer antibody against gastrin 17 than the G17P64K fusion protein immunogen, which could inhibit the growth of the tumor cell SW480.

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Key words: Gastrin; P64K protein; therapeutic vaccine

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INTRODUCTION

The peptide hormone gastrin, released from the G cells of the antrum and duodenum, is known to stimulate the secretion of gastric acid and act as a trophic factor within gastrointestinal tract[1-3]. In recent years evidence has accumulated that gastrin is a growth factor for gastrointestinal tumors, such as gastric cancer, colon cancer, and pancreatic cancer[3-6]. Therefore it has emerged as a potential anticancer target[7].

There are several forms of gastrin; the precursor molecule preprogastrin is cleaved by an endopeptidase to progastrin, which is further processed to glycine-extended gastrin 34 and to gastrin 34[8]. These 35- and 34-amino acid peptides can be cleaved to form glycine-extended gastrin 17 and gastrin 17 respectively. Cells with gastrin receptors can respond to mature amidated forms of gastrin, as well as precursor forms, particularly glycine-extended gastrin 17. It has become clear that in cancer cells originating in various parts of the gut, the gastrin gene and the genes that encode the gastrin receptors are switched on at a very early stage of oncogenesis. Scientists have demonstrated that the glycine-extended gastrin 17 and gastrin 17 can promote cancer cell division via an autocrine or endocrine pathway[9]. A unique B cell epitope to glycine-extended gastrin 17 and gastrin 17, which is not found on gastrin 34 and CCK, consisting of the 9-amino acid stretch at the amino-terminal end of the molecule, has been identified and mapped. The immunogens comprising this unique epitope result in high levels of anti-gastrin 17 antibodies

that do not cross-react with gastrin 34 and CCK, which share with gastrin 17 a common receptor to carry out their physiologic function.

P64K protein isolated from pathogenic bacterium *Neisseria* is found in the outer membrane of the cell and well recognised in sera from individuals convalescent from meningococcal disease or vaccinated with VA-MENGOC-BC®, a Cuban antimeningococcal vaccine based on outer membrane vesicles[10]. Its high molecular mass and strong immunogenicity have made it a suitable carrier protein for weak immunogens. Recent studies have manifested that P64K protein has a better immune enhancing effect than conventional carrier protein such as BSA, TT[11]. The B cell epitope of gastrin 17 was cross-linked or fused via a peptide spacer to P64K to comprise an immunogen against gastrin 17.

MATERIALS AND METHODS

Materials

P64K gene and protein were conserved in our laboratory. DH5α, BL21(DE3) were purchased from BioDev (Beijing China). Restriction endonucleases, polymerase, T4 DNA ligase and DL2000 were from Takara (Dalian,China). GENECLEAN II kit was ordered from Q.BIOgene (Morgan Irvine,USA). SV Minipreps DNA purification system was provided by Promega (Madison,USA). DT mutant (CRM197) was purchased from Sigma(Ronkonkoma, USA). Polypeptide was synthesized and cross-linked in Institute of Basic Medical Sciences (Academy of Military Medical Sciences, Beijing, China). Low molecular weight calibration kit for SDS electrophoresis was purchased from Amersham Biosciences(Beijing,China). Goat anti-rabbit IgG-HRP was from Bio-LAB(Beijing China). New Zealand white rabbits and BALB/C nude mice were supplied by Laboratory of Animal Center (Academy of Military Medical Sciences, Beijing, China).

SW480 is a human colonic epithelial tumor cell. It could be stimulated to proliferate by gastrin via an autocrine or endocrine pathway and inhibited by gastrin inhibitor[12,13]. It was obtained from The Cell Center of Basic Medical Sciences(Chinese Academy of Medical Sciences, Beijing, China). Cell lines were grown in DMEM (GIBCO-BRL) supplemented with 10% heat-activated fetal bovine serum(FBS) in a humidified incubator at 37 ℃ in an atmosphere of 5% CO_2.

Methods

Construction of recombinant expression plasmid: Cloning of gene, isolation of plasmid and all other molecular biology procedures were carried out according to the standard procedures published. P64K gene was cloned from *Neisseria meningitids* and gastrin17 B cell epitope was designed into 5' upperstream primer[14]. G17P64K gene was cloned by PCR amplification under the following conditions: 30 cycles of 94℃ for 1 min, 50 ℃ for 1 min and 72 ℃ for 2 min with one additional cycle for 10 min at 72 ℃. The reaction components were: 1μg of P64K DNA; 50 pmol of primer 1 (5'- CATGCCATGGAAGGCCCTTGGCTTGAAGAGGAAGAATCTTCACCCCCTCCGCCGCTTTAGTTGAATTGAAAGTG-3') and primer 2 (5'-GGGAATTCTTATTTTTTCTTTTGCGGAG-3'); 200 μmol/L of each deoxynucleotide triphosphates (dNTPs); PCR buffer (10 mmol/L KCl, 20 mmol/L Tris-HCl pH 8.8, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgSO_4$, 0.1%Triton X-100; double distilled water to a final volume of 50 μL and 1 unit per reaction of Pyrobest DNA polymerase. PCR product was purified by agarose gel electrophoresis, digested by *Nco*I and *Eco*RI, then ligated into the pET28a plasmid digested with the same enzymes. The ligation products were then transformed into *E.coli* DH5α. Positive clones were selected by PCR using the conditions described above and subjected to double-stranded DNA sequencing with T7 sequencing primer according to the manufacturer's specifications. Sequence processing was done with the DNAStar software.

Expression of recombinant protein in shake flask cultures: The strain *E.coli* BL21 (DE3) was transformed with G17P64K recombinant plasmid, which was also transformed with plasmid (negative control). A transformed colony from each construct was inoculated into 20 mL Luria Bertani (LB) cultures. Bacteria were grown in a shaker at 37℃. After measurement of the optical density at 600 nm (OD_{600}), IPTG was added to induce recombinant protein synthesis considering OD_{600} of 0.5. Cell culture was allowed to continue incubation for 4 h. Samples were collected and centrifuged at 10 000 g for 10 min at 4 ℃. The bacterial pellet was washed twice with 10 mmol/L Tris and 1mmol/L EDTA, pH8 (TE), and then resuspended in 1 mL TE. Cells were destructed by sonication for 15 min. After centrifugation of the lysate at 27 000 g for 30 min at 4℃, supernatants and pellets from destruction were collected. All protein samples were subjected to 15% SDS-PAGE according to the standard procedure of Laemmli. Protein bands were visualized by staining with Coomassie brilliant G-250. Gels were scanned and the purity was estimated by densitometry using the Molecular Analysis software.

Production of recombinant protein in bioreactors: The colony containing recombinant plasmid was inoculated into 5 mL cultures of LB containing kanamycin(30 μg/mL). Cells were grown in a shaker at 37 ℃ to an OD_{600} of 0.8. The sample was transferred into 200 mL LB culture and continued to grow for 12 h at 37 ℃ with shaking (200 rpm). This culture was used to inoculate 3 L of rich media supplemented with 30 μg/mL kanamycin. When OD_{600} reached 20, expression of G17P64K gene was induced by addition of IPTG to a final concentration of 1 mmol/L and allowed to take place at 37 ℃ for 7 h. One milliliter of the bacteria was taken out at 0, 1, 2, 3, 4, 5, 6 and 7 h after induction. Cells were harvested by centrifugation at 10 000 g for 20 min and stored at -20 ℃. Five microliters of bacteria were electrophoresed on a 15% SDS-PAGE for evaluation of protein expression.

Purification of the recombinant protein: The supenatant obtained after centrifugation of the lysate was precipitated by adding solid ammonium sulfate to 20%, 30%, 40%, 50% saturation respectively. And the condition of the most optimal purification effect was ascertained. The pellet was resuspended after centrifugation at 27 000

Table 1 The dosage of rabbit immunization

Polypeptide (mg/rabbit)	G17P64K protein (mg/rabbit)	G17(9) : P64K (mg/rabbit)	G17(9) : DT (mg/rabbit)	P64K protein (mg/rabbit)
0.25	2.0	0.5	0.5	0.25

g for 20 min at 4 ℃. The resuspended solution was applied to the following chromatography steps similar to P64K.

The first stage was a phenyl-sepharose hydrophobic interaction column. After the sample was loaded, the column was washed with loading buffer(2.0 M ammonium sulfate, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, pH7.2) and eluted with a 1.5-0 mol/L ammonium sulfate gradient. G17P64K protein was eluted as a major peak in about 0.8M ammonium sulfate and G17P64K-containing fractions were pooled by adding solid ammonium sulfate to 100% saturation. The second step was a G200 Sephadex column. The concentrated sample was loaded on the column, re-equilibrated and eluted with PBS. G17P64K was eluted in the major peak and both sides contained only small amounts of contaminants. The last chromatography step was a Q-Sepharose anion exchange column. The sample from the previous G-200 sephadex column was diluted 10 times and loaded onto the anion exchange column. After washing the column with loading buffer (20 mmol/L KPB, 5mmol/L NaCl, 1mmol/L EDTA, pH6.0), G17P64K was eluted with 5-500 mmol/L NaCl gradient. In this step, the G17P64K-containing peak was the only major peak. The samples were subjected to 15% SDS-PAGE under denaturing conditions as previously described.

Chemical synthesis and crosslink of polypeptide: Polypeptide with the following amino acid sequences were synthesized: pyro-Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Gly-Gly-Gly-Gly-Ser-Cys. Synthetic polypeptide contained a B-cell epitope consisting of residues 1 to 9 of gastrin 17, a five amino acid peptide spacer, and an N-terminal cysteine. It was produced by Fmoc solid-phase synthetic chemical method and its purity was detected by high-performance liquid chromatography(HPLC) analysis method. The polypeptide was conjugated to amino group present on a carrier protein such as P64K and CRM-197(DT mutant) via the terminal peptide cysteine residue utilizing heterobifunctional linking agent-m-maleimidobenzoyl-N-hydroxysuccinimide ester(MBS). After desalting? and concentrating over a PM 10 ultrafiltration membrane, the conjugate was lyophilized and stored desiccatedly at -20℃ until use.

Preparation of rabbit anti-gastrin 17 antibody: New Zealand rabbits were immunized with the immunogens including synthetic polypeptide, G17P64K recombinant protein, polypeptide-DT conjugate and polypeptide-P64K conjugate. Four groups of 2 rabbits were immunized with one of the immunogens. Each animal was injected subcutaneously with 1.5 mL of immunogen. An additional group of 2 rabbits was immunized with P64K protein as a blank immunization control. Each group of rabbit was immunized as shown in Table 1. Rabbits were vaccinated by subcutaneous injection of immunogens emulsified with CFA/PBS for the first immunization and IFA/PBS for the subsequent 2 booster injections at 3-wk intervals. Blood samples were collected from rabbits at wk 0, 2, 5, 8 and 12.

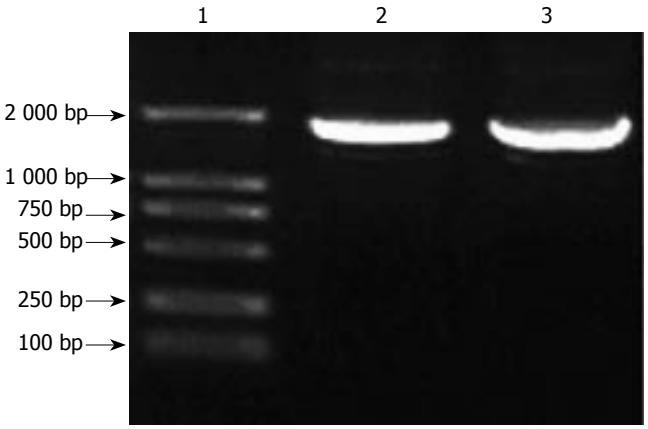


Figure 1 PCR products on agarose electrophoresis. Lane1: DL2000 marker; Lane 2, 3: PCR products.

Detection of antibody titer*:* Antibodies levels in sera were determined by enzyme linked immunosorbent assay (ELISA). To detect anti-gastrin 17 antibodies titer, 96-well plates were coated with 200 μL/well of corresponding peptide(10 μg/mL) in carbonate buffer(0.05 mol/L $NaCO_3$, pH 9.6) for 2 h at 37 ℃. Bovine serum albumin (2%) was used as a blocking reagent. After three washes in /0.05% PBS Tween 20 (PBST), 100 μL of serial dilution of each serum (starting dilution 1:100) was added to the plates, which were incubated for 2 h at 37 ℃. All sera were analyzed in four replicates. Serum anti-gastrin 17 antibody levels were expressed as their absorbance (492 nm) values in ELISA and used for statistical analysis.

Evaluation of the antibody in vitro: SW480 cells were seeded into 96 flat bottomed well plates(Costar) at a concentration of 5×10^4 per well in a 100 μL volume in the serum free medium. Affinity-purified immunoglobulins(IgG) raised against gastrin 17 and normal rabbit control IgG were normalized to standard protein concentration of 0.5 mg/mL. IgG dilutions from 1/2 to 1/256 were prepared in serum free medium and added to the cells in 100 μL volume per well. Each condition was performed with four replicates. Cell proliferation was assessed after 72 h by the methylthiazoyl tetrazolium(MTT) assay.

Statistical analysis

Statistics evaluation of *in vitro* data was performed by a one way analysis of variance on the SPSS package.

RESULTS

Construction of recombinant expression plasmid

After amplification of G17P64K gene by PCR, there was a band of about 1.8kb by agarose gel electrophoresis(Figure 1). The fragment was purified, digested by *Nco*I and *Eco*RI

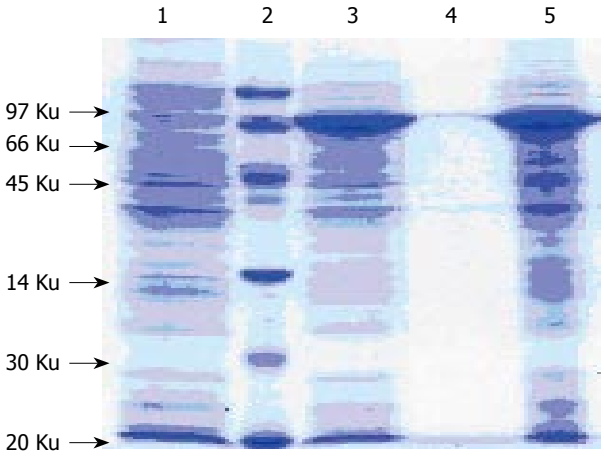


Figure 2 15% SDS-PAGE analysis of expression products. Lane 1: Non-recombinant bacteria; Lane 2: Low molecular protein marker; Lane 3: Recombinant bacteria; Lane 4: Deposition after sonication; Lane 5: Supernatant after sonication.

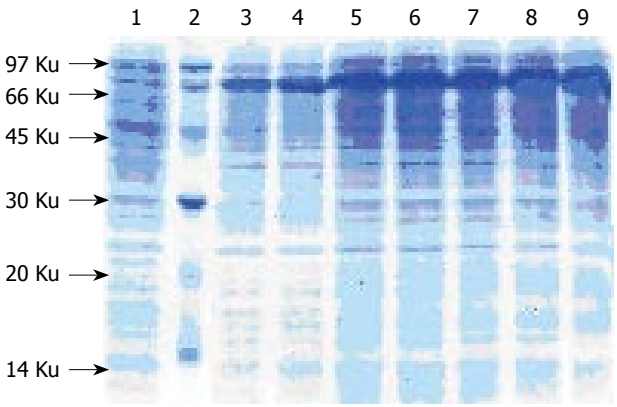


Figure 3 The expression of G17P64K protein in bioreactor. Lane 1: Low molecular protein marker; Lane 2: Sample before IPTG induction; Lanes 3-9: Sample after induction 1-7 h.

and cloned into the pET28a. The resulting plasmid was designated as pET28a-G17P64K. Sequencing revealed that the G17P64K gene was fused to the correct reading frame with the coding sequence.

Expression of recombinant protein in shake flask cultures

SDS-PAGE was carried out with the sample from BL21(DE3), which transformed with pET28a- G17P 64K (Figure 2). An approximate 70KD protein was solubly expressed in BL21(DE3) and accounted for about 30% of the total cellular protein.

Production of recombinant protein in bioreactors

Recombinant bacteria were grown in 5-L fermentors to investigate whether the G17P64K protein could be expressed and the yields could be amplified using a scale-up cultivation. The 5-L fermentors strategy allowed cultures to grow to final OD_{600} of 33. At 5-L fermentors culture of 5 h, induction of the T7 promotor and production of G17-P64K protein was initiated. The G17P64K protein was gradually increased per OD_{600} until the end of fermentation (Figure 3).

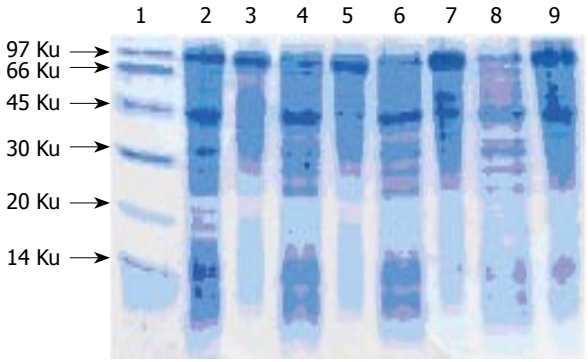


Figure 4 15% SDS-PAGE analysis of sample after ammonium sulfate deposition. Lane 1: Low molecular protein marker; Lanes 2, 4, 6, 8: Supernatant after ammol/ Lonium sulfate deposition; Lanes 3, 5, 7, 9: Deposition after 20%, 30%, 40%, 50% ammonium sulfate deposition.

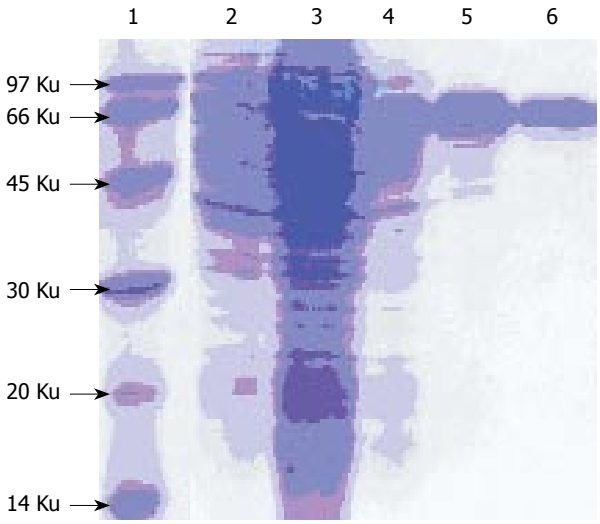


Figure 5 15% SDS-PAGE analysis of purified G17P64K fusion protein. Lane 1: Low molecular protein marker; Lane 2: Total protein after induction; Lane 3: Supernatant after sonication; Lane 4: Sample after hydrophobic chromatography; Lane 5: Sample after gel filtration chromatography; Lane 6: Sample after anion exchanger chromatography.

Purification of the recombinant protein

The G17P64K production system described in this work may permit the protein to be purified using commercially available, conventional method. SDS-PAGE manifested that 30% saturation of ammonium sulfate could most efficiently remove the contaminants (Figure 4). The first step consisted of a phenyl-sepharose hydrophobic interaction column chromatography and used a high con-centration to enhance G17P64K capture. A G200 Sep-hadex column chromatography was used next to further minimize host cell protein contaminants, followed by a Q-Sepharose anion exchanger column chromatography to help eliminate the residual contaminants. The results of this purification scheme are shown in Figure 5. After four steps of purification, the final product was judged to be more than 90% of purity. And the result of N-terminal amino acid sequencing (MEGPW) was in accordance to

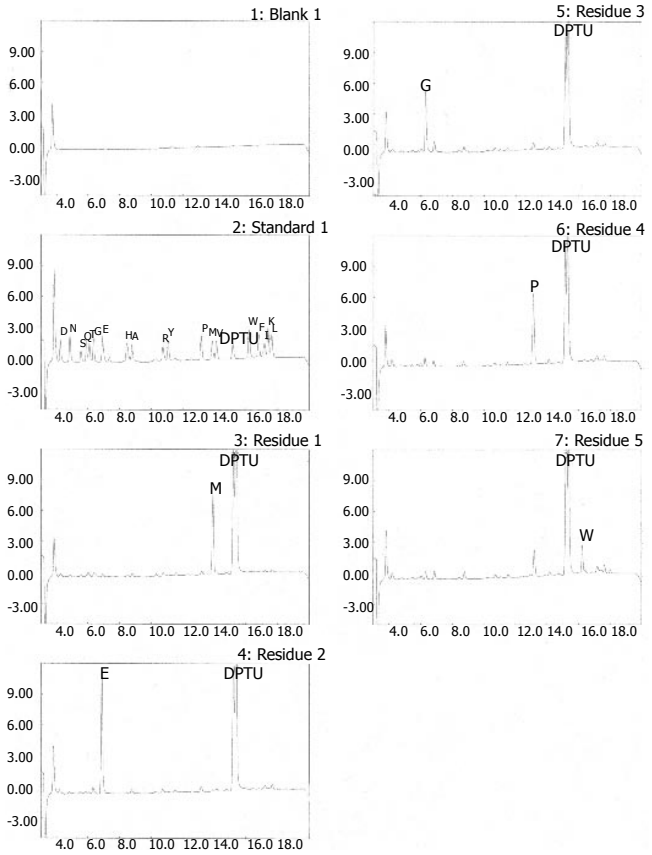
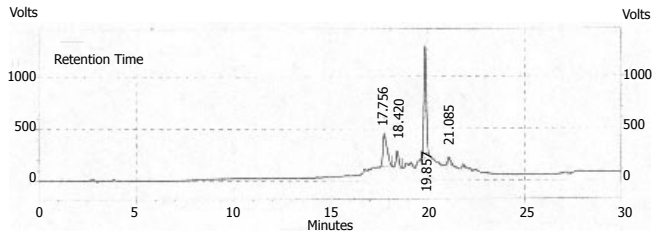


Figure 6 N-terminal amino acid sequence of G17P64K protein.



200 UV Results

Retention Time	Area	Area (%)	Height	Height (%)
17.756	4493678	27.37	312380	19.19
18.420	1530656	9.32	158889	9.76
19.857	9748167	59.37	1087743	66.84
21.085	646928	3.94	68440	4.21
Totals	16419429	100.00	1627452	100.00

Figure 7 HPLC identification of the purity of synthetic polypeptide.

the anticipated sequence (Figure 6).

Chemical synthesis and crosslink of polypeptide

Polypeptide was synthesized by Fmoc solid-phase synthetic chemical methods. The total weight of synthetic polypeptide was determined to be 60mg and the purity was detected to be 66.84% by HPLC (Figure 7). The synthetic peptide was conjugated to carrier protein via MBS cross-linking reagent. The conjugate was lyophilized and stored desiccatedly at -20 ℃ until use.

Table 2 Rabbit anti-gastrin 17 antibody levels

	Polypeptide	G17P64K protein	G17(9) : DT	G17(9) : P64K
D 0	0	0	0	0
D14	100	100	800	800
D35	800	800	6400	6400
D54	3200	3200	25600	25600
D84	6400	6400	51200	51200

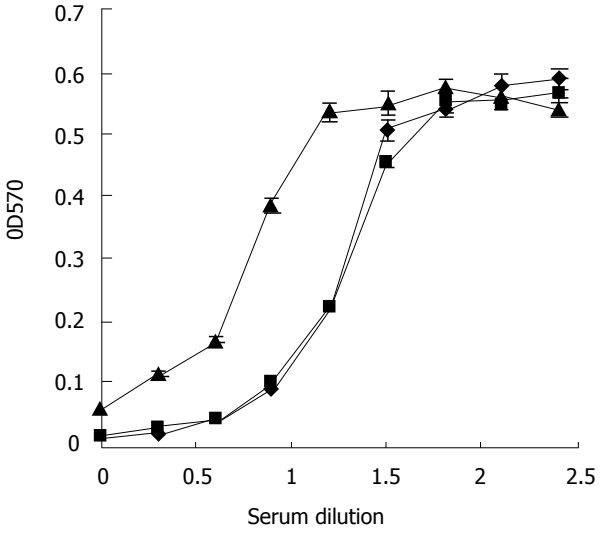


Figure 8 Effects of rabbit anti-gastrin 17 antibody on the *in vitro* growth of SW480 ◆: anti-gastrin 17(9): P64; ■Red: rabbit anti-gastrin 17(9): DT; ▲ : rabbit control IgG

Detection of antibody titer

Anti-gastrin 17 antibody levels in rabbit sera were determined by ELISA (Table 2). High levels of antibody were obtained against synthetic polypeptide conjugated to protein carriers DT and P64K. The result also revealed that the immunogenicity of G17P64K fusion protein was as weak as the single synthetic peptide.

Assessment of the activity of antibody in vitro

MTT uptake was used to measure *in vitro* proliferation which assesses the oxidative ability of the cell to convert a soluble tetrazolium compound into an insoluble colored crystalline product which can be dissolved and assessed colorimetrically. This has previously been shown to correlate with direct cell counts in many cases. The characterization of SW480 has revealed that endocrine/autocrine gastrin–mediate pathways may be operational in the growth of the cells. Figure 8 shows the MTT uptake of SW480 in the presence of increasing dilutions of rabbit ani-gastrin 17 IgG. In experiment, the growth of SW480 was significantly reduced by treatment with rabbit anti-gastrin 17(9) : DT and anti-gastrin 17(9) : P64K from 1/1 to 1/16 dilution, and modestly inhibited at dilutions of 1/32 and 1/64 ($P < 0.05$, one way analysis of variance). The effect of rabbit anti-G17(9) : P64K treatment on the *in vitro* growth of SW480 was not significantly different from rabbit anti-G17(9) : DT from 1/1 to 1/64.

DISCUSSION

Immunization against specific cancer promoting hormones may be useful in the treatment and prevention of cancer. Gastrin has been identified as the central trophic factor for gastrointestinal cancers and has therefore emerged as a potential anticancer target. There are many optional inhibitors of gastrin production[15]. Gastrin-receptors antagonists have been evaluated, but failed to produce any survival benefit in clinical trials[16-18]. Anti-gastrin antibody was demonstrated to be able to bind to gastrin peptides and prevent their interaction with gastrin receptors in preclinical research. And *in vivo* animal studies showed that infusion of anti-gastrin antibodies could inhibit the growth of human colorectal xenografts[19-22]. However, passive infusions of antibodies have serious drawbacks for the patient. Antibodies given passively to patients are not long-lasting and 1 or 2-h infusion would be required every week. In addition, antibody titer would have to be in excess of those required to neutralize normal gastrin secretion so that tumor-associated gastrin would be targeted. Active immunization was likely to work better. A novel immunotherapy has now completed phase III clinical trials of "Gastrimmune" as monotherapy in patients with pancreatic cancer and reported positive results[23-25]. Aphton Corp. has filed for marketing approval in Australia, Canada and New Zealand in 2004. Therefore targeting gastrin as an active-immunotherapeutic approach is appropriate.

There are several forms of gastrin. The glycine-extended gastrin 17 and gastrin 17 are the central trophic factor of cancer cell, while gastrin 34 and CCK are necessary for human body to maintain normal operation. A unique B cell epitope to glycine-extended gastrin 17 and gastrin 17 that is not found on gastrin 34 and CCK, consisting of the 9-amino acid stretch at the amino-terminal end of the molecule, has been identified and mapped. The immunogens comprising this unique epitope result in high levels of anti-gastrin 17 antibodies that do not crossreact with gastrin 34 and CCK, and could effectively decrease the side-effect of vaccine.

An important drawback for the use of the conventional carrier protein in different conjugate vaccines is carrierinduced hapten-specific suppression. It has been demonstrated when mice, presensitized with keyhole limpet hemocyanin (KLH) and subsequently vaccinated with KLH-dinitrophenol(DNP) conjugates, elicited a reduced anti-DNP IgG response[26]. Being a novel carrier protein, P64K protein has its superiority to BSA, TT and DT. This study evaluated the effect of P64K presensitization on its ability as a carrier, by comparing the hapten-specific humoral immune response elicited in rabbit. It proved that P64K protein can help to activate humoral immune system and enhance immune reaction against gastrin 17 B cell epitope as DT.

There are two problems for the immunogen comprising G17 recombinant protein associated with the low level of anti-gastrin 17 antibody because the immunogen is made up of single synthetic polypeptide. First, the excess methionine at the N-terminal of the G17P64K recombinant protein remodels the conformation of the B cell epitope of gastrin 17, and thereof disturbs the recognition of the B cell. Second, polypeptide could provide more B-cell epitope than the G17P64K recombinant protein in the experiment of preparation of anti-gastrin 17 antibody.

A series of tests should be done to assess the inhibitory effect and specificity of the P64K-gastrin immunogen, including the *in vivo* experiment and the adjustment between the inhibitory effect and the gastrin 17 levels. Furthermore, if there were a suitable cell line, an *in vivo* experiment could be done directly, but not human-rabbit-mice? conversion. Another test is the quality control of the crosslink immunogen of P64K and synthetic peptide. Other kinds of cancer cell line should be considered.

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BASIC RESEARCH

Effect of Chinese medicine Qinggan Huoxuefang on inducing HSC apoptosis in alcoholic liver fibrosis rats

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Abstract

AIM: To investigate the effect of Qinggan Huoxuefang (QGHXF) on improvement of liver function and pathology in rats, and to analyze the mechanism.

METHODS: Wistar rats were divided into three groups at random: normal control group (12), micro-amount carbon tetrachloride group (CCl_4)(12) and model group A (60). The model group A was ingested with the mixture (500 mL/L alcohol, 8 mL/kg per day; corn oil, 2 mL/kg per day; pyrazole, 24 mg/kg per day) once a day and intraperitoneal injections of 0.25 mL/kg of a 250 mL/L solution of CCl_4 in olive oil twice a week for 12 wk. The CCl_4 group received intraperitoneal injections only. At the end of 8 wk the model group A (60) was divided into 5 subgroups: model group, Xiaochaihu Chongji (XCH) group, QGHXF high dose group, moderate dose group and low dose group, and were given the drugs respectively. At the end of 12 wk, all the rats were killed and blood samples collected, as well as liver tissue. Blood samples were used for evaluation of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (γ–GT). Liver specimens were obtained for routine HE, apoptosis gene array and flow cytometry analysis.

RESULTS: A liver fibrosis animal model was successfully established. Fibrosis was obviously reduced in QGHXF high dose group, and no fibrosis formed in CCl_4 group. Compared with model group the QGHXF group and XCH group could obviously decrease the level of ALT, AST, ALP, and GGT ($P<0.05$). QGHXF high dose group was better than XCH group in ALT (615±190 *vs* 867±115), and AST(1972±366 *vs* 2777±608). Moreover, QGHXF could reduce liver inflammation, fibrosis-induced hepatic stellate cell (HSC) apoptosis and regulate apoptosis gene expression. The HSC apoptosis rates of QGHXF groups were 22.4±3.13, 13.79±2.26 and 10.07±1.14, higher than model group, 6.58±1.04 ($P<0.05$). Compared to model group, 39 genes were up-regulated, 11 solely expressed and 17 down-regulated in high dose group.

CONCLUSION: QGHXF can improve liver fibrosis and induce HSC apoptosis.

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Key words: Qinggan Huoxuefang; Alcoholic liver fibrosis; Apoptosis; Gene array

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INTRODUCTION

Alcohol abuse and dependence have been a public problem all over the world. In the United States, 45 billion dollars were spent on alcoholic intemperance and its related problems[1,2]. In recent years morbidity rate of alcoholic liver diseases (ALD) has risen rapidly. It has become the second liver disease after viral hepatitis in China[3,4]. Liver disease in the alcoholics is due not only to malnutrition but also to ethanol's hepatotoxicity linked to its metabolism by means of the alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) pathways and the resulting production of toxic acetaldehyde[5-8]. Alcoholic liver disease is a major source of alcohol-related morbidity and mortality. Heavy drinkers and alcoholics may progress from fatty liver to alcoholic hepatitis to cirrhosis, and it is estimated that 10 to 15 percent of alcoholics will develop cirrhosis[9]. Alcoholic liver fibrosis (AF) is one of the alcoholic liver diseases. It is the excessive accumulation of extracellular matrix (ECM) proteins including collagen that occurs in chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation[10]. However, recent evidence indicates that even ad-

focus of researches. Fibrogenesis in human ethanol injury is due to the activity of stellate cells, Kupffer cells, and to a lesser extent, to endothelial cells[14]. Above all of the factors, hepatic stellate cell (HSC) is the key to fibrosis. Activated HSCs are the source of collagen deposition in the liver[15]. This study aimed to explore the mechanism of QGHXF (a traditional Chinese herb) on AF through examination of liver function, and histopathology, detection of HSC apoptosis and gene array, in an effort to search for the experimental basis for traditional Chinese medicine in AF therapy.

MATERIALS AND METHODS

Materials

Jianzhuang Alcohol (52°) and corn oil were purchased from Lianhua Supermarket. Formaldehyde, 400 g/L, olive oil, carbon tetrachloride, hematoxylin and eosin were supplied by Shanghai Chemicals Company. The test kit of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (γ-GT), and alkaline phosphatase (ALP) were purchased from Shanghai Rongsheng Biotech Co. Ltd. QGHXF (bupleurum root 9 g, scutellaria root 9 g, red sage root 15 g, carapax trionycis 9 g, *Radix puerariae* 15 g), concentrated to 2.6 kg/L were processed by Department of Pharmacy, Longhua Hospital, Shanghai University of Traditional Chinese Medicine. XCH was from Shanghai Shikang Technology Co. Ltd (ZZ-3484 No.081006). In situ cell death detection kit (Cat. No. 1684817) and DAB substrate (Cat. No.1718096) were purchased from Roche Diagnostics Ltd.

Animal preparation[16-20]

Eighty-four specific pathogen free (SPF) male Wistar rats weighing 150 ± 20g were purchased from Shanghai Experimental Animal Co. Ltd. All the rats were randomly assigned into three groups: normal group (12), micro-amount CCl_4 group (12) and model group A (60). The model group A was ingested the mixture (500 mL/L alcohol, 8 mL/kg per day; corn oil, 2 mL/kg per day; pyrazole, 24 mg/kg per day) once a day and intraperitoneal injections of 0.25 mL/kg of a 25% solution of CCl_4 in olive oil twice a week for 12 wk. The CCl_4 group received intraperitoneal injections only. Normal group was ingested saline (10 mL/kg per day). At the end of 8 wk the model group A (60) was divided into 5 subgroups: model group, XCH group, QGHXF high dose group, moderate dose group and low dose group, and drugs were given respectively. Model group was given saline (5 mL/kg per day); QGHXF high dose group was given QGHXF 2.6 g/kg, 5 mL/kg; moderate dose group was given 1.3 g/kg, 5 mL/kg; low dose group was given 0.65 g/kg, 5 mL/kg; XCH group was given XCH (3 g/kg per day,); and model group and CCl_4 group were given saline. At the end of 12 wk all the rats were anaesthetized and killed. Blood sample and liver tissue specimens were collected. A portion of liver was fixed for histopathology. Another portion was for flow cytometry assay and the remaining tissue stored at -80°C until assayed.

Serum ALT, AST, ALP and GGT determination

ALT, AST, ALP and GGT were evaluated in samples of serum obtained at the end of the experiment. The activity was evaluated by using a commercial clinical test kit (Shanghai Rongsheng Biotech Co. Ltd.) according to instructions of the kit.

Histopathology and estimation criterion

Liver tissue was instantly fixed in 40 g/L phosphate buffered formaldehyde, processed by routine histology procedures, embedded in paraffin, cut into 5 μm section and mounted on the slide. The samples were stained with hematoxylin and eosin (HE) for histopathological examination[21]. Histopathological criteria refer to the report[22].

Flow cytometry assay[23,24]

Six liver specimens were obtained randomly from each group. The liver tissue was rapidly removed, weighed, and placed into 10 mL of ice-cold PBS containing 2 g/L bovine serum albumin (BSA) (Sigma), 0.01 mol/L EDTA, and 10 g/L deoxyribonuclease I (Sigma), and then the tissue was broken in a glass homogenizer and passed through a 40 μm nylon cell stainer (Becton Dickinson). The suspension was centrifuged at 500 g for 10 min at room temperature. The pellet was resuspended in 500 μL of PBS with BSA and transferred into a fresh tube. The cells obtained by the above method were fixed with ice-cold 700 mL/L ethanol in PBS at 4°C for 8 h, then incubated with RNase (20 mg/L) for 30 min at 37°C and labeled with propidium iodide (50 mg/L). DNA contents were measured by an FACSCalibur cytometer (Becton Dickinson). Multicycle software (CellQuest software, Becton Dickinson) was used to produce histograms of DNA content frequency. Sub-diploid DNA peaks were quantified from the DNA content data.

The tests were completed in Flow Cytometry Laboratory of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay

Tissue sections were dewaxed and rehydrated according to standard protocols. They were incubated for 15-30 min at 21-37 ℃ with proteinase K working solution (10-20 mg/L in 10 mmol/L Tris/HCl, 7.4-8). Slides were rinsed twice with PBS. Fifty microliter TUNEL reaction mixture was added to the sample tissue and incubated for 60 min at 37 ℃ in a humidified atmosphere in the dark. Slides were rinsed 3 times with PBS. Fifty microliter Converter-POD was added to the sample. Slides were incubated in a humidified chamber for 30 min at 37 ℃, rinsed 3 times with PBS, then DAB substrate was added and counterstained with hematoxylin, mounted under glass coverslip and analyzed under light microscope.

Apoptosis gene array[25-29]

Three liver specimens were taken from each group of QGHXF high dose group, model group and normal group, then homogenized and mixed for apoptosis gene array, including RNA isolation, RNA yield and quality assessment, probe synthesis, hybridization, chemiluminescent detection, image and data acquisition and analysis. The

Table 1 Impact of QGHXF on ALT, AST, ALP, and -GT in AF rats (mean ± SD)

Group	*n*	ALT (nkat/L)	AST (nkat/L)	ALP (nkat/L)	GGT (nkat/L)
Normal	12	464 ± 90 [b]	1156 ± 250[b]	1065 ± 315[b]	360 ± 98[b]
CCl_4	12	674 ± 172	1678 ± 293[a]	1695 ± 406[a]	748 ± 242[a]
Model	10	926 ± 154	3344 ± 330	2806 ± 639	1376 ± 215
Low dose	11	747 ± 113	2552 ± 388[b]	1748 ± 462[a]	982 ± 236[a]
Moderate dose	10	718 ± 156	2215 ± 650[b]	1632 ± 502[a]	869 ± 303[a]
High dose	10	615 ± 190[bc]	1972 ± 366[bc]	1570 ± 497[a]	770 ± 240[b]
XCH	10	867	2777 ± 608[a]	2413 ± 609[a]	768 ± 292[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group; [c]$P < 0.05$ *vs* XCH.

data were analyzed and calculated by Gearray Analyzer software.

Statistical analysis

Data were expressed as mean ± SD and analyzed by the ANOVA and post-hoc test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Rat condition

When the rats were given alcohol they became excited and ran around the cage. After that they could not walk, and at last fell into a profound sleep. As the time prolonged spoor time changed from 1~2 h to about 5 h. The weight of rats fell obviously and rats maintained cachexia. Conditions of the rats of QGHXF groups and XCH group were better than those of model group and CCl_4 group.

Liver function

Serum levels of ALT, AST, ALP, and γ-GT in model group were higher than those in normal group. All the indexes were improved in each medicine control group ($P < 0.05$ or 0.01), especially in QGHXF high dose group each index was better than that in XCH group. Details of the data are shown in Table 1.

Effects of QGHXF on liver pathology

The normal group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). The establishment of the model group was successful with marked fatty degeneration, slight confluence, inflammatory infiltrates of monocytes, portal inflammation and necrosis, obvious collagen deposition, and decreased density of hepatocytes (Figure 1B). The CCl_4 group only developed micro- and moderate steatosis. Inflammation and fibrosis could not be found (Figure 1C). The treatment group (QGHXF high dose group) could markedly improve those pathological parameters. Inflammation and collagen deposition decreased obviously, and steatosis could nearly not be found (Figure 1D).

Apoptosis of HSC

Apoptosis was measured in the liver tissue with FC-AS (Figure 2). All QGHXF groups had increased apoptosis rate of HSC, and decreased proliferation compared with the model group. The differences were significant (Table 2).

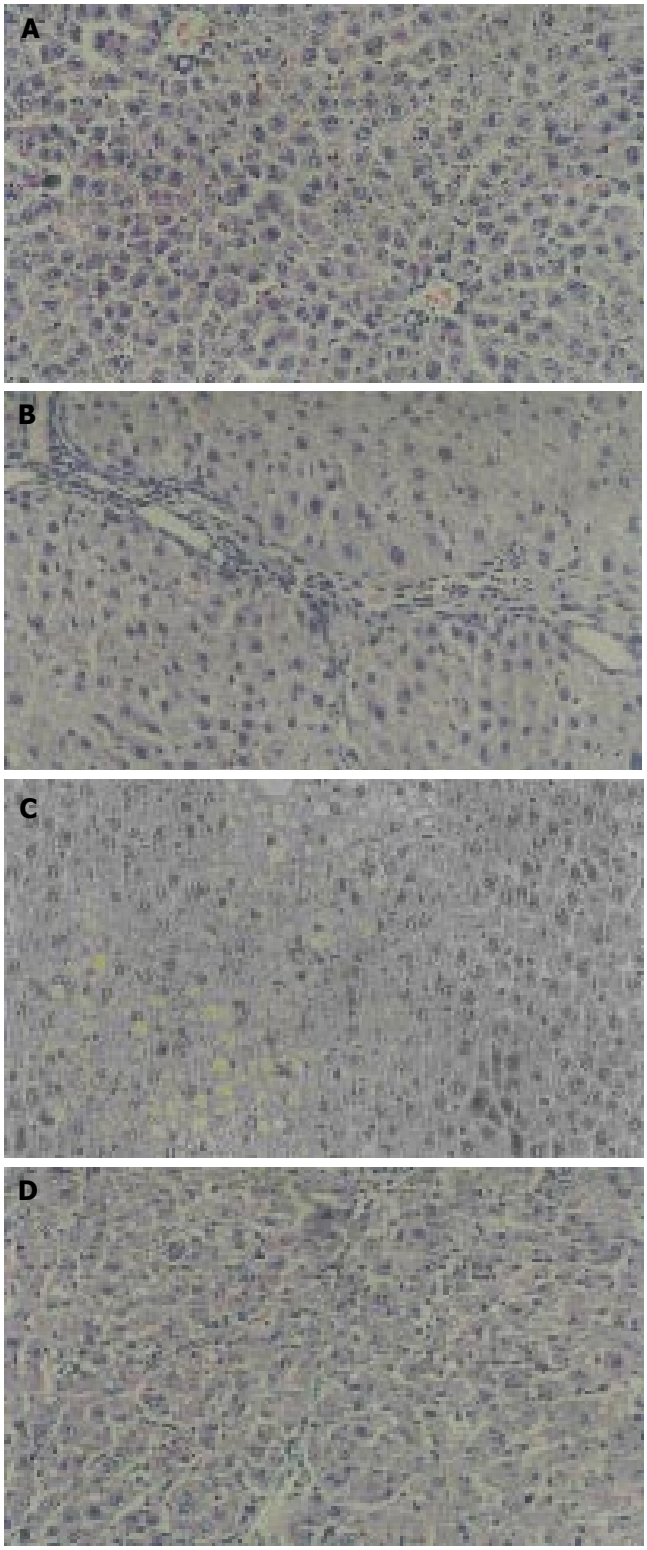

Figure 1 Analysis of liver pathology of each group (HE × 200). **A**: normal group; **B**: model group; **C**: CCl_4 group; **D**: high dose group.

TUNEL assay

TUNEL assay demonstrated standard apoptotic HSC in space of Disse and collagenous fibers in high dose group (Figure 3A), and in model group apoptotic hepatocytes

Table 2 Effect of QGHXF on proliferation and cell cycle of HSC ($n = 6$; mean ± SD)

Group	Apoptotic rate (%)	Cell cycle			
		G_0/G_1	S	G_2/M	PI value (%)
Normal	4.47 ± 0.8	79.30 ± 1.03	1.69 ± 0.73	19.02 ± 1.67	20.71 ± 1.03
Low dose	10.07 ± 1.14[a]	37.47 ± 0.68[b]	1.46 ± 0.46[a]	61.07 ± 0.33[b]	62.53 ± 0.68[b]
Moderate dose	13.79 ± 2.26[a]	68.31 ± 0.92[a]	2.11 ± 0.46[a]	29.58 ± 1.38	31.69 ± 0.92[a]
High dose	22.4 ± 3.13[b]	71.25 ± 1.05[b]	2.70 ± 0.46	26.06 ± 1.47[b]	28.75 ± 1.05[b]
Model	6.58 ± 1.04	64.55 ± 1.53	3.69 ± 0.66	31.76 ± 0.87	35.45 ± 1.53

Proliferative index = 100 % × (S+G_2/M)/(G_0/G_1+S+G_2/M). [a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

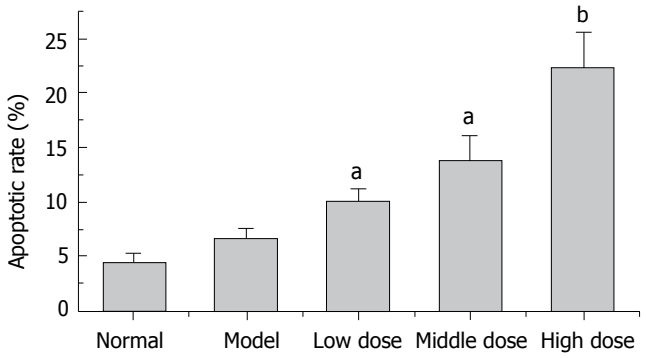

Figure 2 QGHXF induced apoptotic rate of HSC ($n = 6$, mean ± SD). There were significant differences between model group and QGHXF groups. [a]$P < 0.05$ *vs* model group. [b]$P < 0.01$ *vs* model group.

could be seen (Figure 3B). There were no obvious apoptotic cells in normal group (Figure 3C).

Gene array analysis

There were 112 genes in the apoptosis chip, and difference was defined when the same gene ratio was more than 2 or less than 0.5 between different groups. Compared to model group 39 genes were up-regulated (ratio > 2) in high dose group, 11 solely expressed and 17 down-regulated (ratio < 0.5). Compared with normal group 16 genes were up-regulated (ratio > 2) in model group, 6 solely expressed and 35 down-regulated(ratio < 0.5). Compared with normal group 38 genes were up-regulated (ratio > 2) in high dose group , 9 solely expressed and 21 down-regulated (ratio < 0.5). These different expression genes were mainly of TNF receptor family, Bcl-2 family, caspase family, and p53 and ATM pathway. Moreover, in model/normal group chip, only a few genes were consistent with those of high/model group chip, and they were Bad, Bak, Bik, NAIP1, 4-1BBL, TRIP (up-regulated) and TRAF2 (down-regulated). However, in high/model group compared with high/normal group, the up-regulated genes were nearly the same. The unique expressive genes in high/model group were Bar, Bcl-x, Bid, Casper, Caspase-7, chk1, DAPkinase, TNFb Mcl-1, OPG, FASL, and CD30L. In high/normal group they were Apaf-1, ATM, Bax, Bim, Bruce RAD53/chk2, TNFR1, TNFS, F11, and P53. Among down-regulated genes one fourth

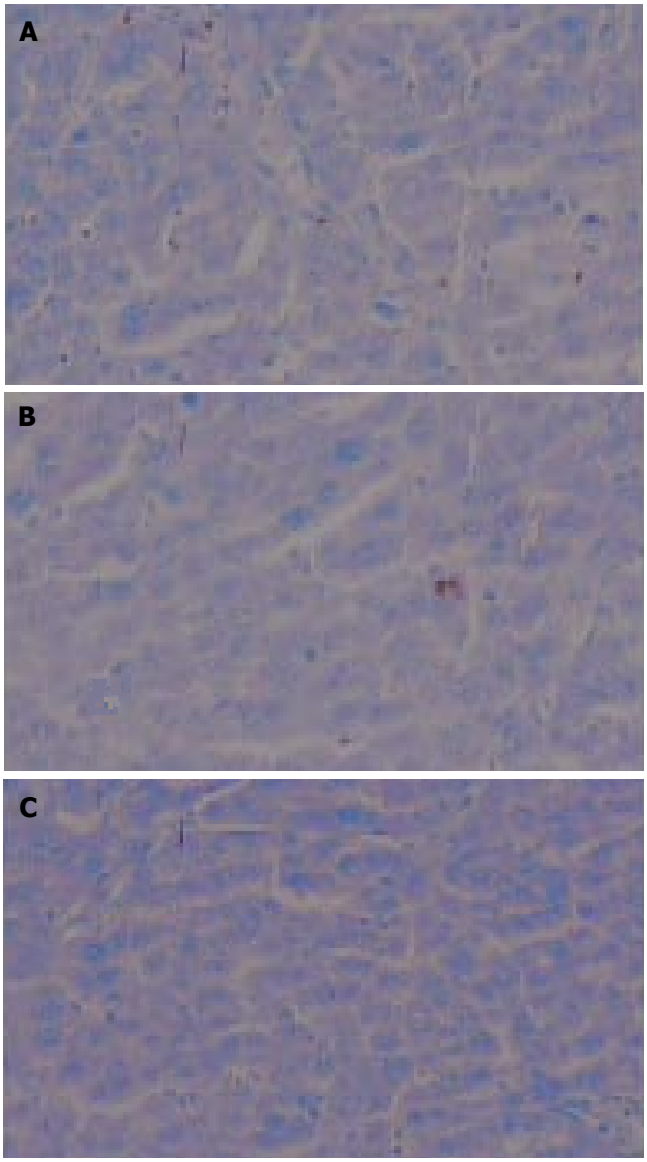

Figure 3 TUNEL assay of each group. **A**: High dose; **B**: Model; **C**: Normal; Counterstained with hematoxylin (original magnification, ×400).

were similar. They were Caspase-1, NOP30-like, RIP, TNFRF11A, and TRAF2. In comparison of model/normal group with high/normal group we found the up-regulated gene expression trend resembled the former result. The same genes were Bcl-2, hrk, IAP2, IAP3 Caspase-11, Caspase-14, and CD30. Down-regulated genes only expressed in high/normal group were CRAF1, CD27, TNFRF11A, RIP, NOP30-like, and Caspase-1 (Figure 4).

DISCUSSION

In contrast with the traditional view that cirrhosis is an irreversible disease, recent evidence indicates that even advanced fibrosis is reversible[11] . It has been demonstrated that apoptosis is the major mechanism by which activated HSCs are removed during recovery from fibrosis[30-32]. As a prophase of AC the reversibility of AF has been reported[33]. The toxicity of ethanol, acetaldehyde, reactive oxygen and other metabolic products are the main causes of AF. These factors lead to hepatocyte inflammation,

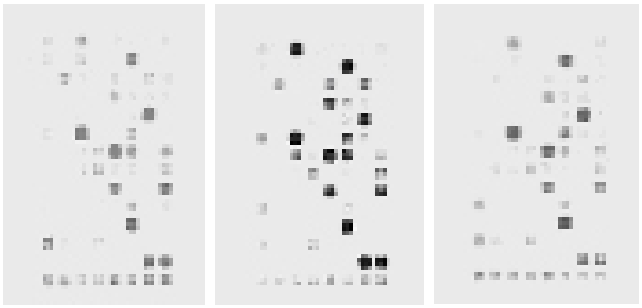

Figure 4 Gene expression profile of each group.

necrosis and apoptosis. AF was historically thought to be a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue. Currently, it is considered a model of the wound-healing response to chronic liver injury. HSCs comprise 15% of the total number of resident liver cells, but they play a very important role in the course of AF[34]. They have been identified as the main collagen-producing cells in the liver. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cell, produce large amounts of extracellular matrix (ECM) and prevent ECM degradation[34,35]. So it is beneficial to induce HSC apoptosis in order to reduce its proliferation and ECM accumulation.

The aim of this study was to explore the mechanism of QGHXF on AF through induction of HSC apoptosis. According to TCM theory, "alcohol is pungent and hot". "Jingyue Quanshu" has already pointed out: if a man drinks too much he will be "Shuigu". Through our long-term clinical practice and epidemiological research we find dampness, heat and gore are the major pathogenesis of ALD, which is in consistent with TCM theory. Therefore, we used QGHXF to clear dampness and heat of the liver and eliminate the gore of the blood. There are five components in QGHXF: bupleurum root, scutellaria root, red sage root, *Carapax trionycis*, and *Radix puerariae*. Bupleurum root (bitter and pungent in flavor, slightly cold in property, acting on liver and gallbladder channel) can expel pathogenic factors from the exterior to reduce fever, sooth the depressed liver and invigorate the spleen-*yang*. Scutellaria root (bitter in flavor, cold in property, acting on the lung, gallbladder, stomach and large intestine channels) can clear away heat and dampness, purge fire, remove toxins from the body, stop bleeding, and prevent miscarriage. Red sage root (bitter in flavor, slightly cold in property, acting on the heart, pericardium and liver channel) can invigorate blood circulation, remove blood stasis, cool the blood, treat carbuncles and tranquilize the disturbed mind by nourishing the blood. *Radix puerariae* (sweet and pungent in flavor, cool in property, acting on the spleen and stomach channels) can dispel pathogenic factors from the superficial muscles to allay fever, promote the production of fluid to quench thirst, promote the eruption of measles and invigorate the spleen-yang to stop diarrhea. carapax trionycis (salty in flavor, slightly cold in property, acting on the liver and kidney channels) can nourish *yin* and suppress hyperactive *yang*, reducing fever and resolving hard lumps. So the QGHXF can clear dampness and heat of the liver and eliminate the gore of the blood.

In this experiment the levels of ALT, AST, GGT, and ALP were obviously decreased in XCH group and each of QGHXF groups($P < 0.05$) and QGHXF high dose group had the best effect. Moreover, QGHXF high dose group had better effect than XCH group on ALT and AST($P < 0.05$). In addition, histopathology proved the effect of the drug. In model group we could see marked fatty degeneration, slight confluence, inflammatory infiltrates of monocytes, portal inflammation and necrosis, obvious collagen deposition, fibrosis and hepatocyte loosening. The treatment group (QGHXF groups) could markedly improve those pathological changes. Inflammation and collagen deposition decreased obviously, and steatosis could nearly not be seen. To clarify that the fibrosis was caused by alcohol but not CCl_4, we established the CCl_4 group in which only micro- and moderate steatosis were found, while inflammation and collagen deposition were not found. It suggested that alcohol was the real reason for fibrosis. To analyse mechanism of the effect we used FCAS and TUNEL assay. The results showed QGHXF groups could not only increase apoptosis rate of HSC but also inhibit it from proliferation. It partly proved our hypothesis that QGHXF could induce HSC apoptosis to reverse liver fibrosis. However, the HSC proliferation rate of low dose group was higher than model group, the reason for which needs to be further studied.

What excited us was, from the FCM analysis, the double regulating function of Chinese medicine. Considering the multi- target effect of Chinese medicine we selected a gene chip for further investigation. According to gene chip result, the gene expression of *p53* and ATM pathway, Caspase family, Bcl-2 family, TNF receptor family, death effector domain family all changed in each group. It meant either alcohol or drug could affect the apoptosis gene expression of liver. As a mixed cell tissue of liver, the types and apoptosis pathway of apoptotic cells were so different that both up-regulated and down-regulated genes in model/normal groups were quite different from that of high/model groups. As we all know, alcohol can induce hepatocytic apoptosis so many apoptotic genes expressed in model/normal group, but these genes were not expressed in high/model group. Thus we deduced the types of apoptotic cells were not the same, and the result of TUNEL also supported this point.

By combination of the TUNEL results with that of FCM analysis (apoptotic rate of HSC was the lowest in model group, while it was the highest in high dose group), we could conclude that QGHXF can induce HSC apoptosis, prevent hepatocytic apoptosis and necrosis. As regards which pathway plays the key role in HSC apoptosis more profound studies are needed. QGHXF has been proven effective on AF from this experiment.

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CLINICAL RESEARCH

Changes in growth factor and cytokine expression in biliary obstructed rat liver and their relationship with delayed liver regeneration after partial hepatectomy

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Abstract

AIM: To study the effects of obstructive jaundice on liver regeneration after partial hepatectomy.

METHODS: Hepatocyte growth factor (HGF), its receptor, c-Met, vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) mRNA expression in both liver tissue and isolated liver cells were investigated after biliary obstruction (BO) by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler. Immunohistochemical staining for desmin and α-smooth muscle actin (α-SMA) was also studied. Regenerating liver weight and proliferating cell nuclear antigen (PCNA) labeling index, and growth factor expression were then evaluated after 70% hepatectomy with concomitant internal biliary drainage in BO rats or sham-operated rats.

RESULTS: Hepatic TGF-β1 mRNA levels increased significantly 14 days after BO, and further increased with duration of cholestasis. Meanwhile, HGF and VEGF tended to increase, but was not significant. In cell isolates, TGF-β1 mRNA was found mainly in the hepatic stellate cell (HSC) fraction. Immunohistochemical studies revealed an increased number of HSCs (desmin-positive cells) and activated HSCs (α-SMA-positive cells) in portal areas after BO. In a hepatectomy model, liver regeneration was delayed in BO rats, as compared to sham-operated rats. TGF-β1 mRNA was significantly up-regulated up to 48 h after hepatectomy, and the earlier HGF mRNA peak was lost in BO rats.

CONCLUSION: BO induces HSCs proliferation and activation, leading to up-regulation of TGF-β1 mRNA and suppression of HGF mRNA in livers. These altered expression patterns may be strongly involved in delayed liver regeneration after hepatectomy with obstructive jaundice.

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Key word: Biliary obstruction; Liver regeneration; Hepatocyte growth factor; Transforming growth factor-β; Hepatic stellate cells; Hepatectomy

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INTRODUCTION

Recently, major hepatectomy has been performed for treatment of advanced hepatic and biliary carcinomas [1]. However, major hepatectomy associated with obstructive jaundice is often complicated by hepatic failure [2, 3], suggesting that biliary obstruction (BO) may influence liver regeneration and cause hepatic failure after major hepatectomy. Although several previous studies regarding liver regeneration after hepatectomy with obstructive jaundice have been reported [4-6], it is not clear how BO affects liver regeneration. Aronson *et al* [4] reported that extrahepatic cholestasis inhibits liver regeneration after hepatectomy, whereas Mizuno *et al* [5], demonstrated that it has no effects on liver regeneration. Thus, the effect of BO on liver regeneration after hepatectomy is still open to discussion even in an experimental model. In clinical cases, whether preoperative biliary drainage before surgery is beneficial or not is also a matter of debate.

In the liver regeneration process, several growth factors are reported to play a crucial role in regulation of regeneration by providing either stimulatory or inhibitory signals for hepatocytes [7]. Epidermal growth factor (EGF),

transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF) stimulate DNA synthesis in hepatocytes *in vivo* and in culture, but HGF is known to be the most powerful mitogen of hepatocytes [8, 9]. In liver, HGF is produced by nonparenchymal cells, mainly hepatic stellate cells (HSCs), and acts on hepatocytes in a paracrine manner via its receptor, c-Met [10-12]. Vascular endothelial growth factor (VEGF) is also reported to be the only angiogenic factor that stimulates proliferation of sinusoidal endothelial cells (SECs) [13-15]. On the other hand, transforming growth factor-β1 (TGF-β1) is a potent growth inhibitor of hepatocytes [16-19]. TGF-β1 mRNA levels are very low or undetectable in normal liver, but increase significantly after partial hepatectomy [20-22]. In cell isolates from regenerating normal liver, the TGF-β1 mRNA was relatively abundant in SECs, Kupffer cells, and HSCs [23]. Meanwhile, in a liver injury model, induced by carbon tetrachloride or D-galactosamine administration, TGF-β1 mRNA expression was up-regulated mainly in HSCs [23-25].

HSCs are known to be located in the space of Disse, below the SECs lining, in close contact to and partially intercalated between hepatocytes, with their long processes extending along sinusoids [26]. HSCs express desmin, a cytokeletal intermediate filament characteristic of muscle cells [27], but once HSCs are activated, they transform into myofibroblast-like cells, and express α-smooth muscle actin (α-SMA). Furthermore, myofibroblast-like cells derived from HSCs produce large amounts of TGF-β1, which stimulates activated HSCs to produce more TGF-β1 in an autocrine manner [28, 29]. On the other hand, activated HSCs lose HGF productivity, although HGF is primarily produced from non-activated HSCs [10].

At present, the influence of BO on HSCs phenotype, especially their TGF-β1 expression during cholestasis and their influence after hepatectomy, is not yet determined. In this study, HGF, c-Met, VEGF and TGF-β1 mRNA expression were investigated after BO in both liver tissue and isolated liver cells, by means of *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these growth/inhibitory factors. Immunohistochemical staining with desmin and α-SMA antibody was also studied to evaluate the number of HSCs and their activation status. To determine the effect of BO on liver regeneration, we also investigated changes in hepatic HGF, c-Met, VEGF and TGF-β1 mRNA expression after 70% hepatectomy with concomitant internal biliary drainage in BO rats, and compared them to those in sham-operated rats. Regenerating liver weight and PCNA labeling index were also studied to evaluate the relationship between growth factor expression and liver regeneration after hepatectomy.

MATERIALS AND METHODS

Animals

Male Wister rats (SLC, Inc. Shizuoka, Japan), weighing 200 to 300 g were used in this study. All animals were housed in a temperature- and humidity-controlled environment with a 12-h light dark cycle, and allowed to drink water and eat *ad libitum*. All surgical procedures were performed under light diethyl ether anesthesia. The operative procedure was carried out using clean, but not sterile technique. Experiments with the animals followed our institution's criteria for the care and use of laboratory animals in research, which conform to National Institutes of Health guidelines.

Experiment 1

The rats were subjected to BO or sham surgery (sham-operated control). In the BO rats, the extrahepatic bile duct was isolated and a polyethylene tube with an outer diameter of 0.8 mm (Natsume, Tokyo, Japan) was inserted into the extrahepatic bile duct, according to the cut-down technique. The other end of the tube was then ligated to induce BO. In the sham-operated rats, the extrahepatic bile duct was isolated but was not occluded. The liver was carefully excised before surgery, and 14 and 21 d ($n = 10$ at each time point) after surgery. HGF, c-Met, VEGF and TGF-β1 mRNA levels in liver tissue were investigated by quantitative reverse-transcription polymerase chain reaction (RT-PCR), using a LightCycler (Roche Diagnostics, Mannheim, Germany). Furthermore, to determine potential cellular sources of these growth factors, liver cells (hepatocytes, SECs, Kupffer cells and HSCs) were isolated from liver tissue 14 d after BO, by means of *in situ* collagenase perfusion and counterflow elutriation. The levels of HGF, c-Met, VEGF and TGF-β1 mRNA in each cell fraction were then investigated by quantitative RT-PCR. Immunohistochemical staining with anti-desmin and anti-α-SMA antibody was also performed, to evaluate the number and activation status of HSCs.

Experiment 2

Fourteen days after BO, rats were subjected to 70% hepatectomy with concurrent internal biliary drainage. The tied end of the polyethylene tube was released and embedded in the duodenum. 70% of the liver was then resected according to the method of Higgins and Anderson [30]. In the sham-operated group, rats underwent internal biliary drainage with concurrent 70% hepatectomy 14 d after sham operation. For the assessment of the HGF, c-Met, VEGF and TGF-β1 expressions, the right inferior lobe of the liver was carefully excised before and 6, 12, 24, 48, 72, 120, 168, and 240 h ($n = 10$ at each time point) after hepatectomy. The levels of HGF, c-Met, VEGF and TGF-β1 mRNA in liver tissue were investigated by quantitative RT-PCR. In addition, remnant liver weight ratio and PCNA labeling index were also evaluated and compared between BO and sham-operated rats.

Quantitative RT-PCR analysis of HGF, c-Met, VEGF, and TGF-β1 mRNA expression

Total RNA was extracted from liver tissues or freshly isolated liver cells by the acid guanidium-thiocyanate/phenol /chloroform method, and 1 mg of extracted total RNA was subjected to a reverse transcription reaction, using Ready To Go™ T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA from 33 ng of total RNA was used as a template. HGF, c-Met, VEGF, and TGF-β1 mRNA levels were quantified by means of a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I. Details of the primers used in this study are summarized in Table 1. The PCR condition was as fol-

Table 1 Primer Sequences for RT-PCR

Gene		Primer Sequence	T (℃)
HGF	Sense	5′-TTATGGGGAATGAGAAATGC	60
	Antisense	5′-TCGAACAAAAATACCAGGAC	
c-Met	Sense	5′-CAGACGCCTTGTATGAAGT	60
	Antisense	5′-CATAAGTAGCGTTCACATGG	
TGF-β1	Sense	5′-ATGACATGAACCGACCCTTC	60
	Antisense	5′-TGTGTTGGTTGTAGAGGGCA	
VEGF	Sense	5′-AATTGAGACCCTGGTGGACA	56
	Antisense	5′-TAGTGACGTTGCTCTCCGAC	
GAPDH	Sense	5′-TGAACGGGAAGCTCACTGG	60
	Antisense	5′-TCCACCACCCTGTTGCTGTA	
β-actin	Sense	5′-CCTGTATGCCTCTGGTCGTA	60
	Antisense	5′-CCATCTCTTGCTCGAAGTCT	

T : annealing temperature .

lows: initial denaturation at 95 ℃ for 10 min, followed by 45 cycles of denaturation at 95 ℃ for 15 s, annealing for 10 s, and extension at 72 ℃ for 20 s. The expression level of each angiogenic factor was adjusted using the level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as ratio to GAPDH mRNA.

Isolated liver cells from liver tissue

Liver cells 14 d after BO were isolated, to determine potential cellular sources of TGF-β1 and HGF mRNA. Rat hepatocytes were isolated according to the methods of Gumucio *et al* [31]. The SECs, Kupffer cells and HSCs were also isolated by means of in situ collagenase perfusion and counterflow elutriation, as described by Knook *et al* [32], with minor modification. A JE-5.0 elutriator rotor (Beckman Instruments, Palo Alto, CA) was used in a J6-MI Beckman centrifuge. The separation process was started by adding the nonparenchymal cell suspension to a sample-mixing chamber. The HSCs were eluted at flow rates of 16 to 18 mL/min, and at a speed of 3 200 r/min. The SECs were then eluted at flow rates of 23 to 26 mL/min, and Kupffer cells at flow rates of 36 to 39 mL/min, at a speed of 2500 r/min. The purity of HSCs was > 90%, as assessed by their positive staining for desmin [33]. The purity of SECs and Kupffer cells was > 90% and > 92%, respectively, as assessed by typical cobblestone morphology and positive staining for ED-1 [34], respectively. To evaluate expression of HGF and TGF-β1 mRNA in each cell fraction, freshly isolated cells were used for total RNA extraction.

Immunohistochemical staining

Resected liver tissue specimens from rats were fixed in 4% paraformaldehyde (Wako Chemical Co. Osaka, Japan) in phosphate-buffered saline, washed with phosphate-buffered saline, dehydrated with 30%, 70%, 95%, and 100% ethanol and xylene, and then embedded in paraffin. Four-micrometer sections were cut and mounted onto superfrosted slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan). Sections were incubated with methanol-1% hydrogen peroxide to destroy endogenous peroxidase, and blocked with nonspecific staining blocking reagent (Dako, Glostrup, Denmark). After overnight incubation at 4 ℃ with mouse monoclonal anti-desmin antibody (diluted 1 ∶ 100; Dako), or mouse monoclonal anti-α-SMA antibody (diluted 1 ∶ 100; DAKO) sections were processed according to the standard immunoperoxidase method, using a streptavidin biotin peroxidase complex kit (Dako LSAB + Kit/HRP; Dako). The peroxidase reaction was then developed with diaminobenzidine (Dako).

PCNA labeling index

Immunohistochemical staining for PCNA was performed on formalin-fixed and paraffin-embedded liver tissue with anti-PCNA antibody as previously described [35, 36]. A three-step immunoperoxidase method using strept-avidin biotin complex (Dako, Copenhagen, Denmark) was performed, according to the procedure described by Hall *et al* [36]. PC-10 monoclonal antibody (Dako, Copenhagen, Denmark) was used at a dilution of 1 ∶ 100, with overnight incubation at 4 ℃. Evaluation of PC-10 immunostaining was performed based on the percentage of positive nuclei of 500 hepatocytes at high power (400 ×), and was expressed as a PCNA labeling index.

Statistical analysis

The results were expressed as mean ± SD. The Mann Whitney test was used for statistical analysis of unpaired data, and differences were considered significant at $P < 0.05$.

RESULTS

HGF, c-Met, VEGF and TGF-β1 mRNA expression after biliary obstruction

The expression of TGF-β1 mRNA (Figure 1D) was at low levels before BO, but increased significantly at 14 d ($P < 0.05$ *vs* sham) and further increased at 21 d ($P < 0.03$ *vs* sham) after BO. Meanwhile, the expression of HGF (Figure 1A) and VEGF (Figure 1C) mRNA tended to increase at 14 and 21 d after BO, but no significant differences were found, as compared to the sham-operated control. The expression of c-Met mRNA (Figure1B) was lower at 14 and 21 d after BO, but was not significantly different from the sham-operated control.

HGF and TGF-β1 mRNA expression in isolated specific cell populations

To determine cellular sources of TGF-β1 mRNA during extrahepatic cholestasis, liver cells were isolated at 14 d after BO, because the expression of TGF-β1 mRNA was strongly induced by that time. In cell isolates, the mRNA for TGF-β1 was found mainly in the HSC fraction (Figure 2). On the other hand, the HGF mRNA expression was found in the nonparenchymal cell fraction, especially in the SEC fraction.

Immunohistochemical staining for desmin and α-smooth muscle actin

In the sham-operated control, several desmin-positive cells, presumed to be HSCs, were seen around the portal area (Figure 3A). But 14 d after BO (Figure 3B), the number

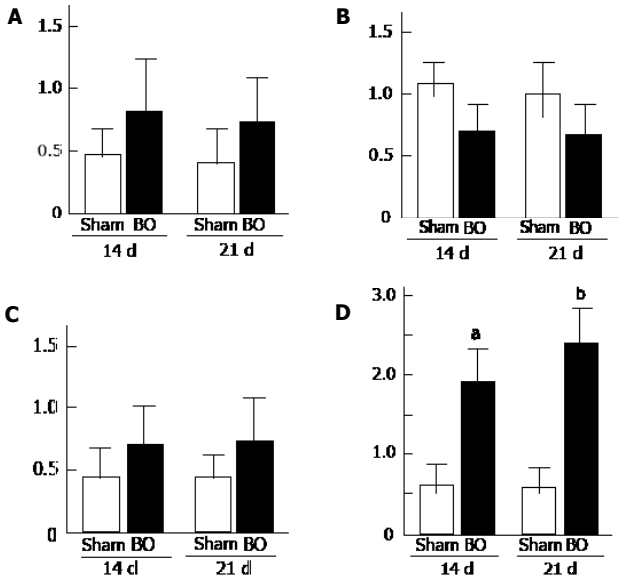

Figure 1 **A:** Hepatocyte growth factor (HGF); **B:** c-Met; **C:** vascular endothelial growth factor (VEGF); **D:** transforming growth factor-β1 (TGF-β1) mRNA expression in rat liver at 14 and 21 d after biliary obstruction (BO) and sham-operation (sham). ($^aP < 0.05$ *vs* sham-operated control, $^bP < 0.03$ *vs* sham-operated control). Results are expressed as mean ± SD of $n = 10$ for each period in each group.

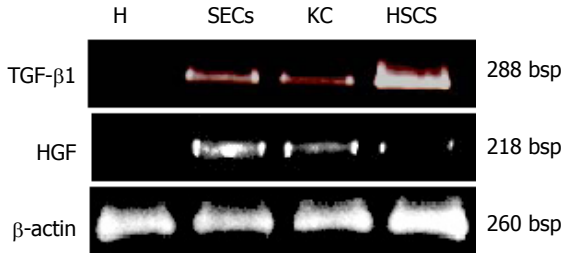

Figure 2 Hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) mRNA expression in isolated rat liver cells (H: hepatocytes; SECs: sinusoidal endothelial sells; KC: Kupffer cells; HSCs: Hepatic stellate cells) 14 d after biliary obstruction (BO).

of positive cells increased, and further increased at 21 d (Figure 3C). On the other hand, α-SMA-positive cells were hardly seen in the sham-operated controls (Figure 3D), whereas α-SMA-positive cells, presumed to be activated HSCs, were prominent in the surrounding portal areas 14 d after BO (Figure 3E).

Remnant liver weight and PCNA labeling index

The ratio of remnant liver to whole liver weight after 70% partial hepatectomy (PH) was approximately 30%. In the sham + PH group, the remnant liver weight ratio started to increase 24 h after hepatectomy (Figure 4A). A significant increase was found from 48 h to 120 h after surgery, as compared with the BO+ PH group. Thereafter, no significant differences were found between the two groups. The remnant liver weight in the BO+ PH group reached the same levels as that ~~in~~ attained by the sham + PH group 240 h after hepatectomy, but required a longer period.

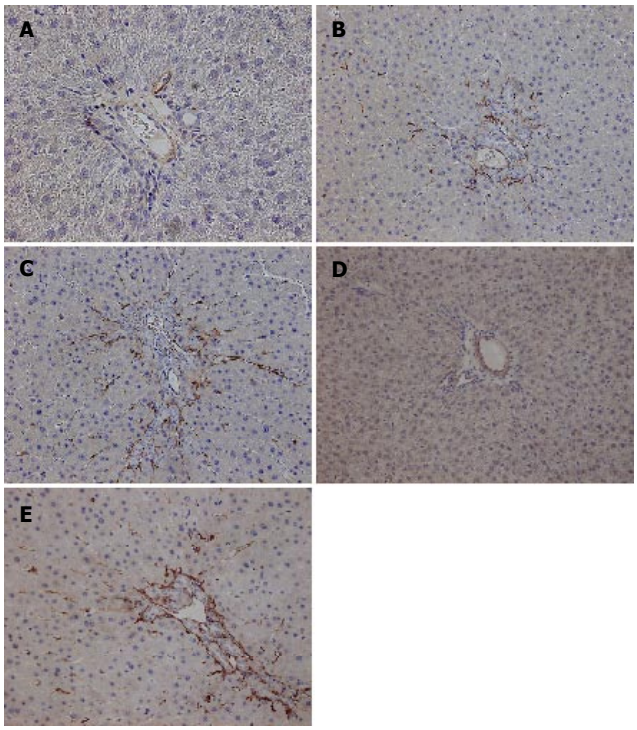

Figure 3 Immunohistochemical staining for desmin **A:** 14 d after sham operation; **B:** 14 d after biliary obstruction (BO); **C:** 21 d after biliary obstruction. Immunohistochemical staining for α-smooth muscle actin (α-SMA); **D:** 14 d after sham operation; E: 14 d after biliary obstruction.

Figure 4B shows changes in PCNA labeling index after hepatectomy. In the sham+ PH group, the PCNA labeling index was less than 5% in hepatocytes before hepatectomy. However, a dramatic increase in the PCNA labeling index of hepatocytes was observed 12 h after hepatectomy, reaching a peak 24-48 h after surgery ($P < 0.03$ *vs* BO+ PH group). In the BO + PH group, the PCNA labeling index was higher than in the sham+ PH before hepatectomy, and was then increased gradually, with a peak 120 h after hepatectomy. Thereafter, the PCNA labeling index decreased to the baseline value 240 h after surgery.

HGF, TGF-β1, VEGF, and c-Met mRNA expression after 70% hepatectomy

The expression of HGF mRNA in the sham + PH group started to increase 6 h after hepatectomy, with a peak at 12 h and 24 h (Figure 5A). Meanwhile, in the BO+ PH group, a small increase of HGF mRNA was observed up to 72 h after surgery, but no earlier peak was observed. The expression of c-Met mRNA in the BO+ PH group was lower, when compared with the sham+ PH control between 0 and 72 h after hepatectomy, but there was no significant difference between the groups (Figure 5B). The expression of VEGF mRNA in the BO+ PH group was somewhat higher up to 24 h after surgery, as compared to the sham+ PH (Figure 5C), but no significant differences were found at any point in this experiment. Meanwhile, the expression of TGF-β1 mRNA (Figure 5D) in the BO+ PH group was significantly up-regulated up to 48 h after hepatectomy, as compared to the sham+ PH group ($P < 0.05$). Thereafter, TGF-β1 mRNA expression was not significantly different between the groups.

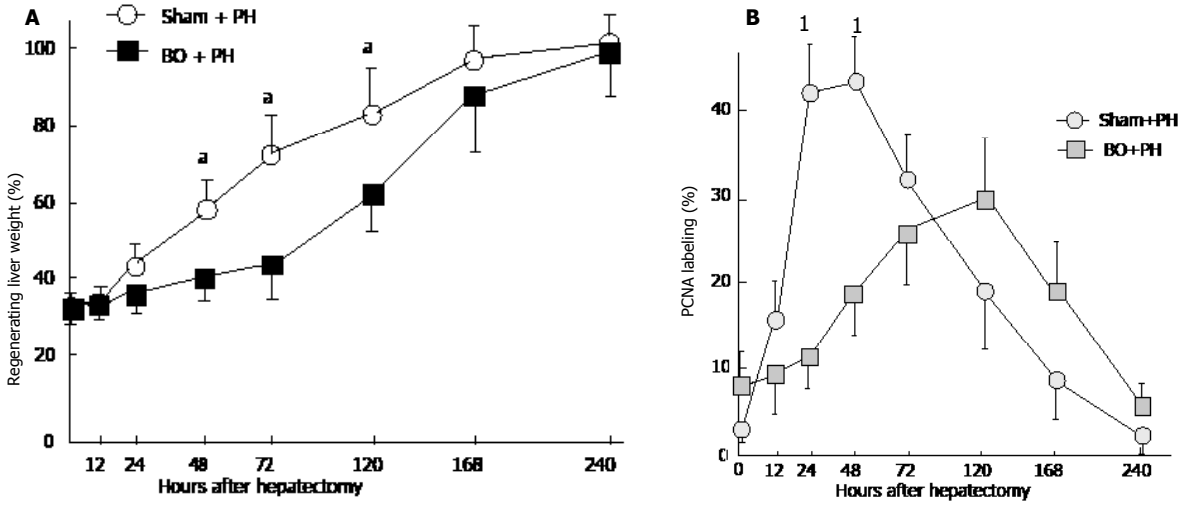

Figure 4 A: Changes in regenerating liver weight ratio after 70% partial patectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^aP <0.05 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group. B: Changes in the hepatocyte PCNA labeling index after 70% hepatectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^1P < 0.03 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group.

DISCUSSION

In the present study, we have clearly shown that hepatic TGF-β1 mRNA levels increased with length of cholestasis. Moreover, in cell isolates from BO liver tissue, TGF-β1 mRNA expression was found mainly in the HSC fraction. Immunohistochemical study also revealed an increased number of HSCs (desmin-positive cells) and activated HSCs (α-SMA-positive cells) in portal areas in proportion to the length of cholestasis. In the hepatectomy model, liver regeneration rate in the BO rats was delayed, as compared to sham-operated rats. TGF-β1 mRNA was also significantly up-regulated up to 48 h after hepatectomy in the BO rats, and no earlier peak of HGF mRNA expression was observed, despite a small increase in HGF mRNA during extrahepatic cholestasis. These findings suggest that increased TGF-β1 secreted from activated HSCs, and earlier suppression of HGF production, may greatly contribute to delayed liver regeneration in a paracrine manner. Earlier suppression of HGF after hepatectomy may be also related to HSCs activation, because activated HSCs reportedly lose their ability to express HGF mRNA [37].

Although several previous studies regarding liver regeneration after hepatectomy with obstructive jaundice have been reported, it is not clear how BO affects liver regeneration. Aronson *et al* [4] reported that extrahepatic cholestasis inhibits liver regeneration after hepatectomy, but they studied liver regeneration in the presence of postoperative BO. In this study, restoration of bile flow was performed simultaneously with hepatectomy, as in clinical cases. According to our results, the PCNA labeling index of hepatocytes, HGF and VEGF mRNA were somewhat higher in BO livers than in sham-operated livers. These results are consistent with previous studies [4, 6, 38, 39] indicating that BO induces DNA replication of hepatocytes. Furthermore, we clearly demonstrated that TGF-β1 mRNA was significantly up-regulated after BO. This phenomenon might be related to the hepatic repair process that compensates for hepatocyte injury caused by BO. This is also suggested by the fact that serum AST levels increased shortly after BO (data not shown). In cell isolates from BO liver, TGF-β1 producing cells were found to be mainly HSCs. Furthermore, an increased number of desmin-positive cells (HSCs) were found in portal areas, and α-SMA-positive cells, presumably activated HSCs, were progressively extended around portal areas in proportion to the length of BO. Activated HSCs reportedly lose their ability to express HGF mRNA, but produce a large amount of TGF-β1. These findings suggest that HSCs in portal areas were gradually activated into myofibroblast-like cells during BO. Activated HSCs produce TGF-β1, and TGF-β1 stimulates activated HSCs to produce more TGF-β1 in an autocrine manner, whereas activated HSCs hardly produce any HGF in the BO liver. HGF mRNA increased, to some extent, after BO, but HGF producing cells were mainly SECs, rather than HSCs. This may be related to the proliferative response of hepatocytes and bile duct cells during cholestatic liver injuries.

We clearly demonstrated that liver regeneration was significantly delayed after hepatectomy in the BO rats, as compared to the sham-operated rats. Moreover, TGF-β1 mRNA was significantly up-regulated up to 48 h after hepatectomy in the BO rats, and high levels of HGF mRNA were not induced in the earlier phase of hepatectomy. Meanwhile, c-Met expression in the BO liver was not significantly different from the sham-operated rats. These results suggest that delayed regeneration may be associated with the initial high expression of TGF-β1, and the suppression of HGF induction after hepatectomy. It is obvious that initial up-regulation of TGF-β1 has a non-beneficial effect on liver regeneration, because TGF-β1 is the most important cytokine controlling the inhibition of hepatocyte proliferation. Furthermore, although HGF mRNA increased to some extent in BO rats after hepatectomy, the amount may be insufficient to induce the initial stimulus for DNA synthesis of in hepatocytes for adequate

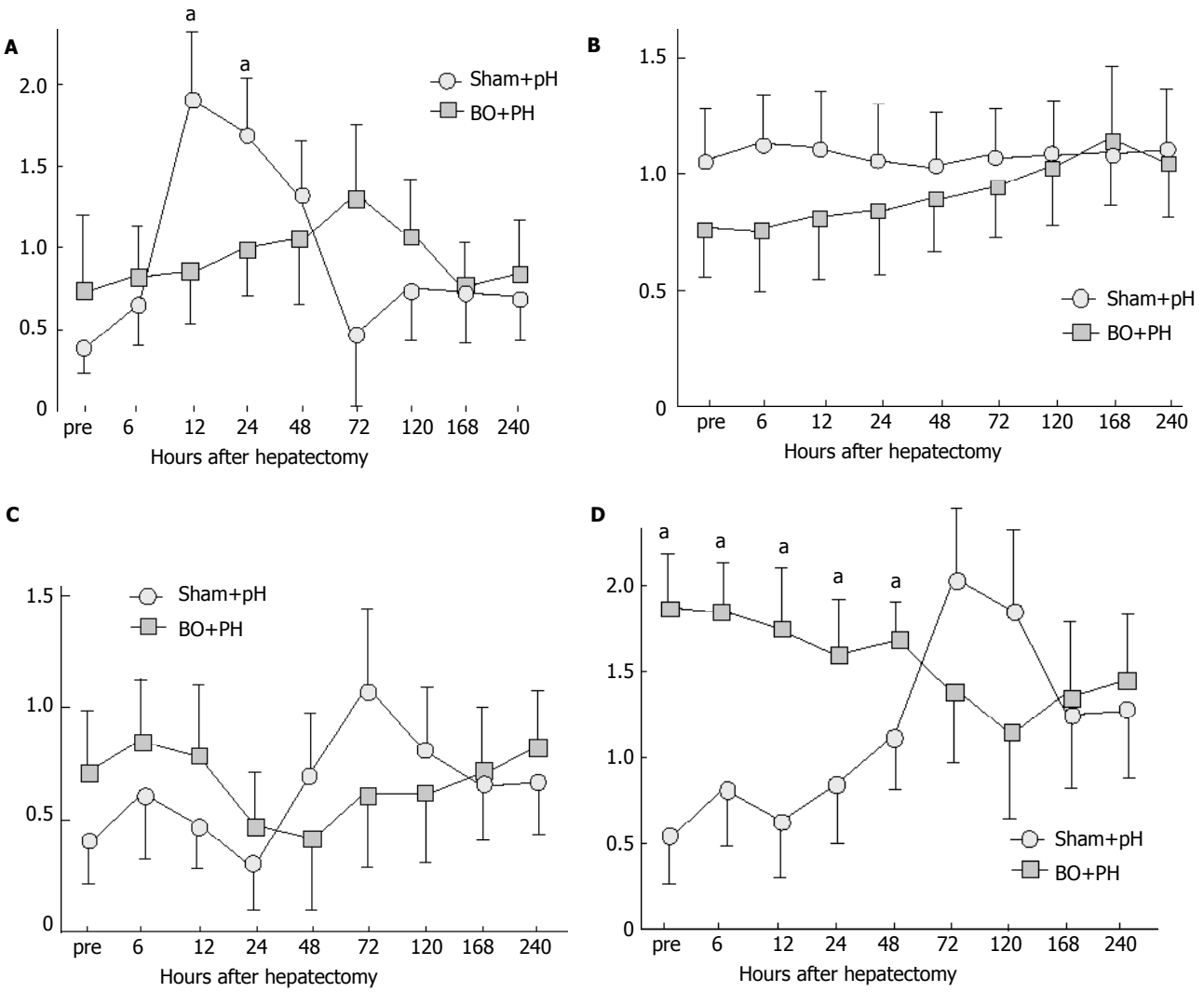

Figure 5 Changes in **A:** hepatocyte growth factor (HGF); **B:** c-Met; **C:** vascular endothelial growth factor (VEGF); **D:** transforming growth factor-β1 (TGF-β1) mRNA expression after partial hepatectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^aP <0.05 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group.

liver regeneration.

In conclusion, BO induces proliferation and activation of HSCs, resulting in up-regulation of TGF-β1 and negative regulation of HGF expression. The altered expression patterns may be involved to a considerable degree in delayed liver regeneration after hepatectomy in rats with obstructive jaundice. These findings may provide clues for the treatment of impaired hepatic regeneration after major hepatectomy with obstructive jaundice. That is, regulation of HSCs activation or restoration of an altered growth/inhibitory expression pattern might have beneficial effects on liver regeneration following major hepatectomy with obstructive jaundice.

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CLINICAL RESEARCH

Advanced gastrointestinal stromal tumor patients with complete response after treatment with imatinib mesylate

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Abstract

AIM: Most gastrointestinal stromal tumors (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). Partial response occurred in almost two thirds of GIST patients treated with Glivec. However, complete response (CR) after Glivec therapy was sporadically reported. Here we illustrated advanced GIST patients with CR after Glivec treatment.

METHODS: Between January 2001 and June 2005, 42 advanced GIST patients were treated with Glivec. Patients were administered 400 mg of Glivec in 100-mg capsules, taken orally daily with food. The response of the tumor to Glivec was evaluated after one month, three months, and every three months thereafter or whenever medical need was indicated. Each tumor of patients was investigated for mutations of kit or PDGFRA.

RESULTS: The median follow-up time of the 42 ad-vanced GIST patients treated with Glivec was 16.9 months (range, 1.0 - 47.0 months). Overall, 3 patients had complete response CR (7.1%), 26 partial response (67.8%), 5 stationary disease (11.9%), and 3 progressive disease (11.9%). The median duration of Glivec administration for the three patients was 36 months (range, 23-36 months). The median time to CR after Glivec treatment was 20 months (range, 9-26 months). Deletion and insertion mutations of c-kit exon 11 and insertion mutation of c-kit exon 9 were found in two cases and one case, respectively.

CONCLUSION: Complete response (CR) can be achieved in selected advanced GIST patients treated with Glivec. The median time to CR after Glivec treatment was 20 months. Deletion and insertion mutations of kit exon 11 and insertion mutation of kit exon 9 contribute to the genetic features in these selected cases.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are soft-tissue sarcomas primarily arising from mesenchymal tissue in the gastrointestinal (GI) tract and abdomen. They are rare neoplasms, estimated to represent 0.1 to 3% of all GI tract tumors[1]. However, GISTs are the most common mesenchymal malignancy of the GI tract with precise incidence unknown[2]. GISTs appear to be related to the interstitial cells of Cajal of the mesenteric plexus[3]. These cells are considered GI pacemaker cells, from the interface between the automatic innervation of the bowel wall and its smooth muscle[4,5]. GISTs express the cell-surface transmembrane receptor kit with a tyrosin kinase activity and is the protein product of the kit proto-oncogene. There are frequent gain-of-function mutations of kit in GISTs. These mutations result in constitutive activation of kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. It has been recently reported that kit activation occurs in all cases of GISTs, regardless of the mutation status of kit.

Surgical resection remains the mainstay of therapy for GIST. However, recurrence is common; the 5-year survival rates after complete resection range from 40 to 65%[6-10]. Unresectable or metastatic GIST is a fatal disease that resists conventional chemotherapy. In a recently reported series, the response rate to doxorubicin therapy was less than 5%[11]. The effectiveness of radiation therapy for unresectable or metastatic GIST has not been proved. The median length of survival for patients with a metastatic GIST is approximately 20 months, and 9 to 12 months for patients with local recurrence[3]. Before the development of Glivec, the outlook for patients with advanced GIST was extremely poor. A significantly large number of patients with initial resection of GIST eventually experience

Imatinib mesylate (formerly STI571, now referred to as Gleevec in the United States and Glivec in Europe [Novartis]) selectively inhibits certain protein tyrosin kinases: intracellular ABL kinase, chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukemia, transmembrane receptor kit, and platelet-derived growth factor (PDRGF) receptors[12-15]. Glivec induced a sustained objective response in more than half of patients with advanced GISTs[16]. However, complete response (CR) induced by Glivec on GIST patients has been sporadically reported. We report herein our experience on three GIST patient treated with Glivec achieving complete response.

MATERIALS AND METHODS

Patients

During January 2001 to May 2005, 42 histologically confirmed, unresectable or metastatic GIST patients ex-pressing CD117 (a marker of kit-receptor tyrosine kinase) and CD34 treated at Department of Surgery, Chang Gung Memorial Hospital, Taiwan were enrolled in this study. Metastatic disease was defined as that occurring at structures noncontiguous with the primary tumor site. Criteria for inclusion were as follows: at least one measurable tumor; adequate hepatic, renal, and cardiac function; an adequate platelet count; and an Eastern Coo-perative Oncology Group (ECOG) performance status of 3 or less. Patients could have previously received che-motherapeutic regimens (the last chemotherapy treatment must have been at least four weeks before the study entry) and undergone radiotherapy, or surgery, or both. R0 resection means curative resection without microscopic evidence of tu-mor. R2 resection means resection with macroscopic evidence of tumor. The study was approved by the Local Institutional Review Board of Chang Gung Memorial Hospital and written informed consent for drug administration and the analysis of tumor-associated genetic alteration was obtained from each patient.

Study design

A prospective, non-randomized, and single center trial was conducted to evaluate the role of Glivec in inducing objective response in GIST patients. Patients were administered 400 mg of Glivec in 100-mg capsules, taken orally daily with food. Patients had regular physical examinations and evaluations of performance status, body weight, complete blood count, and serum chemistry. The administration of each dose and any adverse events were recorded for each patient. Standard computed tomography (CT) was performed on each patient every three months to assess patient response. Standard [18F] fluoro-2-deoxy-D-glucose positron-emission tomography (PET) scanning was performed on selected patients to complement standard CT and assess changes in the metabolic profiles of the tumors.

Efficacy and safety evaluation

The response of the tumor to Glivec was evaluated after one month, three months, and every three months thereafter or whenever medical need was indicated. Assessments were performed according to the standard Southwest Oncology Group (SWOG) criteria and based solely on CT or PET[17]. Responses were classified as follows: complete response (CR) (disappearance of all disease that could be measured and evaluated); partial response (PR) (> 50% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, the absence of progression, and the absence of new lesions); stationary disease (SD) (a response that did not qualify as a complete response, a partial response, or disease progression); and disease progression (DP) [> 50% increase or an increase of 10 cm (whichever was smaller) in the sum of the products of the perpendicular diameter of all measurable lesions, worsening of a lesion that could be evaluated, the reappearance of any lesion or the presence of a new lesion, or failure of the patients to return for evaluation because of disease progression]. Toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria[18].

Analysis of KIT and PDGFRA mutations

Sections were prepared from formalin-fixed, paraffin-embedded pretreatment specimens trimmed to enrich tumor cells. Polymerase chain reaction amplification of genomic DNA for KIT and PDGFRA was performed and amplification was analyzed for mutations as previously described[19].

RESULTS

Age and sex

The investigation comprised one male patient and two female patients with ages ranging from 45 to 56 years (median: 51 years) (Table 1). All three patients had grade 0 ECOG status.

Tumor location, size, and treatment

Table 1 summarizes the size and location of each tumor. One patient underwent laparotomy with excisional biopsy and the other two had curative segmental resection of jejunal GIST previously. Tumors of all three patients di-splayed strong positive kit staining with the tumor size ranging from 10 cm to 20 cm (median: 10 cm). The inter-val between diagnosis of GIST and tumor recurrence ranged from 0 to 15 months (median 7 months). All three patients displayed peritoneal carcinomatosis and two had liver metastasis.

Treatment

All three patients were administered 400 mg Glivec after diagnosis of metastasis was made. The duration of Glivec administration ranged from 24 to 36 months (median: 36 months). The side effect of Givec treatment was grade II to III edema.

Genetic investigations of tumors from GIST

The sequencing analysis of the tumor from the three patients exhibited mutation in c-kit gene. Two displayed deletion and insertion mutation in exon 11 and one insertion mutation in exon 9 (Figures 1, 2, and 3).

Time to response and follow-up outcome

Since 2000, Glivec has been administered to advanced GIST patients. Forty-two patients with advanced stages

Table 1 Clinicopathological and mutational status of three advanced and metastatic GIST patients treated with imatinib meslyate with CR

Patient	1	2	3
Age (yr)	57	45	51
Gender	F	M	F
ECOG	Grade 0	Grade 0	Grade 0
Tumor origin	Jejunum	Stomach	Jejunum
Tumor size (cm)	20	10	10
Previous treatment	Operation	Laparotomy and excisional biopsy	Operation
Resection	R0	R2	R0
Site of tumor recurrence	Liver, locoregional, and peritoneum	Liver, peritoneum, and retroperitoneum	Peritoneum
Interval between previous treatment and recurrence (mo)	15	0	18
Glivec dose/ duration (mo)	400/36	400/23	400/36
Side effect	Grade II edema	Grade III edema	Grade II edema
Mutation status	Deletion and insertion mutation at codons 563-572 in exon 11	Deletion and insertion mutation at codons 556-557 in exon 11	Insertion AY at codons 502-503 in exon 9
Time to CR (mo)	20	9	26
CT	CR	CR	CR
PET	CR without activity	CR without activity	CR without activity
Duration of response (mo)	16	14	10
Overall survival (mo)	40	24	54
Status	Free of disease	Free of disease	Free of disease

ECOG: Eastern Cooperative Oncology Group; mo: months; CR: complete response; CT: computed tomography; PET: positron emission tomography.

of the disease were given 400 mg Glivec per day. The median follow-up duration was 16.9 months (range, 1.0-47.0 months). Overall, 3 (7.1%) patients had complete response (CR), 26 (67.8%) partial response, 5 (11.9%) stationary disease, 3 progressive disease, and 3 (7.2%) patients were unavailable to evaluate. The time to CR after Glivec treatment ranged from 9 to 26 months (median, 20 months) as illustrated by CT first and confirmed by PET without any metabolic activity (Figures 1, 2 and 3). The median follow-up period of the three advanced GIST patients treated with Glivec with CR was 40 months (range, 24 - 54 months).

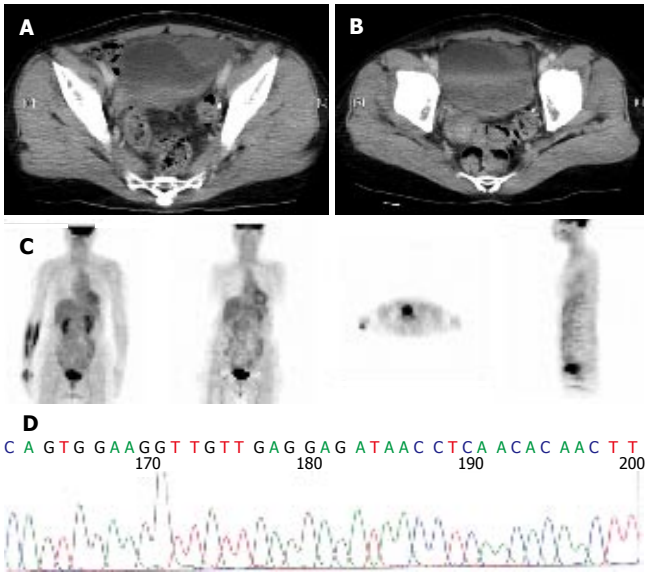

Figure 1 (**A**) Abdominal CT showing a tumor located near the urinary bladder (arrow); (**B**) abdominal CT showing complete response without tumor at the same level as Figure 1A; (**C**) [18F] fluoro-2-deoxy-D-glucose positron-emission tomography (PET) scanning PET revealing no tumor with metabolic activity in the whole body; (**D**) Direct sequencing analysis of DNA from patient 1 showed deletion and insertion mutation at codons 563-572 in exon 11 (arrow).

DISCUSSION

Before the introduction of Glivec, poor responses to radiation and chemotherapy made surgery the only realistic treatment to cure the primary lesion[5,10,20-22]. A substantial number of patients with initial resection of GISTs eventually experience recurrence. There has been no effective treatment for advanced GISTs and the outlook for patients is extremely poor.

Therapeutic responses to targeted inhibition of activated tyrosine kinases have been demonstrated for certain types of leukemia, sarcoma, and breast cancer[19]. The mechanisms of kinase activation vary considerably among these cancers, but there is little information available in literature about the influence of these mechanisms on drug response[19]. The GISTs, in particular, present a variety of genomic mutations across two different receptor tyrosine kinase genes. The KIT or PDGFRA mutation in Asian clinically advanced small bowel GIST patients was examined in this study. The kit kinase oncoproteins were intrinsically sensitive to Glivec, accounting for the excellent overall clinical response to Glivec, and coincident with results obtained by Heinrich *et al*[19]. Similar to the report by Demetri's *et al*[16], the CR and PR rates for Glivec in this

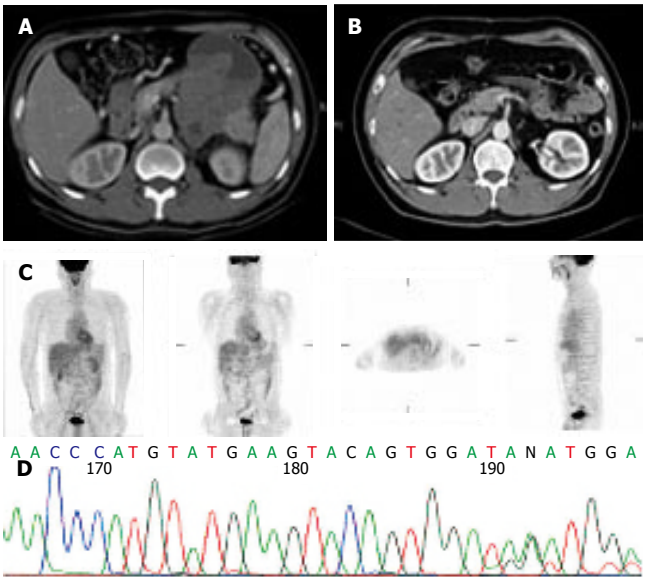

Figure 2 (**A**) Abdominal CT showing a huge retroperitoneal tumor invading the pancreas (arrow); (**B**) abdominal CT showing complete response without tumor at the same level as Figure 2A; (**C**) PET showing no tumor with metabolic activity in the whole body; (**D**) direct sequencing analysis of DNA from patient 2 showed deletion and insertion mutation at codons 556-557 in exon 11 (arrow).

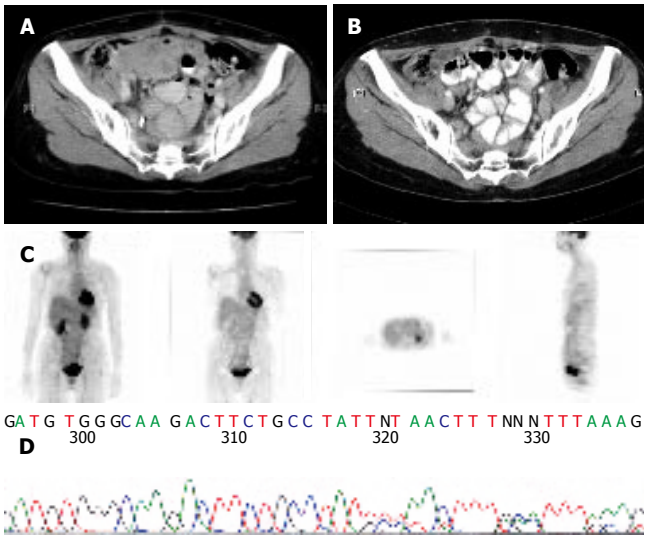

Figure 3 (**A**) Abdominal CT showing a tumor located near the ileum (arrow); (**B**) abdominal CT revealing complete response without tumor at the same level as Figure 3A; (**C**) PET showing no tumor with metabolic activity in the whole body; (**D**) direct sequencing analysis of DNA from patient 3 showed insertion AY at codons 502-503 in exon 9 (arrow).

study was 68.0%. Glivec induced a sustained objective response in more than half of the patients with advanced GISTs[16]. However, CR induced by Glivec on GIST patients was sporadically reported. In US Intergroup S0033 phase III study on 751 metastatic or unresectable GIST patients receiving 400 or 800 mg Glive per day[23], CR rate was 3%. Moreover, in the EORTC 62005 phase III study, the CR rate was 4.76% for 923 metastatic or unresectable receiving 400 or 800 mg Glive per day. Contrast to the aforementioned two studies, the CR rate in this study was 7%. The experience on CR after Glivec treatment for advanced or metastatic GIST patients in this study may justify the use of Glivec as neoadjuvant or adjuvant treatment in the future. FDG PET has been proven to be highly sensitive in detecting early response[24]. Stroobants *et al*[24] demonstrated that the CR rate increased to 52.3% (11/21), however, discrepancy was noted between the CT and PET results. In this study, CR was diagnosed according to SWOG criteria by CT scan first. PET scan was used to confirm its metabolic activity by FDG uptake on PET scan thereafter.

Regarding further use of Glivec for GIST patients with CR after Glivec treatment, no consensus was made. A recently reported randomized trial has shown that Glivec interruption after 1 year is associated with a high risk of relapse, even for patients with CR[25]. So, Glivec might be administered in the three patients until intolerance or patient refusal. The further use of Glivec for GIST patients with CR after Glivec treatment needs investigation.

In conclusion, CR can be achieved in selected patients with advanced GIST treated with Glivec. Deletion and insertion mutations of kit exon 11 and insertion mutation of kit exon 9 contribute to the genetic features in these selected cases.

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RAPID COMMUNICATION

Oxidative damage, pro-inflammatory cytokines, TGF-α and c-myc in chronic HCV-related hepatitis and cirrhosis

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Abstract

AIM: To assess whether a correlation exists between oxidative DNA damage occurring in chronic HCV-related hepatitis and expression levels of pro-inflammatory cytokines, TGF-α and c-myc.

METHODS: The series included 37 patients with chronic active HCV-related hepatitis and 11 with HCV-related compensated cirrhosis. Eight-hydroxydeoxyguanosine in liver biopsies was quantified using an electrochemical detector. The mRNA expression of TNF-α, IL-1β, TGF-α and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR.

RESULTS: TNF-α levels were significantly higher in hepatitis patients than in cirrhosis patients ($P = 0.05$). IL-1β was higher in cirrhosis patients ($P = 0.05$). A significant correlation was found between TNF-α and staging ($P = 0.05$) and between IL-1β levels and grading ($P = 0.04$). c-myc showed a significantly higher expression in cirrhosis patients ($P = 0.001$). Eight-hydroxydeoxyguanosine levels were significantly higher in cirrhosis patients ($P = 0.05$) and in HCV genotype 1 ($P = 0.03$). Considering all patients, 8-hydroxydeoxyguanosine levels were found to be correlated with genotype ($P = 0.04$) and grading ($P = 0.007$). Also multiple logistic regression analysis demonstrated a significant correlation among the number of DNA adducts, TNF-α expression and HCV genotype ($P = 0.02$).

CONCLUSION: In chronic HCV-related liver damage, oxidative DNA damage correlates with HCV genotype, grading and TNF-α levels. As HCV-related liver damage progresses, TNF-α levels drop while IL-1β and c-myc levels increase, which may be relevant to liver carcinogenesis.

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Key words: Oxidative DNA damage; Chronic HCV-related hepatitis; Inflammatory mediators

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INTRODUCTION

Oxidative damage may affect a number of cell targets, including DNA[1-2]. Eight-hydroxydeoxyguanosine (8OHdG), a modified DNA base generated by genomic material interacting with reactive oxygen species, is a mutation that causes G-C to T-A transversion at DNA replication[3]. This adduct is a marker of oxidative DNA damage and one of the most widely-investigated lesions, since its consequences may well be linked to carcinogenic mechanisms[4-6].

Oxidative damage in general and 8OHdG accumulation in particular, have been described in experimental and clinical HCV infection, with HCV-related oxidative damage playing a major part in the induction of liver diseases[7-9]. Although it is well known that reactive oxygen species induction lies at the center of a complex network of tissue and inflammatory responses involving the expression of cytokines, growth factors and oncogenes, this network has not been thoroughly investigated in HCV-related liver diseases.

Liver injury is reportedly associated with a chronic inflammatory response involving tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), etc. The former plays a central role in liver injury, triggering the production of other cytokines that in turn recruit inflammatory cells, promote fibrogenesis and further activate oxidative burst[10]. The initiation of a number of intracellular signal pathways involving apoptotic and/or anti-apoptotic signals should also be included amongst the effects of TNF-α[11] and HCV infection is indeed associated with an increase in TNF-α production, and the expression of viral proteins apparently results in more severe liver injury and hepatocyte death[12-14].

On the other hand, IL-1β gives rise to the cascade of

the inflammatory response and recent reports have shown that its levels are higher in HCV-related liver diseases than in other forms of liver damage[15]. Its polymorphisms behind are related to the risk of progression to HCC[16-17].

Our hypothesis is that oxidative DNA damage prompted by pro-inflammatory cytokines and/or by a specific effect of HCV core protein[18-19], and associated with an imbalance between apoptosis and cytoproliferation[20], is a fundamental event in HCV-related liver carcinogenesis.

Since a number of additional mediators are involved in liver carcinogenesis, including oncogenes such as c-myc that controls hepatocyte proliferation[21] and growth factors such as TGF-α involved in controlling liver regeneration and tumoral progression[22], particularly when they are co-expressed[23], the present study was to seek any correlations between oxidative DNA damage and the levels of pro-inflammatory cytokines like TGF-α and of c-myc in chronic HCV-related liver damage.

MATERIALS AND METHODS

Patients

Forty-eight patients consecutively recruited (34 M / 14 F, mean age 42 ± 12 years) with liver disease characterized by abnormal serum transaminase levels for more than 6 months, were admitted to the Division of Gastroenterology for diagnostic liver biopsy. Informed consent was obtained from all patients. Patients taking medication or vitamins capable of interfering with oxidative balance or liver damage were excluded from the study. The study was approved by the Human Research Committee of the University of Padova. Thirty-seven patients (24 M / 13 F, mean age 40.5 ± 11 years) were assigned to chronic active HCV-related hepatitis (CAH) group and 11 patients (7 M / 4 F, mean age 50.5 ± 14 years) were assigned to HCV-related compensated (Child class A) cirrhosis (CIRR) group. Before biopsy, each patient was tested to measure HCV antibodies using a second-generation ELISA and all positive sera were confirmed by RIBA II assay. In all patients, anti-HCV seropositivity was confirmed by positive HCV-RNA levels using the Amplicor HCV test (Amplicor PCR Diagnostic, Hoffman-La Roche, Basel Switzerland). A standardized genotyping assay (Inno-Lipa HCV III, Innogenetics, Gent, Belgium) was used. HCV genotypes were classified as genotype 1, subtypes 1a and 1b, genotypes 2, 3 and 4 and their subtypes. All the following studies were performed prior to any treatment.

Morphological evaluation

Biopsies (one per patient) were taken using a 16-17 gauge modified Menghini needle under ultrasound guidance and local anesthesia. Only patients whose biopsy material was adequate (i.e. about 4 cm long) were included in the study to avoid taking a second biopsy. At least 2 cm of biopsy material was cut, fixed in 10% buffered formaldehyde and handed over to the pathologists. The tissue was embedded in paraffin, cut and routinely stained with H&E and PAS for routine evaluation. Together with the overall diagnosis, the pathologist (who was unaware of the clinical diagnosis) also gave a semi-quantitative score (0-3) for the presence and extent of macro- and micro-vesicular steatosis and the Knodell index[24], as modified by Ishak *et al*[25], including both a grading and a staging of hepatic disease.

Biochemical findings

Serum levels of ferritin, transaminases and γ-glutamyl transpeptidase (γGT) were determined as part of the routine clinical procedure. The tissue for biochemical determination was around 15 mg wet weight. Samples were processed immediately and stored at -80℃.

Quantification of 8OHdG from hepatic biopsies

Liver biopsy specimens obtained at endoscopy were stored at -80℃ for no longer than 3 wk. Preliminary experiments indicated that storage under these conditions could not affect the results of the assessment obtainable with unfrozen samples and the samples might remain stable for as long as 1 month (data not shown).

After thawing, the specimens were homogenized in separation buffer (75 mM NaCl, 10 mM Tris/Cl pH 7.5, 5 mM EDTA pH 6, 0.5% sodium dodecyl sulfate) and proteinase K at 55℃ overnight. After treatment with ribonuclease A, the DNA was purified according to Fraga *et al*[26]. Following nuclease P1 and alkaline phosphatase hydrolysis, samples were filtered through 0.22 μm nylon filter units (Scientific Resources, Inc., Alfatech, Genova - Italy), and approximately 20 μg of DNA per sample was injected into the HPLC (Shimadzu, Kyoto, Japan). 8OHdG and normal deoxynucleosides were separated in a 3 μm Supelcosil LC-18-DB analytical column (7.5 cm × 4.6 mm, Supelco, Bellefonte, PA) equipped with a 5 μm SupelguardTM LC-18-DB guard column cartridge. The solvent system consisted of an isocratic mixture of 90% 50 mmol/L potassium phosphate (pH 5.5) and 10% methanol at the 1 mL/min flow rate. 8OHdG was detected using an electrochemical detector (ECD; ESA Coulochem II 5200A, Bedford, MA) equipped with a high-sensitivity analytical cell model 5011 with the oxidation potentials of electrodes 1 and 2 adjusted to 0.15 V and 0.35 V, respectively. 8OHdG levels were referred to the amount of deoxyguanosine (dG) detected in the same sample by UV absorbency at 254 nm. The amount of DNA was determined according to a calibration curve versus known amounts of calf thymus DNA. 8OHdG levels were expressed as the number of 8OHdG adducts per 10^5 dG bases. An 8OHdG standard (Sigma) prepared immediately before determination, was injected before any set of samples. The coefficient of variation was <10% and the amount of DNA required for the assay (expressed in μg of DNA) was 100 μg. Samples with lower amounts of DNA were rejected, since the risk of methodological error was only acceptable above this cut-off.

TNF-α, IL-1β, TGF-α and c-myc determination

The mRNA expression of TNF-α, IL-1β, TGF-α and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR. Total RNA extracted from frozen liver tissue (stored at -80℃) by acid guanidium thiocyanate-phenol-chloroform according to the Chomczynski and Sacchi method[27], was quantified spectrophotometrically. Integrity of the RNA sample was assessed by electrophoresis on 2% agarose gel (FMC Bio Product, Rockland,

Table 1 Eight-hydroxydeoxyguanosine levels in chronic HCV-mediated liver damage (mean ± SD)

	n° 8OHdG/10^5 dG		*n*° 8OHdG/10^5 dG
CAH	42.3±25.3	**HCV genotype 1**	74±36
CIRR	73.64±28.2	**Other genotypes**	46.9±23
P	0.05		0.03

Table 2 TNF-α, IL-1β, TGF-α and c-myc expression in chronic HCV-mediated liver damage (mean ± SD)

	TNF-α/β-actin	IL-1β/β-actin	TGF-α/β-actin	c-myc/β-actin
CAH	0.7 ± 0.2	1.1 ± 0.3	0.41 ± 0.1	0.09 ± 0.05
CIRR	0.5 ± 0.2	1.4 ± 0.6	0.43 ± 0.2	0.46 ± 0.2
P	0.05	0.05	N.S.	0.001

MC, USA). One μg of RNA was reverse transcribed in cDNA in the presence of 1× PCR buffer, 1 mM each of dNTPs, 1 U RNase inhibitor, 2.5 μM random exomers and 2.5 U of murine leukemia virus. cDNA was amplified in a final volume of 50 μL of PCR buffer, 2 U Amplitaq DNA polymerase, 0.056 μmol/L of Taq Start antibody, 0.2 mM of each of the dNTPs, 0.4 μmol/L of each primers for TNF-α, IL-1β, TGF-α, c-myc and β-actin. PCR products underwent a vertical electrophoresis on polyacrilamide gel. Electrophoretic bands were stained with silver nitrate and scanned on a densitometer image analyzer system (Quantity-one Biorad, Hercules, CA, USA). The results were expressed as the optical density ratio of TNF-α, IL-1β, TGF-α and c-myc to control β-actin.

Statistical analysis

The data were examined statistically by one-way ANOVA and Student's *t* - test, Kruskal-Wallis and linear regression. Multiple logistic regression analysis was also used by including the following variables: 8OHdG levels, diagnosis, age, expression of TNF-α, IL- 1β, TGF-α and c-myc, genotype.

RESULTS

Patient characteristics

No difference in the patients' age or gender distribution was observed between the CAH and CIRR groups. ALT levels were significantly higher in patients with chronic hepatitis than in patients with cirrhosis (87.9 ± 49 *vs* 50.5 ± 14, $P = 0.002$ by *t*), while AST levels were significantly higher in CIRR group than in CAH group (178 ± 95 *vs* 52 ± 23, $P = 0.002$ by *t*) . Serum ferritin and γGT levels did not differ significantly between the two groups of patients.

According to the classification Ishak *et al*[25], the stages of disease were, by definition, significantly higher in CIRR than in CAH patients (5.25 ± 0.4 *vs* 2.5 ± 0.8, $P = 0.0001$ by *t*), while grading was similar in the two groups. All patients were HCV-RNA positive. Type 1 (1a/1b) infection was the most prevalent (45%), followed by subtype 3a (29%), type 2 (17%) and finally type 4 (3%).

Oxidative DNA damage, TNF-α, IL-1β, TGF-α and c-myc expression

8OHdG levels were significantly higher in CIRR patients ($P = 0.05$ by *t*) and when oxidative damage was correlated with different HCV genotypes, 8OHdG levels were higher in HCV genotype 1 hepatitis than in the other genotypes ($P = 0.03$ by *t*). The results of 8OHdG are shown in Table 1. Considering all patients, 8OHdG levels correlated significantly with genotypes ($P = 0.04$ Spearman's rank correlation) and grading ($P = 0.007$ Spearman's rank correlation). The results for pro-inflammatory cytokines, TGF-α and c-myc are shown in Table 2. TNF-α expression was significantly higher in CAH group than in CIRR group (0.7 ± 0.2 *vs* 0.5 ± 0.2, $P = 0.05$ by *t*), whereas IL-1β expression was significantly higher in CIRR group than in CAH group (1.4 ± 0.6 *vs* 1.1 ± 0.3, $P = 0.05$ by *t*). The previously mentioned higher oxidative DNA levels in genotype 1 HCV infection correlated with TNF-α ($P = 0.04$). A significant correlation was also found between IL-1β levels and grading ($P = 0.04$), and between TNF-α and staging ($P = 0.05$). No significant correlations were found between pro-inflammatory cytokine levels, steatosis score or genotype. TGF-α levels were similar in the two groups of patients (0.41 ± 0.18 *vs* 0.43 ± 0.21, $P =$ NS by *t*), while c-myc expression was significantly higher in patients with cirrhosis (0.46 ± 0.2 *vs* 0.09 ± 0.05, $P = 0.001$ by *t*). No significant correlations were found between c-myc, steatosis score or genotype. Finally, multiple logistic regression analysis confirmed the previously reported significant correlation among the number of DNA adducts, TNF-α expression and genotype ($P = 0.02$).

DISCUSSION

We have previously reported that oxidative DNA damage in the liver is, at least to some degree, a specific feature of HCV infection, in which it reaches its maximal levels[7]. Even though it occurs in the early stages too, 8OHdG accumulation parallels the progression of the disease and is more striking in subjects with HCV genotype 1b infection[28].

This paper provides data on patients with HCV-related liver damage, partly describing the complex network of relationships between DNA oxidative damage, cytokine synthesis and release, c-myc and TGF-α expression that may both be strongly involved in liver cancerogenesis[29-31]. Numerous data link oxidative damage (and the parameters considered here) with the progression of liver disease and the onset of liver cancer. In primary murine hepatocyte cultures, TNF-α expression causes 8OHdG formation and an increase in cell cycle progression indicates a possible role of TNF-α in early malignant transformation of hepatocytes[32].

The first set of our results was related to TNF-α and IL-1β which plays a direct role in causing growth arrest and a chronic role in inducing TNF-α expression[10-11]. This effect was not confirmed in our series, since a correlation between IL-1β and TNF-α was not detected. On the other hand, IL-1β expression was higher in the later stages of HCV-related liver disease, as previously dem-

onstrated by Gramantieri *et al*[33], while the opposite was true of TNF-α, whose levels of expression were higher in CAH patients. We have previously reported that the balance between cytoproliferation and apoptosis is disrupted in HCV infection[20]. It is worth stressing that both TNF-α and IL-1β are involved in controlling the above described balance, thus taking part in determining the liver cell's fate and progression to liver cancer. In fact, the binding of TNF-α and IL-1β to their receptors leads to the activation of transcriptional factors, such as NFkB and AP-1, again involved in controlling cell proliferation[10]. What role does persistent oxidative stress play in this scenario? The overproduction of oxidative species, linked to over-expression of inflammatory cytokines (as shown by the positive correlation between TNF-α and 8OHdG levels in the liver), might be responsible for inhibiting the apoptotic process, most likely by activating the NFkB-dependent pathway[34].

Last but not the least, oxidative damage may be related to the expression of proto-oncogenes, such as c-myc[35]. In our study, c-myc transcript levels were significantly higher in cirrhotic than in non-cirrhotic tissues, indicating that tissue damage progression from hepatitis to cirrhosis, with the related cell growth changes, may be mediated to some degree by c-myc, which indeed is considered one of the activators of cell proliferation[36]. In this series, we could detect no relationship between 8OHdG and c-myc, suggesting that they have different and independent proto-oncogene activation mechanisms.

It was reported that TGF-α /c-myc double transgenic mice exhibit enhanced cell proliferation and build up extensive oxidative DNA damage which possibly accounts for massive DNA damage and accelerated neoplastic development in the liver[37]. In the present study, all liver samples with or without cirrhosis, expressed low levels of TGF-α mRNA and revealed no correlation with any of the other parameters investigated. This may not be totally surprising, since a strong and prominent localization of TGF-α in ground-glass hepatocytes of HBV-related liver disease in association with HBV pre-S1 antigen has been reported and this may mean that TGF-α is more involved in HBV than in HCV liver disease[38].

In our study DNA oxidative damage correlated with TNF-α over-expression in chronic HCV-mediated liver damage. Evolution to cirrhosis was characterized by an increased oxidative DNA damage, c-myc expression and IL-1β release. When disease activity was severe, it was paralleled by an increased expression of IL-1β and c-myc associated with genotype 1b infection and accumulation of 8OHdG. The above findings suggest that chronic HCV-mediated oxidative DNA damage in the liver may have an impact not only on hepatocyte proliferation rate through c-myc activation but also on cell proliferation and apoptosis through TNF-α activation.

In conclusion, HCV infection is associated with increasing cell proliferation unaccompanied with any substantial increase in apoptosis[20], while TNF-α activation in this scenario has more to do with cell proliferation rather than with cell apoptosis.

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RAPID COMMUNICATION

Prevention of *de novo* HBV infection by the presence of anti-HBs in transplanted patients receiving core antibody-positive livers

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Abstract

AIM: To analyze whether the presence of anti-HBs in liver transplant recipients is effective in preventing HBV infection.

METHODS: Twenty-three patients receiving anti-HBc positive liver were studied. Nine recipients were anti-HBc positive as a result of previous HBV infection. Of them, one also received HBV vaccine during the pre-liver transplantation period. Fourteen recipients were anti-HBs positive due to HBV vaccine administered during the pre-transplant period. Liver biopsy was obtained in 10/14 anti-HBc negative/anti-HBs positive recipients and in 4/9 anti-HBc positive recipients.

RESULTS: After a mean follow-up period of 46 months, 1 recipient with protective serum anti-HBs levels developed *de novo* HBV infection as a consequence of immune escape HBV mutants. Among the 14 vaccinated anti-HBc negative/anti-HBs positive recipients, 1/10 patients with available liver biopsy (10%) had liver HBV-DNA at 13 mo post-liver transplantation without serum viral markers and did not develop *de novo* HBV infection. The vaccinated anti-HBc positive recipient without HBV vaccine response was HBV-DNA positive in serum and liver, viral DNA was continuously negative in the following tests, so a spontaneous seroconversion was diagnosed.

CONCLUSION: The presence of anti-HBs as a result of HBV vaccine or past HBV infection seems to be effective at protecting patients receiving livers from anti-HBc positive donors. However, the emergence of immune escape HBV mutants, which can evade the anti-HBs protection, should be considered as a risk of HBV infection.

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Key words: HBV vaccine; Liver transplantation; De novo HBV infection; Hepatitis B core antibody

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INTRODUCTION

It has been reported that the incidence of hepatitis B virus (HBV) infection is high in recipients after liver transplantation (LT) from hepatitis B surface antigen (HBsAg) negative but anti-core antibody (anti-HBc) positive donors[1,2]. The frequency of HBV transmission depends on the HBV serological recipient status, while the presence of anti-HBc and anti-HBs in organ recipients may confer resistance to HBV infection. Their absence results in *de novo* HBV infection[3-5]. To avoid the occurrence of *de novo* HBV infection in recipients without serum HBV markers, use of passive immunization with hepatitis B immune globulin (HBIG) in combination with lamivudine is necessary[6,7]. Another possibility to make use of these anti-HBc positive organs is to direct liver grafts to patients with anti-HBc and/or anti-HBs as a consequence of past HBV infection or HBV vaccination[8].

It is possible that HBV transmission from anti-HBc

Table 1 Features of donors and liver recipients

Pt.	Age (yr)	Sex	Liver transplant indications	Donor HBV status		Recipient pre-LT HBV status		Liver HBV-DNA	Latest	Follow-up
				anti-HBc	anti-HBs	anti-HBc	anti-HBs	(mo after LT)	anti-HBs levels	(mo)
1	58	M	HCV cirrhosis	+	-	-	+ V (>10)	13 + (RC)	ND	66
2	46	F	Cryptogenic cirrhosis	+	-	-	+ V (>10)	1 -	-	69
3	62	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	70
4	66	M	HCV cirrhosis	+	+	+	+	0 -	ND	71
5	55	F	HCV cirrhosis	+	+	-	+ V (>10)	0 - 12 - 36 -	-	51
6	44	M	HCV cirrhosis	+	-	-	+ V (>100)	4 - 12 -	+	63
7	63	M	HCV cirrhosis	+	+	-	+ V (>100)	1 -	ND	74
8	54	M	HCV cirrhosis	+	-	+	+	2 - 28 -	+	76
9	41	M	HCV/alcoholic cirrhosis	+	+	+	- V (<10)	23 + (RC)	-	66
10	52	F	Alcoholic cirrhosis	+	+	-	+ V (>50)	ND	-	52
11	40	M	HCV/alcoholic cirrhosis	+	-	-	+ V (>50)	0 -	-	56
12	65	M	Alcoholic cirrhosis/ HCC	+	+	-	+ V (>10)	0 -	-	61
13	50	F	Cryptogenic cirrhosis	+	+	-	+ V (>10)	ND	-	39
14	67	F	HCV cirrhosis/HCC	+	+	-	+ V (>10)	14 -	+	36
15	67	M	HCV cirrhosis	+	+	-	+ V (>10)	ND	ND	36
16	60	M	Alcoholic cirrhosis	+	+	-	+ V (>10)	1 -	ND	32
17	62	M	HCV/HCC	+	ND	+	-	6 -	-	31
18	67	M	Alcoholic cirrhosis	+	-	-	+ V (>10)	ND	ND	28
19	46	F	HCV cirrhosis	+	+	+	-	ND	ND	24
20	58	M	Alcoholic cirrhosis	+	-	+	+	ND	ND	18
21	49	M	HCV/alcoholic cirrhosis/HCC	+	+	-	+ V (>10)	5 -	ND	18
22	56	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	17
23	57	M	Alcoholic cirrhosis	+	+	+	+	ND	+	16

HCV: hepatitis C virus; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; V: HBV vaccine; RC: partially double-stranded HBV DNA genome; ND: not determined.

donors to recipients via LT may be due to the persistence of HBV in tissue in a state of replication-competent that can be reactivated to form infectious particles. During initiation of HBV infection, the partially double-stranded HBV DNA genome (rcDNA) becomes a covalently closed circular DNA molecule (cccDNA) that serves as a template for viral transcription[9] and is regulated and amplified by an intracellular pathway[10]. This process establishes a pool of nuclear cccDNA, which persists in the nuclei of infected cells as long as hepatocytes survive, explaining the requirement for long-term antiviral therapies[11]. This fact could explain the reactivation of HBV under certain conditions such as administration of immunosuppressive drugs[12]. The genomic organization of the HBV direct repeat region (DR) provides a strategy to distinguish rcDNA from cccDNA using PCR primers flanking the DR region.

The purpose of this study was to analyze whether past HBV infection or positive response to HBV vaccine is effective in preventing *de novo* HBV infection in patients receiving a liver from anti-HBc positive donors.

MATERIALS AND METHODS

Patients

Anti-HBc screening is a general practice performed in organ donors at our hospital. Between February 1999 and February 2004, 31 HBsAg negative patients received anti-HBc positive liver. Thirty-one donors were anti-HBc positive and 16 of them were also anti-HBs positive (51.6%). Of the 31 recipients, 8 patients had a second LT or were under lamivudine treatment because they were anti-HBc and anti-HBs negative, so they were excluded from the study. The remaining 23 liver recipients were studied. Of them, 14 anti-HBc negative recipients developed protective anti-HBs levels after vaccination (40 mg/dose intramuscularly administered on days 0, 15 and 30; Engerix B, Smith-Kline Beechman, Belgium) and 9 were anti-HBc positive. Immunosuppression treatment included was of steroids and cyclosporin or tacrolimus. Characteristic and virological features of both donors and liver recipients are shown in Table 1.

Serum samples for detection of HBV markers anti-HBs and serum HBV-DNA were collected at the time of liver graft and during the post-transplant follow-up period. Liver tissues when possible were collected for analysis of HBV-DNA by PCR.

Hepatitis B virus markers

Serum samples were tested for HBsAg, anti-HBs and anti-HBc with commercially available radioimmunoassays kits (Abbott Laboratories, N Chicago, IL). Detection of viral DNA in serum was carried out by Abbott hybridization assay. Biopsy specimens were examined with immunohistochemical techniques for HBsAg and HBcAg

detection.

DNA extraction from paraffin-embedded liver tissues was performed using the MasterPure complete DNA purification kit (Epicentre, Madison, WI). Liver DNA was assayed for HBV DNA by 2 different sets of primers corresponding to the surface[13] and X genes. The sequences of the primers amplifying the X gene are as follows: HBVfe (TCTTGGACTCTCAGCAATGTCA nt 1438-1456), HBVre (GGTGAAAAAGTTGCATGGTGC nt 1583-1603), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), HBVri (CCAATTTATGCCTACAGCCTCC nt 1550-1571). PCR was started with the hot-start technique. The first round of PCR was performed with the outer primers for 30 cycles (at 95℃ for 15 s, at 55℃ for 10 s, and at 72℃ for 30 s) followed by an extension at 72 ℃ for 5 min. The second round was carried out with the inner primers for 30 cycles at 57℃ as the annealing temperature.

The selective detection of cccDNA was carried out by a nested PCR procedure as previously described with some modifications to distinguish between rcDNA genome present in virions and ccc HBV-DNA found in hepatocytes[14]. The rcDNA contains a single-stranded gap at the 5´end of the minus strand DNA. Since this region is sensitive to mung bean nuclease (MBN) leading to a disruption in the viral genome, no PCR product could be obtained using this rcDNA as a template. However, since cccDNA is a double-stranded covalently closed molecule and resistant to MBN, PCR could yield a fragment when this DNA was used as a template. MBN reaction was done as previously described[14]. The whole reaction was used as a template for the first round of nested PCR. The primers used to amplify cccDNA were HBVdr-s (TTACGCGGACTCCCCGT nt 1410-1424), 1900AS (GGTCAATGTCCATGCCCCAA nt 1769-1790), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), and HBVdr-as (GACATGAACAAGAGATGATTAGGCA nt 1706-1730). As a positive control, a PCR fragment containing the nick region of rcDNA was cloned into the pMosBlue vector (pMOSBlue blunt ended cloning kit, Amersham Pharmacia Biotech, UK). HBV DNA extracted from serum of HBsAg-positive individuals was used as a negative control. Both positive and negative controls were treated with MBN.

RESULTS

Only one patient developed *de novo* HBV infection after a follow-up of 45 ± 20.81 months (Table 1). This patient was vaccinated and developed a low anti-HBs response with anti-HBs title between 10 and 100 IU/mL. Seven months after LT, her routine biochemical tests showed abnormal level of liver enzymes (AST 97 U/L, ALT 151 U/L, GGT 136 U/L) but liver ultrasound was normal. Besides, virological analysis was found to be positive for HBsAg and serum HBV-DNA, even if her serum anti-HBs titer was 17 IU/mL. These virological tests did not reveal any other viral infections (CMV, CEV, HCV, HAV). Based on these results, *de novo* HBV infection was diagnosed and the patient was treated with lamivudine (100 mg/d). Serum HBV-DNA still remained positive, so a combined therapy of lamivudine and adefovir was administered 32 mo after LT. A second liver biopsy revealed marked steatosis with positive immunostaining for HBcAg (in nuclei and cytoplasm) and HBsAg (in cytoplasm and membrane). No tissue was obtained for the analysis of HBV-DNA.

It is known that the "a" determinant located within HBsAg is the target of immune response providing immunity against HBV infection[15]. The emergence of HBV with surface gene mutations is able to escape immune response against HBV vaccine, causing infection. In fact, HBV envelope mutants associated with the "a" determinant after HBV vaccination have been identified[16]. To know whether this might be the reason why *de novo* HBV infection occurred in this liver recipient, a serum sample taken 36 months after LT was used for HBV-DNA extraction, PCR amplification of the "a" determinant of the S gene and PCR fragment sequencing. Viral sequence revealed that this patient harbored a HBV variant with 2 point mutations at amino acid positions 127 and 145 of HBsAg. The first mutation resulted in a substitution of proline or lysine for threonine (Pro or Lys 127 Thr). The second mutation was a substitution of glycine for alanine (Gly 145 Ala). Several HBV mutants with amino acid changes in the "a" determinant have been reported in the post-transplant situation[16,17]. Among those mutations, the particular and almost invariably change is Gly 145 Arg, which can cause persistent infections[18]. Our patient also presented this change at position 145. However, the sequence analysis revealed the presence of an Ala instead of an Arg. This amino acid change has never been previously found in the literature. The change of Pro 127 Thr has been described in liver-transplanted patients [16].

A total of 18 biopsies taken at different post-LT time points from 14 recipients were obtained for the analysis of HBV-DNA in liver tissues. Open circular HBV-DNA (RC) was found in 2/14 patients (Table 1) and no cccDNA was detected in any of them. These results were reproducibly obtained in 3 repeated sets of PCR experiments. The anti-HBc negative patient with HBV-DNA in tissue (Table 1) had no detectable HBV-DNA and HBsAg in serum throughout a follow-up period of 66 mo. The anti-HBc positive recipient with viral DNA in liver (Table 1) was found to be serum HBV-DNA positive in one of his routine virological tests but this viral marker was continuously negative in the following tests. HBsAg was all negative during the whole follow-up period (66 months). No recurrent HBV infection could be considered in this patient.

DISCUSSION

Accumulating evidence suggests that HBV can be transmitted to the organ recipients from anti-HBc-positive donors through LT[19,20]. HBV transmission fluctuates among different studies between 50 % and 90 %. In our study only one patient receiving an anti-HBc positive liver developed *de novo* HBV infection as a consequence of an immune escape HBV mutant associated with the "a" determinant while in the absence of HBV mutants, none of the recipients developed HBV infection, suggesting that the presence of anti-HBs during LT, as a consequence of HBV vaccination or past HBV infection together with

anti-HBc, can protect against HBV infection transmitted by anti-HBc positive liver grafts.

Although less than 50% of the patients with cirrhosis due to HBV infection respond to HBV vaccine[21], a large number of patients can be considered as liver transplant recipients.

Anti-HBs levels >10 IU/mL due to HBV vaccine are considered protective in immunocompetent patients[22]. We consider this anti-HBs titer protective in those vaccinated patients[21].

We were aware of the short-term persistence of anti-HBs in these patients. Only 5 of them still maintained anti-HBs titers over the follow-up period while the rest of the patients lost this marker (Table 1). Since anti-HBs response is reduced in immunosuppressed patients[21,23], it is accepted that this rapid drop of anti-HBs levels is the consequence of their immunosuppressive therapy. Importantly, these results suggest that even with loss of anti-HBs during the post-LT period, HBV vaccination is effective as prophylaxis for the prevention of HBV infection in LT, which is in agreement with previous studies[24]. Another interesting finding that supports the efficacy of HBV vaccine against HBV infection is the continuous negativity for serum HBV DNA after LT during the follow-up period.

It is known that viral genome can persist in hepatocytes as a rcDNA molecule and as a cccDNA molecule, the later is required for viral replication[9]. Detection of intrahepatic cccDNA may indicate the possible ongoing viral replication[25]. Thus, its presence could explain the reactivation of HBV replication in patients receiving a liver from donors with anti-HBc. The only viral form detected in 2 recipients (1 anti-HBc negative, 1 anti-HBc positive) was rcDNA. The anti-HBc negative patient who had no history of previous HBV infection received HBV vaccine prior to LT with anti-HBs titers >10 IU/L. It is interesting to know that this patient, even maintaining HBV DNA in liver after one year of LT, did not show any virological evidence of *de novo* HBV infection during the follow-up period of 66 months (Table1). Moreover, he lost anti-HBs titers. The histopathological study at this time showed stage III fibrosis, confirming recurrence of HCV infection. The data may suggest that HBV graft infection may be infrequent. Likewise, in the anti-HBc positive recipient with viral DNA in liver tissue 23 months after LT (Table 1) and after 66 months of follow-up, no recurrent HBV infection occurred although he had positive serum HBV-DNA in one of his routine virological tests, suggesting that spontaneous seroconversion occurs in him.

However, even if the presence of anti-HBs in liver recipients seems to prevent recurrent or *de novo* HBV infection, the latest risk can still occur as seen in one of the recipients. Nevertheless, in this recipient HBV infection was not prevented by anti-HBs response because the cause was a circulating HBV mutant. The presence of circulating surface antigen-mutated HBV was proved when mutations in the "a" determinant region of the surface antigen were identified in this patient. In our study, the prevalence of HBV surface antibody escape mutants after liver transplantation was 6.6%, which is consistent with other studies[16, 26].

Our results are in agreement with an earlier study[27]. However, other reports have provided clear evidence that HBV genome is detectable in most anti-HBc positive donors[28]. The validity of our amplification method was confirmed by our control experiments using negative and positive controls. One possibility could be that DNA molecules isolated from preserved paraffin-embedded liver tissues are generally of poor quality because of the high degree of DNA degradation in these samples. However, HBV detection may be reduced beyond detectable levels but focal distribution of HBV infection cannot be excluded.

In conclusion, the presence of anti-HBs in liver recipients at the time of LT can prevent HBV recurrence or *de novo* HBV infection. Although we have described a new vaccine HBV mutant in a liver transplant recipient causing *de novo* HBV infection, the efficacy of HBV vaccine in organ recipients could not be considered as universal due to the development of immune escape HBV mutants associated with the "a" determinant which can evade the anti-HBs protection. Administration of HBV vaccine is mandatory in patients with chronic liver pathology potentially needing liver transplantation. Although these results are promising, the limited patient number may lead to an erroneous interpretation of the data. Most extensive studies including a large number of recipients need to be done.

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RAPID COMMUNICATION

Prognosis following transcatheter arterial embolization for 121 patients with unresectable hepatocellular carcinoma with or without a history of treatment

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Abstract

AIM: To retrospectively evaluate the prognosis of patients with hepatocellular carcinoma (HCC) with or without a history of therapy for HCC following transcatheter arterial embolization (TAE).

METHODS: One hundred and twenty-one patients with HCC treated with TAE from 1992 to 2004 in our hospital were enrolled in this study. Eighty-four patients had a history of treatment for HCC, while 37 did not. At the time of entry, patients with extra-hepatic metastasis, portal vein tumor thrombosis, or Child-Pugh class C were excluded. TAE was repeated when recurrence of HCC was diagnosed by elevated tumor markers, or ultrasonography or dynamic computed tomography findings.

RESULTS: Tumor size was larger and the number of tumors was fewer in patients without past treatment ($P < 0.01$). However, there were no differences in tumor node metastasis (TNM) stage or survival rate between the 2 groups. A bilobular tumor and high level of α-fetoprotein (AFP) (>100 ng/mL) were factors related to a poor prognosis in patients with a history of HCC.

CONCLUSION: The prognosis following TAE is similar between HCC patients with and without past treatment. Early diagnosis of HCC or recurrent HCC and obtaining good local control against HCC before entry to a repeated TAE course can improve prognosis.

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Key words: Unresectable hepatocellular carcinoma; Prognosis; Repeated transcatheter arterial embolization

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INTRODUCTION

Liver transplantation is recognized as an effective therapy for hepatocellular carcinoma (HCC)[1]. However, a shortage of donors in Japan has led to the general use of transcatheter arterial embolization and transcatheter arterial chemoembolization (TAE) in patients with unresectable HCC without an indication of surgery and percutaneous therapy. Although disappointing results are published[2-4], the usefulness of TAE has been reconfirmed recently as some studies found that the procedure reduces the overall 2-year mortality rate and improves the survival rate of patients with unresectable HCC[5-8]. Past reports regarding the prognosis of patients with HCC are usually limited to the initial therapy, including surgery[9], percutaneous ethanol injection therapy (PEIT)[10], radiofrequency ablation (RFA)[11,12], and TAE. In previous studies of TAE, the subjects had no history of treatment for HCC. However, most patients with HCC have no indication for therapy such as surgery, PEIT and RFA due to multiple recurrences finally. No reports have evaluated prognosis and its related factors of patients with a history of HCC following a repeated TAE course. In the present study, we retrospectively evaluated the prognosis of HCC patients with or without a history of therapy for HCC following TAE.

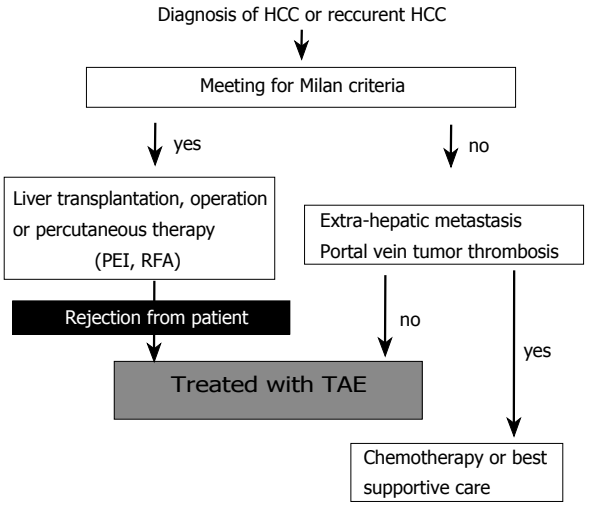

Figure 1 Strategy for treatment of HCC employed at our institution. Nearly all patients with HCC, which were outside of the Milan criteria, were recommended for a repeated TAE course.

MATERIALS AND METHODS

This was a single-center retrospective study conducted at Ehime University Hospital. One hundred and twenty-one patients with advanced HCC treated with TAE from 1992 to 2004 were enrolled in the study. After informed consent was obtained, the entry date was considered the day of the first TAE therapy after enrollment. The diagnosis of HCC was based on histological and cytologic findings or findings of dynamic computed tomography (CT). Tumor stage was established by dynamic CT, ultrasonography (US), angiography, chest CT, and bone scintigraphy examinations.

Patients with extra-hepatic metastasis, portal vein tumor thrombosis (PVTT), or Child-Pugh class C at entry were excluded from this study since the existence of PVTT and a high Child-Pugh score are poor prognostic markers and TAE can not improve these patients[5]. As a result, 37 patients with no history of treatment for HCC and 84 with such a history of surgery, PEIT, or RFA, were studied. During the 13-year study period, a total of 121 patients underwent 435 courses of TAE, with 99 (82%) outside of the Milan criteria[1]. In 37% of 121 patients, HCC was confirmed by histological examination. Others were diagnosed based on the increasing course of α-fetoprotein (AFP) and dynamic CT. All HCC nodules treated with TAE were hypervascular.

The patients treated with TAE were evaluated based on our strategy (Figure 1). TAE was repeated when HCC recurrence was diagnosed by the elevation of tumor markers, or US or dynamic CT examination findings. Experienced radiologists performed all the TAE procedures. A microcatheter was inserted into the artery feeding the tumor super-selectively after conventional hepatic angiography or angiography CT, and then a segmental or subsegmental TAE procedure[13] was performed. Before the procedure, antegrade flow in the portal vein and no obstruction of the main trunk of the portal vein were confirmed by US, dynamic CT, and portography findings via the superior mesenteric artery. Lipiodol and a gelatin sponge (Gelfoam, Upjohn, Kalamazoo, MI, USA) were used for embolization, and epirubicin hydrochloride was used together with Lipiodol in 25% of the cases. The goal of embolization was disappearance of tumor staining without complete obstruction of the hepatic artery. Patients that underwent additional chemotherapy via a subcutaneously implanted injection port, surgery, PEIT, or RFA for the purpose of reducing the size of the tumor after undergoing TAE were excluded from this study.

Table 1 Backgrounds of patients without or with history of treatment

	Patients without history of HCC ($n=37$)	Patients with history of HCC ($n=84$)	P value
Age (yr)	66.4 ± 9.9	67.0 ± 8.1	NS
Sex (male ： female)	32 ： 5	64 ： 20	NS
Frequency of positive for anti-HCV	72%	81%	NS
TNM stage (II ： III)	12 ： 25	35 ： 49	NS
Tumor size (mm)	46.4 ± 23.5	27.7 ± 16.1	$P<0.01$
Number of tumors (≤3 ： >3)	21 ： 16	23 ： 61	$P<0.01$
Monolobular ： bilobular	17 ： 20	27 ： 57	NS
Child-Pugh class (A ： B)	27 ： 10	48 ： 36	NS
Alanine transferase (IU/L)	63.8 ± 45.1	82.1 ± 64.3	$P=0.07$
AFP (≤100 ： >100 ng/mL)	23 ： 14	53 ： 31	NS
TAE with or without anti-cancer medication	13 ： 24	17 ： 67	NS
Average number of past treatments	-	2.9 ± 2.2	-
History of hepatectomy	-	18%	-
Average observation period (d)	557.6 ± 377.0	493.6 ± 390.6	NS

Anti-HCV: hepatitis C virus antibody; AFP: α-fetoprotein; TNM stage: tumor node metastasis stage.

The backgrounds of both groups at study entry are shown in Table 1. The group of patients without past treatment consisted of 32 males and 5 females, of whom 12 and 25 patients were in tumor node metastasis (TNM) stage[14,15] II and III, respectively. Furthermore, 27 were Child-Pugh class A and 10 were class B, of whom 72% were positive for the hepatitis C virus antibody (anti-HCV) and 14% for the hepatitis B surface antigen (HBs Ag).

As for the group of patients with treatment history, 64 were male and 20 female, of whom 35 and 49 were TNM stages II and III, respectively. Forty-eight patients in this group were Child-Pugh class A and 36 class B, of whom 81% were positive for anti-HCV and 16% for HBs Ag.

Determination of markers of hepatitis viruses

The presence of anti-HCV and HBs Ag was determined precisely using enzyme immunoassay kits (Imcheck-FHCV, Kokusai-Shiyaku, Kobe, Japan; AxSYM HBs Ag, Dainabot, Tokyo, Japan), according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were carried out using a personal computer with StatView version 5.0 (SAS Institute, Inc., Berkeley, CA, USA). Analyses were conducted using Student's t-test, Mann-Whitney U test, Cox's proportional hazards regression model, logrank test, and the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Table 2 Univariate analysis of patients with past treatment for HCC ($n=84$)

Factors	Number	Hazard ratio	95% CI	*P* value
Age (= and <65 ： >65)	38：46	0.99	0.95-1.02	NS
Sex (male ： female)	64：20	1.02	0.54-1.93	NS
Anti-HCV (positive ： negative)	67：17	1.31	0.64-2.67	NS
TNM stage (II ： III)	35：49	1.57	0.83-3.00	NS
Tumor size (mm)	-	1	0.98-1.02	NS
Number of tumors (≤3 ： >3)	23：61	0.88	0.47-1.67	NS
Monolobular : bilobular	27：57	2	1.04-3.86	$P<0.05$
Child-Pugh class (A ： B)	48：36	1.07	0.59-1.95	NS
AFP (≤100 ： >100 ng/mL)	53：31	1.9	1.03-3.48	$P<0.05$
History of hepatectomy (negative ： positive)	69：15	1.63	0.72-3.70	NS
Number of past treatments	-	1.05	0.93-1.19	NS

CI: confidence interval; anti-HCV: hepatitis C virus antibody; AFP: α-fetoprotein; TNM stage: tumor node metastasis stage.

Table 3 Multivariate analysis of patients with past treatment for HCC ($n=84$)

Factors	Hazard ratio	95% CI	*P* value
Existence of bilobular tumors	2.37	1.19-4.71	$P<0.05$
AFP (>100 ng/mL)	2.24	1.19-4.23	$P<0.05$

CI: confidence interval; AFP: α-fetoprotein.

RESULTS

There were no significant differences in the background findings between patients with or without past treatments for HCC, except for tumor size and the number of tumors ($P<0.01$) (Table 1). There was also no significant difference in patient distribution for TNM staging between the groups. None of the patients died due to the TAE procedure. For patients with treatment history, the average number of past treatments for HCC was 2.9±2.2 (range 1-10) and a hepatectomy was performed before entry to the repeated TAE course in 18% of these patients.

The survival rate was not significantly different between the 2 groups (Figure 2). The 1-, 2-, and 3-year survival rates were 90%, 57%, and 20% respectively in patients without past treatment, and 75%, 43%, and 25% respectively in those with past treatment. The factors related to poor prognosis in the 84 patients with past treatment for HCC were evaluated. Seventy-four of them (88%) were outside of the Milan criteria. According to univariate analysis, variables significantly associated with survival were tumor location (bilobular) and a high concentration of AFP (>100 ng/mL) ($P<0.05$). There were no relationships between the prognosis of patients with a history of treatment for HCC and other factors, including history of past hepatectomy and the number of past treatments for HCC (Table 2). Multivariate analysis showed that the existence of bilobular HCC and high concentrations of AFP (>100 ng/mL) were the factors for poor prognosis ($P<0.05$, Table 3). The survival rate of patients without both risk factors was better than that of those with both risk factors ($P<0.01$, Figure 3). In all 121 patients, the existence of bilobular HCC was related to poor prognosis ($P<0.01$), while a high concentration of AFP ($P=0.059$) and other factors including past treatments, were not related to poor prognosis.

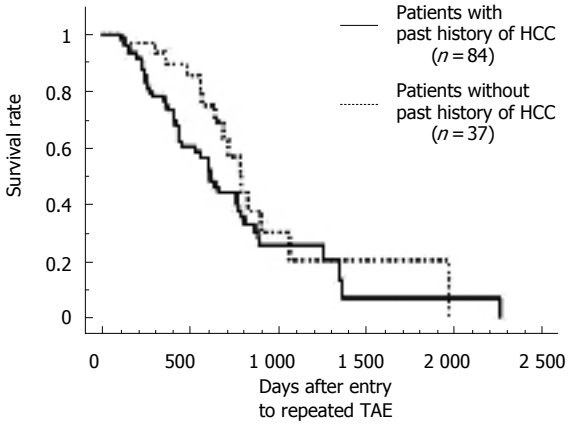

Figure 2 Survival rates of HCC patients with or without past treatment for HCC. There was no significant difference between the 2 groups. Survival rates after 1, 2, and 3 years were 90%, 57%, and 20% respectively for patients without past treatment, and 75%, 43%, and 25% respectively for patients with past treatment.

DISCUSSION

The prognosis of a patient with HCC is dependent on the hepatic reserve function and HCC staging[16,17]. A repeated TAE course is widely used for patients with unresectable HCC[18,19], though it was reported that TAE is not effective for improving the prognosis of such patients[2,3,4]. The reason for the disappointing results is that TAE is repeated within a fixed period of time although the liver reserves function and the patients have or have no recurrence of HCC. When TAE is repeated after a fixed period of time, embolization from the main trunk of the hepatic artery can lead to liver atrophy and deterioration of hepatic reserve function. Recently, the efficacy of TAE for patients with HCC has been reported, with good improvement of survival results[5-8]. Caturelli *et al*[20] reported that repeated TAE does not induce long-term deterioration of hepatic reserve function in HCC patients with Child-Pugh A and B but without PVTT.

Since repeated TAE for a fixed period without recurrence can lead to a reduction in hepatic reserve function, we think that it is important to perform TAE at the time

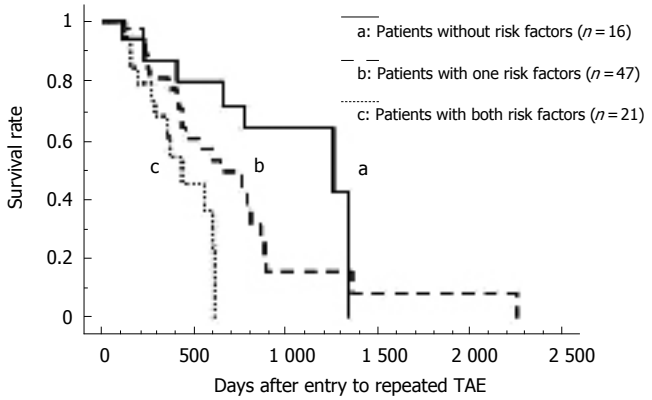

Figure 3 Survival rates of HCC patients with past treatment and with or without the 2 risk factors found in the present study. Significant differences were shown between "**a**" and "**b**" and between "**b**" and "**c**" ($P<0.01$ and $P=0.01$), while there were no significant differences between "**a**" and "**b**" ($P=0.08$). **a**: Patients without either factor [existence of bilobular tumors and high concentration of AFP (greater than 100 ng/mL; $n=16$)]; **b**: Patients with one of the factors ($n=47$); **c**: Patients with both factors ($n=21$).

when HCC recurrence is diagnosed by the elevation of tumor markers, or based on US or dynamic CT findings [21,22]. Recently, diagnostic progress of US and CT has made it easier to accurately diagnose new and recurrent HCC with low levels of AFP. Dynamic CT can offer detailed information about tumor vascularity and dynamic CT is useful to distinguish cholangiocarcinoma from HCC[23,24]. In our study metastatic liver tumor was denied from clinical course in all cases. In a large number of patients, HCC develops to an unresectable condition during the course of therapy and becomes outside of the Milan criteria. To our knowledge, the prognosis of patients with a history of HCC and factors for poor prognosis have not been reported after a repeated TAE course. In the present study, though the tumor maximum size and the number of tumors were different between the patients with or without past treatment, the survival rates of both groups after undergoing TAE did not show a significant difference, which might be due to no significant difference in TNM staging distribution between the groups.

As for the patients with past treatment, a past hepatectomy and the number of past percutaneous therapies (e.g. PEIT and RFA) did not influence the prognosis after repeated TAE in regard to maintaining liver reserve function. A high concentration of AFP and tumor location (bilobular) each had a significant influence. The elevation of AFP and existence of bilobular HCC are dependent on the malignant potential of HCC [25] and intra-hepatic metastasis, respectively.

Prognosis was not significantly different between HCC patients with or without a history of HCC following TAE. Our results show that diagnosis of HCC or recurrent HCC in the early stage and obtaining good local control against HCC before a repeated TAE course can reduce the time bias and improve prognosis.

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RAPID COMMUNICATION

Cryptogenic cirrhosis in the region where obesity is not prevalent

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Abstract

AIM: Recent studies have demonstrated that obesity is the common feature of cryptogenic cirrhosis (CC) and non-alcoholic steatohepatitis. However, there is little information on CC in the region where obesity is not prevalent.

METHODS: The clinical features, and the liver-related morbidity and mortality of CC were analyzed in Japan where the prevalence of obesity is low. Among 652 cirrhotic patients, we identified 29 patients (4.4%) with CC. Of these, 24 CC patients who were followed up for more than 6 months were compared in a case-control study with age-, sex-, and Child-Pugh score-matched controls having cirrhosis of viral etiology.

RESULTS: Obesity (BMI$\geqslant$25 kg/m^2), diabetes mellitus, and hypertriglyceridemia were more frequent, and the visceral fat area was larger in the CC patients than in the controls. The indices of insulin resistance were higher and the serum aminotransferase levels were lower in the CC patients than in the controls. Logistic regression analysis identified the elevated hemoglobin A1c, BMI $\geqslant$ 25 kg/m^2, and normal aminotransferase levels as independent predictors of CC. Kaplan-Meier analysis demonstrated lower occurrence of hepatocellular carcinoma and higher survival rate in the CC than in the controls in contrast to the similar cumulative probability of liver-related morbidity between those groups.

CONCLUSION: CC more frequently presents with the clinical features suggestive of non-alcoholic steatohepatitis compared with controls even in the region where obesity is not prevalent. The lower occurrence of hepatocellular carcinoma and higher survival rate may indicate an indolent clinical course in CC as compared with viral cirrhosis.

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Key words: Cryptogenic cirrhosis; Viral cirrhosis; Non-alcoholic steatohepatitis; Hepatocellular carcinoma; Case-control study

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INTRODUCTION

Liver cirrhosis is the terminal condition of liver diseases resulting from various etiologies. Despite the recent development of diagnostic tools, no recognizable etiology can be detected in approximately 5% to 31% of cirrhotic patients who are therefore diagnosed as having cryptogenic cirrhosis (CC)[1-3]. Although several explanations such as unknown viral infections, occult alcohol abuse, or burnt out autoimmune hepatitis had been proposed as possible causes of CC, they actually induce CC only in some cases[3,4].

Obesity is an independent risk factor for chronic liver diseases, and liver fibrosis can develop in the obese patients without any known causes of liver diseases [5, 6]. Due to the recent increase of the obese population, great attentions have been paid to non-alcoholic steatohepatitis (NASH), which is characterized by obesity and insulin resistance. It has been widely recognized that NASH can progress to liver cirrhosis and hepatocellular carcinoma (HCC)[7]. Several studies have suggested that NASH may be an under-recognized cause of CC, because the prevalence of risk factors for NASH such as obesity and diabetes mellitus is definitely increased in the patients with CC[8-10]. However, these studies have been conducted

in the West where obesity is common, and there is little information on CC in the region where the dietary habits are different from those in the West and the prevalence of obesity is low. Because the key component of the association between NASH and CC is obesity[8-10], whether this association is true even in the non-obese population is an important issue.

In Japan where obesity is not prevalent, the clinical characteristics, and the liver-related morbidity and mortality of CC were investigated with a special reference to the association with NASH in this study. The aim was to clarify the clinical features of CC in the region where the prevalence of obesity is low.

MATERIALS AND METHODS

Patients

We identified 652 cirrhotic patients in whom sufficient data were available to establish the etiology of liver disease from the inpatients' registry of Nara Medical University since 1990. Liver cirrhosis was diagnosed on the basis of compatible clinical and imaging findings, and/or liver histology. The diagnosis of CC was made only after an exhaustive evaluation of the clinical history and laboratory data, from which no specific etiologies were defined. Patients with histological evidence of other defined causes of liver diseases were also excluded from the diagnosis of CC. Of 29 patients with CC, 24 patients who had been followed up for more than 6 months were classified as the CC group in our case-control study. For patients in CC group, 3 patients with cirrhosis of viral etiology [2 hepatitis C virus (HCV)-related and 1 hepatitis B virus (HBV)-related], age- (within 3 years), sex-, and Child-Pugh score-matched, were identified from the corresponding inpatients' registry in a consecutive manner. Finally, 24 patients with CC, 48 with HCV-related cirrhosis, and 24 with HBV-related cirrhosis were enrolled in the present case-control study. This study was approved by the Ethical Committee of Nara Medical University and was performed in accordance with the Declaration of Helsinki.

Methods

All patients underwent an exhaustive re-evaluation of the following clinical information: past or present evidences of diseases including diabetes mellitus, dyslipidemia, and hypertension; personal history of alcohol intake, intravenous drug use, or blood transfusion, and family history of liver diseases. The height and weight were measured, and the body mass index (BMI) was calculated as weight (kg) divided by squared height (m). The cases with ascites were evaluated after the resolution of ascites. HBV markers (surface antigen/antibody and core antibody) and HCV antibody were negative in all patients with CC. The following laboratory data were also collected at enrollment: total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, albumin, γ-globulin, cholinesterase, total cholesterol, triglyceride, prothrombin activity (International normalized ratio: INR), hemoglobin A1c, α1-antitrypsin, iron storage parameters (transferrin saturation and ferritin), copper and ceruloplasmin levels, anti-nuclear antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal antibody. When immune mediated liver disease could not be ruled out, liver histology was evaluated. The fasting blood levels of glucose and insulin were determined, and the homeostasis model assessment parameter of insulin resistance (HOMA-IR) and the quantitative insulin check index (QUICKI) were calculated as the indices of insulin resistance[11,12]. Liver biopsy was performed in 12 of the 24 patients with CC and was used to investigate the histological features suggestive of NASH.

Abdominal fat distribution

Computed tomograms were recorded at the umbilical level, and the visceral fat areas were measured as described previously [13]. Other tomograms were taken at the level where the liver and spleen were observed in the same slice. The Hounsfield unit of the liver and spleen was determined, and the liver/spleen ratio was calculated as an index of the hepatic fat content [14].

Assessment of the outcomes

Clinical information including the tumor markers and the imaging studies was monitored every 6 months until data analysis. In cases which lost to follow-up, up-to-date clinical information was investigated by telephone interview and/or contact with the primary care physician. The following outcomes were evaluated; (1) liver-related morbidity including variceal bleeding, ascites, jaundice, and hepatic encephalopathy, (2) occurrence of HCC, (3) mortality and cause of the death. HCC was histologically confirmed or diagnosed by elevation of α-fetoprotein and/or compatible ultrasonographic or computed tomographic findings. Death was considered to be liver-related when it happened in consequence to hepatic failure, variceal bleeding, and/or HCC.

Statistical analysis

All analyses were performed with StatView 5.0 program (SAS Institute, Cray, NC, USA). Comparisons were made using the two-tailed Student's *t* test for quantitative variables and the Chi-square test for qualitative variables. Kaplan-Meier's method and the log-rank test were used to compare the cumulative probability to liver-related morbidity and mortality. The factors associated with CC were identified using a multivariate logistic regression model. All data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Etiology of liver cirrhosis

Among the 652 patients, cirrhosis was attributed to viral etiology in about 80% (HCV-related cirrhosis: 62.9%, HBV-related cirrhosis: 16.6%) and to alcohol in 9.5%. The prevalence of CC was 4.4%. The other cases resulted from various causes such as autoimmune liver diseases, hemochromatosis, Wilson's disease, Budd-Chiari syndrome, and congestion. The males were predominant in viral and alcoholic cirrhosis, whereas there was no gender difference

Table1 Characteristics of cryptogenic cirrhotic patients and matched controls

	Cryptogenic ($n=24$)	Control; all ($n=72$)	HCV-related ($n=48$)	HBV-related ($n=24$)
Age (yr)	58.2 ± 10.6	58.6 ± 8.8	58.7 ± 8.1	58.3 ± 10.2
Child-Pugh score	6.3 ± 1.4	6.5 ± 1.2	6.5 ± 1.2	6.4 ± 1.3
Follow-up (yr)	5.7 ± 4.2	5.9 ± 4.4	6.5 ± 4.2	4.7 ± 4.5
Transfusion (%)	0	26.8[b]	34.0[b]	12.5
Body mass index (kg/m^2)	25.5± 3.2	22.4±3.0[b]	22.6±3.3[b]	22.0±2.4[b]
ICGR15 (%)	31.5±16.7	27.2±12.3	28.2±12.1	25.0±12.7
Total bilirubin (mmol/L)	22±15	21±10	22±10	19±10
AST (nkat/L)	959±572	1349±772[a]	1410±573[b]	1229±1064
ALT (nkat/L)	860±640	1229±765[a]	1359±748[b]	972±747
AKP (nkat/L)	6518±2942	6638±2976	6695±2577	6529±3692
Albumin (g/L)	40±5	38±5	38±5[b]	38±6
γ-globulin (g/L)	17±3	18±5	19±4	15±5
Cholinesterase (U/L)	242.8±118.2	174.5±67.8[b]	162.1±61.7[b]	198.8±73.7
Total cholesterol (mg/dL)	170.5±43.0	156.5±35.1	151.2±31.3[b]	166.8±40.2
Triglyceride (mg/dL)	103.8±44.2	83.0±25.5[b]	84.5±26.6[b]	80.1±23.8[b]
Prothrombin time (INR)	1.15±0.15	1.19±0.14	1.21±0.14	1.16±0.14
Hemoglobin A1c (%)	6.2±1.3	5.1±0.8[b]	5.3±0.9[a]	4.8±0.7[b]
Blood glucose (mg/dL)	123.1±48.2	96.4±21.0[b]	96.9±23.0[b]	95.2±16.3[a]
Insulin (mU/L)	26.8±15.7	14.2±6.4[b]	15.9±6.6[b]	10.8±4.5[b]
HOMA-R (%)	8.6±4.8	3.6±2.5[b]	4.0±2.9[b]	2.6±1.3[b]
QUICKI	0.29±0.02	0.33±0.03[b]	0.32±0.02[b]	0.34±0.04[b]
Transferrin saturation (%)	36.3±17.5	45.2±29.7	48.5±31.1	40.0±27.8
Ferritin (mg/L)	160.0±155.3	193.5±282.9	225.0±318.1	99.3±104.8
Visceral fat area (cm^2)	102.0±39.8	56.9±35.3[b]	52.1±30.7[a]	64.3±43.9[b]
Liver/spleen ratio	1.08±0.05	1.08±0.06	1.07±0.06	1.09±0.06

[a]$P<0.05$, [b]$P<0.01$ *vs* cryptogenic cirrhosis. ICGR15:indocyanine green retention rate, INR:international normalized ratio, HOMA-R: homeostasis model assessment parameter of insulin resistance, QUICKI: quantitative insulin check index.

Table 2 Prevalence of obesity and complication in cryptogenic cirrhotic patients and matched controls

	Cryptogenic ($n=24$)	Control; all ($n=72$)	HCV-related ($n=48$)	HBV-related ($n=24$)
Body mass index: ≥25kg/m^2	54.2%	20.8%[b]	29.2%[a]	8.3%[b]
⩾30kg/m^2	16.7%	1.4%[a]	2.1%[a]	0%[a]
Visceral fat area: ⩾100cm^2	40.0%	5.3%[b]	3.7%[b]	9.1%[a]
Complication				
Type 2 diabetes mellitus	54.2%	26.4%[a]	35.4%	13.3%[b]
Hypertriglyceridemia (⩾150mg/dL)	20.8%	4.2%[a]	2.1%[b]	13.3%
Hypertension	25.0%	15.3%	18.8%	13.3%

[a]$P<0.05$, [b]$P<0.01$ *vs* Cryptogenic cirrhosis.

in CC (male/female ratio; HCV: 255/155, HBV: 82/26, alcohol: 52/10, CC: 15/14).

Characteristics of patients

The follow-up period was not significantly different between groups. Whereas one-third of the patients with viral cirrhosis were transfused, no patient with CC was transfused. BMI was significantly higher in the CC patients than in the controls of viral etiology. AST and ALT levels were lower in the CC patients than in the controls, although AST was higher than ALT in all groups. Cholinesterase, total cholesterol, and triglyceride were higher in the CC patients than in the controls. The fasting levels of blood glucose, hemoglobin A1c, insulin, and the indices of insulin resistance such as HOMA-IR and QUICKI were also higher in the CC patients than in the controls. Iron storage parameters such as transferrin saturation and ferritin were similar in all groups (Table 1). The visceral fat area was larger in the CC patients than in the controls, whereas the liver/spleen ratio was similar between groups. Obesity was more prevalent in CC patients than in controls (BMI ⩾ 30 kg/m^2: CC 16.7% *vs* controls 1.4%, $P<0.05$) (Table 2). The patients whose visceral fat area was larger than 100 cm^2 constituted 40% of the CC as compared with 5.3% of the controls ($P<0.05$). Type 2 diabetes mellitus and hypertriglyceridemia were more frequent in the CC patients than in the controls. No patient suffered from type 1 diabetes mellitus. The prevalence of hypertension was similar in all groups.

Although liver biopsy was performed in 12 of 24 patients with CC, there was no specific finding to define the etiology of liver disease. Ten of 12 biopsies, however, revealed one or more histological components suggestive of NASH such as macrovesicular steatosis,

Table 3 Factors associated with cryptogenic cirrhosis

	Odds ratio (95% CI)	*P* value
Univariate		
Body mass index (≥25 kg/m^2)	4.2 (1.51-11.48)	0.006
Visceral fat area (≥100 cm^2)	12.8 (2.16-75.32)	0.005
Diabetes mellitus	3.3 (1.26-8.60)	0.015
Dyslipidemia	6.1 (1.33-27.64)	0.020
Hypertension	1.8 (0.60-5.70)	0.285
ALT (<667 nkat/L)	3.8 (1.42-9.91)	0.008
Cholinesterase (≥192 U/L)	3.9 (1.42-10.94)	0.008
Triglyceride (≥150 mg/dL)	5.9 (1.29-26.86)	0.022
Total cholesterol (≥220 mg/dL)	3.4 (0.77-14.62)	0.108
HbA1c (≥6%)	5.5 (1.40-21.75)	0.015
HOMA-R (≥4%)	11.6 (2.46-54.45)	0.002
Ferritin (≥220 ng/dL)	0.4 (0.11-1.82)	0.255
Transferrin saturation (≥40%)	0.4 (0.11-1.82)	0.158
Multivariate		
HbA1c (≥6.0%)	7.8 (1.62-37.55)	0.010
Body mass index (≥25 kg/m^2)	6.8 (1.41-32.87)	0.017
ALT (<40 U/L)	5.0 (1.12-22.43)	0.035

CI: confidence interval, ALT: alanine transaminase, HbA1c: hemoglobin A1c, HOMA-R: homeostasis model assessment parameter of insulin resistance.

ballooning hepatocyte degeneration, neutrophilic lobular inflammation, and Mallory hyaline. Eight cases had macrovesicular steatosis in less than 30% of the hepatocytes. Ballooning hepatocyte degeneration, neutrophilic lobular inflammation, and Mallory hyaline were present in 7, 2, and 2 cases, respectively. Two cases had no inflammatory activity, 7 cases demonstrated minimal activity, and 3 cases had mild-to-moderate activity. The inflammatory infiltrates consisted predominantly of lymphocytes in fibrous bands.

Factors associated with CC

In the univariate analysis, BMI≥25 kg/m^2, visceral fat area ≥ 100cm^2, and the coincidence of diabetes mellitus and dyslipidemia, were significantly associated with CC (Table 3). Moreover, normal ALT levels (<667nkat/L), elevated levels of cholinesterase, triglyceride, and hemoglobin A1c ≥ 6.0%, and HOMA-IR ≥ 4% were significantly associated with CC. The multivariate analysis identified the elevated levels of hemoglobin A1c (OR: 7.8, 95% CI: 1.62-37.55, $P<0.05$) and BMI (OR: 6.8, 95% CI: 1.41-32.87, $P<0.05$), and normal ALT levels (OR: 5.0, 95% CI: 1.12-22.43, $P<0.05$) as independent predictors of CC.

Clinical course of CC as compared with the viral cirrhosis

During a mean follow-up of 5.7 years for the CC group ($n=24$), 2 patients experienced variceal bleeding, and 6 patients developed ascites (Figure 1). Jaundice and hepatic encephalopathy occurred in 5 patients and 6 patients, respectively. The viral group ($n=72$) was followed-up for a mean of 5.9 years, during which variceal bleeding, ascites, jaundice, and hepatic encephalopathy occurred in 12, 27, 20, and 10 cases, respectively. The cumulative probabilities of the liver-related morbidity were not significantly different between the groups. HCC occurred in 9 patients of the CC group in contrast to 53 patients of the controls (HCV: 36 patients, HBV: 17 patients) (Figure 2A). Although the cumulative probability of HCC occurrence was lower in

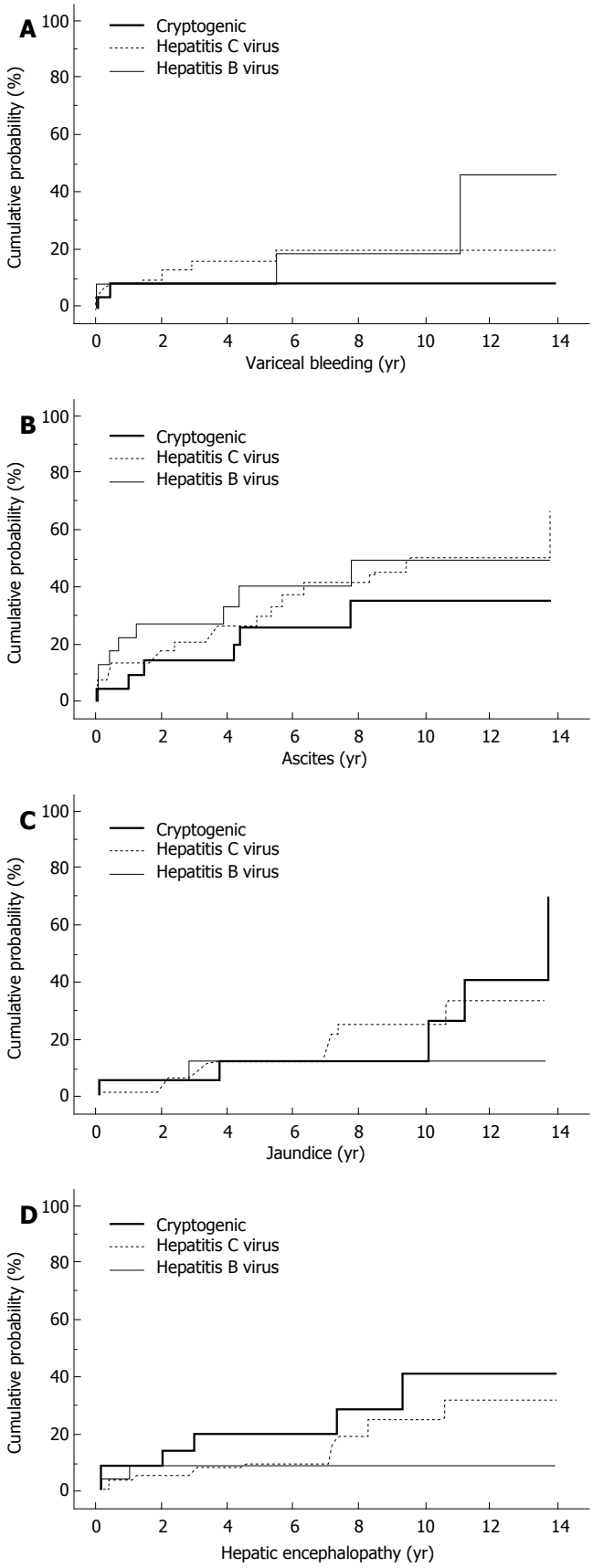

Figure 1 The cumulative probability of the liver-related symptoms for cryptogenic cirrhosis and viral cirrhosis using Kaplan-Meier's method and log-rank test.

the CC group than in the controls of viral etiology ($P<0.01$, CC *vs* HCV and CC *vs* HBV), HCC occurrence in the CC group rapidly increased 8 years after the observation. Of

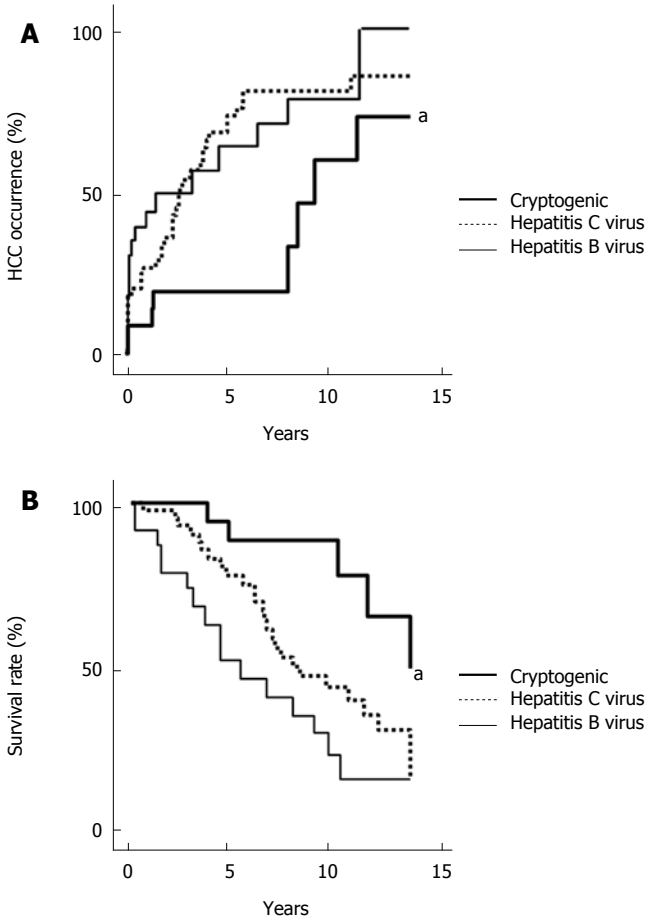

Figure 2 The cumulative probabilities of hepatocellular carcinoma and survival censoring non-liver related deaths for cryptogenic cirrhosis and viral cirrhosis. a: $P<0.01$, *vs* viral origin.

24 patients with CC, 3 died of liver failure and 2 of HCC (Figure 2B). No patient was associated with non-liver-related death. In the controls, 39 patients (24 HCV-related and 15 HBV-related) were associated with liver-related death (liver failure in 10 cases and HCC in 29 cases). The cumulative probability of survival censoring non-liver related deaths were higher in the CC group than in the controls ($P<0.05$, CC *vs* HCV, $P<0.01$, CC *vs* HBV).

DISCUSSION

Recent studies in the West have proposed that NASH may be an under-recognized cause of CC[8-10]. Although obesity is the key component of the association between CC and NASH, whether this association can be true even in the region with the low prevalence of obesity is unknown. The World Health Organization defined obesity as BMI $\geqslant$ 30[15], but in Japan, the prevalence of the population with such BMI is no more than 2%-3% in contrast to the 20%-30% in the West[16-18]. Moreover, the medical examination in 2002 ($n=6360$) in the local district where this study was performed showed that the prevalence of BMI $\geqslant$ 30 was 2.5% (data not published). We, therefore, investigated the clinical features of CC focusing on the association with NASH in Japan. This study demonstrated that in Japan, obesity, diabetes mellitus, and hypertriglyceridemia were more frequent, and insulin resistance, which is closely associated with the pathogenesis of NASH was greater in the CC group than in the controls of viral etiology. These findings are similar to those in the West, indicating that CC has the similar clinical features to NASH regardless of the prevalence of obesity.

The Japanese people as a race are known to suffer from obesity-related disorders even with a mild excess of adiposity[16,19-21]. The definition of obesity is proposed as BMI $\geqslant$ 25 in Japan, because the obesity-related disorders increase with a BMI $\geqslant$ 25[16,19]. This study demonstrated that the prevalence of BMI $\geqslant$ 30 in the CC group was 16.7% in Japan in contrast to 47% in the West. The prevalence of BMI $\geqslant$ 25 in the CC group was 54.2%, which is similar to that of BMI $\geqslant$ 30 in the West[9]. Moreover, our patients with CC, despite of the low prevalence of obesity, were accompanied with diabetes mellitus in 54.2% and hypertriglyceridemia in 20.8%, which is also similar to the reports in the West[8,9]. Although the BMI in viral cirrhosis was also lower in Japan than in the West (22.4 ± 3.0 *vs* 25.1 ± 4.2), the prevalence of diabetes mellitus was similar in these regions (26.4% *vs* 32.0%)[9]. Considering that the NASH patients in Japan are not as obese as those in the West [21], Japanese people, even with mild obesity, may suffer from NASH which may progress to CC.

Many researchers have shown that excess visceral fat is more closely related to the risk of health problems than the BMI itself[22-23]. The contribution of visceral fat area is greater in Japanese in whom the degree of whole fat accumulation is not as severe as in the West. Our present study demonstrated an increased visceral fat deposit in the CC group as compared with viral cirrhosis group. Because the increased visceral fat deposit plays a role in the pathogenesis of NASH via a production of various adipocytokines from the visceral fat tissue[23,24], the larger visceral fat area in CC further supports the association between CC and NASH. On the other hand, the hepatic fat deposit reflected by the liver/spleen ratio was similar in CC and viral cirrhosis groups. The hepatic fat deposit may decrease in the cirrhotic stage because the sinusoidal capillarization impairs the movement of gut-derived lipoproteins into the hepatocytes and the porto-systemic shunt diverts blood-borne lipids away from the liver. In fact, loss of the hepatic fat deposit has been observed in serial biopsies of NASH patients with progression to cirrhosis[7]. Because the excessive steatosis of the hepatocytes induces oxidative stress leading to the death of hepatocytes, loss of the hepatic fat deposit may be associated with lower ALT levels in CC.

This study demonstrated the lower occurrence of HCC and the higher survival rate in the CC than in viral cirrhosis, indicating that CC may take an indolent clinical course. There is a wide variation in the carcinogenic risk of CC among previously studies. Caldwell *et al*[8] showed that HCC developed in one of 71 patients with CC (1.4%). Another group reported higher prevalence of HCC in the CC group than in HCV-related cirrhosis[10]. The reason for these controversial data is unclear, but it may be attributed to the difference of the observation period. Bugianesi *et al*[25] suggested a later onset of HCC in the CC, because the patients with the CC-associated HCC were older as compared with the other cirrhosis-associated HCCs. Interestingly, this study demonstrated that although the

cumulative probability of HCC occurrence was lower in the CC than in the viral cirrhosis, HCC occurrence in the CC rapidly increased 8 years after the observation, indicating the later increase of HCC in CC. The later occurrence of HCC in CC can not be attributed to age and sex difference or degree of liver damage, because of a case-control study with age-, sex-, and Child-Pugh score matched viral cirrhosis. Therefore, HCC occurrence may be a late complication of CC.

In conclusion, the features suggestive of NASH were more frequently observed in the CC patients than in the controls of viral etiology even in Japan where obesity is not prevalent. It indicates that NASH may be a possible cause of CC regardless of the prevalence of obesity. Moreover, HCC developed less frequently and its prognosis was less severe in the CC group, indicating that CC may take an indolent clinical course. Further larger studies are necessary to understand the clinical features of CC.

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RAPID COMMUNICATION

Safety advantage of endocut mode over endoscopic sphincterotomy for choledocholithiasis

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Abstract

AIM: To evaluate whether an automatically controlled cut system (endocut mode) could reduce the complication rate of endoscopic sphincterotomy (EST) and serum hyperamylasemia after EST compared to the conventional blended cut mode.

METHODS: From January 2001 to October 2003, 134 patients with choledocholithiasis were assigned to either endocut mode group or conventional blended cut mode group at the time of sphincterotomy. The two groups were retrospectively compared for the complications after EST and serum amylase level before and 24 h after the procedure.

RESULTS: Of the 134 patients treated, 79 were assigned to conventional blended cut mode group and 55 to endocut mode group. There was no significant difference in age, sex, and serum amylase level before EST between the two groups. Complications were found in 5 patients of the endocut mode group (9%): hyperamylasemia (5 times higher than normal) in 4 and moderate pancreatitis in 1. Complications were found in 13 patients of the conventional blended cut mode group (16%): hyperamylasemia in 12 and moderate pancreatitis in 1. Serum amylase levels were elevated in both groups 24 h after EST ($P < 0.02$). The average serum amylase level 24 h after EST in the conventional blended cut mode group was significantly higher than that in the endocut mode group ($P < 0.05$).

CONCLUSION: Endocut mode offers a safety advantage over conventional blended cut mode for pancreatitis after EST by reducing hyperamylasemia.

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Key words: Endocut mode; Endoscopic sphincterotomy; Choledocholithiasis

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INTRODUCTION

The main complications of endoscopic sphincterotomy (EST) are pancreatitis, hemorrhage, perforation and sepsis[1-4]. Acute pancreatitis is still the most common complication associated with the procedures. Testoni *et al*[5] have shown that pancreatitis is associated with the elevation of serum amylase level 24 h after ERCP/EST.

An automatically controlled cut system, endocut mode (ICC 200 ERBE), can reduce the danger of papillary hemorrhage but may lead to pancreatitis[6]. This study was to evaluate whether the endocut mode could reduce the complication rate of EST and serum hyperamylasemia after EST compared to the conventional blended cut mode.

MATERIALS AND METHODS

From January 2001 to October 2003, 134 patients admitted to Aso Iizuka Hospital due to choledocholithiasis were assigned to either endocut mode group or conventional blended cut mode group at the time of EST. Three gastroenterologists performed the procedure. An Olympus UES-10 electrosurgical generator (Olympus, Japan) was used for conventional cut with the blended current set at output limit 30W and the coagulation current set at output limit 15W. An Erbe ICC200 (Erbe, Germany) was used for endocut with the effect 3 current set at output limit 120W and forced coagulation current set at output limit 30W.

The two groups were retrospectively compared for the complications, pancreatitis, hemorrhage after EST and

Table 1 Age, sex, and serum amylase level before EST

	Number of patients	Yr	Male (%)	Female (%)	Amylase (IU/L)
Conventional cut group	79	73.5±1.3	54	46	102±8
Endocut group	55	70.8±1.8	56	44	101±18

Table 2 EST complications

	Hyperamylasemia (> 5 times upper normal limit)	Pancreatitis
Conventinal cut group	12	1
Endocut group	4	1

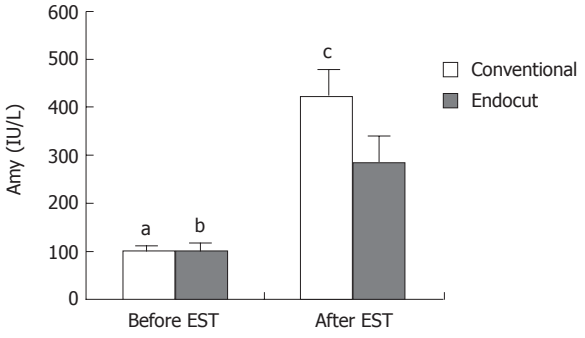

Figure 1 Average serum amylase level before and after EST. Data are expressed as mean ± SE. $^{a}P < 0.02$ *vs* conventional cut group after EST; $^{b}P < 0.02$ *vs* endocut group; after EST; $^{c}P < 0.05$ *vs* endocut group after EST.

serum amylase level before and 24 h after the procedure. Pancreatitis was defined when CT grade was higher than grade II (local pancreas swelling). A bleeding complication was defined when the patient required blood transfusion or had a drop in hematocrit level greater than 5%.

Results were presented as mean ± SE. Complications in the two groups were evaluated using χ^2 test and Fisher's exact test. Differences in serum amylase levels between the two groups were evaluated by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

One hundred and thirty-four patients were evaluated in this study. Seventy-nine patients underwent conventional blended cut and 55 patients underwent endocut. There were no significant differences in age and sex distribution as well as serum amylase level before EST (Table 1).

Complications were found in 5 patients of the endocut mode group (9%): hyperamylasemia in 4 (serum amylase level was 5 times higher than normal) and moderate pancreatitis in 1 patient. On the other hand, complications were found in 13 patients of the conventional blended cut mode group (16%): hyperamylasemia in 12 patients and moderate pancreatitis in 1 patient (Table 2). One patient in each group had mild bleeding not showing hematocrit decrease. No major complications such as perforation were found in both groups. There were no significant differences in the incidence of complications such as pancreatitis, hemorrhage, and hyperamylasemia between the two groups.

Serum amylase levels were elevated in both groups 24 h after EST ($P < 0.02$). The average serum amylase level 24 h after EST in the conventional blended cut mode group was significantly higher than that in the endocut mode group ($P < 0.05$, Figure 1).

DISCUSSION

Since the introduction of EST, the indications for the procedure have grown steadily. The complications of EST are similar to those of diagnostic ERCP but occur more frequently. Freeman *et al*[3] have shown that the incidence of pancreatitis after EST is 5.4% and significant risk factors for pancreatitis are sphincter of Oddi dysfunction, younger age, the number of pancreatic contrast injections, precut sphincterotomy, and difficulty of cannulation. Elta *et al*[7] reported that the type of electrocautery current affects this risk and found that the use of pure cut current is associated with a lower incidence of pancreatitis rather than blended current.

A new high-frequency current generator equipped with an automatically controlled system (endocut mode) has been currently used in EST. With endocut mode, each interval of cutting is automatically triggered by the initial electric arc sensor within the ICC 200 and is thus reproducible in duration. The automatically-fractionated cut and controlled cutting speed can prevent perforation of the major papilla[6].

In this study, we observed the most common complications such as moderate pancreatitis and mild hemorrhage in both groups, which are consistent with the other reports[3,4,6,7]. The endocut mode could reduce hemorrhage after EST[6,9-10], but leads to pancreatitis[6]. On the other hand, Ellahi *et al*[11] reported that the endocut mode does not offer a safety advantage over the conventional blended cut mode. In this study, there were no significant differences in the incidence of complications such as pancreatitis and hemorrhage between the two groups. The rate of hyperamylasemia (serum amylase level was 5 times higher than normal) was 7% in the endocut mode group and lower than 15% in the conventional blended cut mode group. But there was no significant difference in the incidence of hyperamylasemia between the two groups. In this study, the number of patients might be too small to have a statistical significance as for the incidence of complications.

A larger study is needed for the evaluation of usefulness about the endocut mode in EST, because the number of patients was less than 150 in this and other studies[6,9-11]. It was reported that the endocut mode reduces hemorrhage after EST[8]. Our results showed that the average serum amylase level 24 h after EST in the endocut mode group was significantly lower than that in the conventional blended cut mode group ($P < 0.05$), which is consistent with study of Shinozuka *et al*[9]. In this study, in comparison with a conventional blended cut mode

unit, all the examiners noted the smooth, easy EST by the endocut mode unit. Thus it might shorten the procedure time, lower the risk of major papilla's edema as well as the number of contrast injections and the serum amylase level after EST.

The endocut mode offers a safety advantage over conventional blended cut mode for pancreatitis after EST by reducing the hyperamylasemia.

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RAPID COMMUNICATION

Reduction of virus burden-induced splenectomy in patients with liver cirrhosis related to hepatitis C virus infection

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Abstract

AIM: To examine the hepatitis C virus (HCV) levels and immunological markers in cirrhotic patients after splenectomy.

METHODS: HCV RNA titers as well as cellular and humoral immune markers were determined in 20 cirrhotic patients after splenectomy and in 32 cirrhotic controls with an intact spleen.

RESULTS: Serum HCV RNA titers were lower in the splenectomized patients than in the controls ($186 \pm 225 \times 10^3$ copies/mL *vs* $541 \pm 417 \times 10^3$ copies/mL, $P < 0.01$). HCV RNA was judged to have been spontaneously eradicated in 4 splenectomized patients, but in none of the controls. Natural killer cell activity was higher in the splenectomized patients than in the controls (41.2 ± 19.3% *vs* 24.7 ± 15.3%, $P < 0.01$), and natural killer cell activity was negatively correlated to HCV RNA titers in the splenectomized patients except in those with serotype 2-related infection. The CD4/CD8 ratio was significantly lower in the splenectomized patients than in the controls.

CONCLUSION: The findings suggest that splenectomy may diminish virus burden in cirrhotic patients with HCV infection at least in part, through augmentation of natural killer cell activity.

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Key words: Hepatitis C virus; Liver cirrhosis; Natural killer cell; Splenectomy

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INTRODUCTION

Persistent infection with hepatitis C virus (HCV), a parenterally transmitted RNA virus, occurs in 70%-80% of HCV-infected patients. Chronic hepatitis C progresses gradually to liver cirrhosis, a condition frequently associated with hepatocellular carcinoma (HCC)[1-3]. To prevent progression to chronic liver disease, continuous virus burden must be interrupted. However, spontaneous elimination of the virus load rarely occurs[4-8]. HCV-specific cytotoxic T cells are thought to play a principal effector role in host defense against HCV infection[9-11]. In addition, natural killer (NK) cells are believed to participate in the defense against hepatitis viruses, because the human liver contains a significantly higher number of NK cells than peripheral blood or any other organs[12]. NK cells exert various effector functions during the early phase of HCV infection, including induction of apoptosis and production of IFN-gamma and TNF-alpha[13-16]. In addition, NK cells are suggested to play a crucial role in the clearance of HCV in patients undergoing interferon therapy[17,18], but the significance of NK cells in the pathogenesis of chronic hepatitis C remains to be clarified. Although several studies have reported reduced activity of NK cells in cirrhotic patients with a history of alcohol abuse[19,20] and complication of HCC[21-23], little is known whether NK cells vary in patients with HCV-positive liver cirrhosis[24,25].

Splenectomy was a popular surgical procedure for esophageal varices associated with liver cirrhosis until endoscopic sclerotherapy has become the first line treatment[26,27]. Splenectomy may compromise the immune system because the spleen plays an important role in phagocytosis and antibody production[28,29]. On the other hand, there is some evidence that splenectomy can positively affect Kupffer cell functions in the liver, and protects against viral infection[30-33]. Ferrante *et al*[32] have insisted that a compensatory increase in the activity of NK cells in splenectomized patients might offer protection against infection and malignant disease. Pereira *et al*[31] reported that the HCV genome is detectable in spleen specimens obtained from HCV antibody seropositive patients associated with chronic schistosomiasis, suggesting that the spleen is an extrahepatic reservoir of the virus. Splenectomy may therefore influence the immune system and virus load of cirrhotic patients with HCV infection. Changes in immune

mediators and HCV burden in cirrhotic patients after splenectomy remain to be evaluated. In the present study, we retrospectively evaluated the effect of splenectomy on replication of HCV as well as cellular and humoral immunity including NK cell activity and lymphocyte subsets in cirrhotic patients with HCV infection.

MATERIALS AND METHODS

Patients

Twenty Japanese cirrhotic patients positive for anti-HCV antibody after splenectomy were enrolled in the present study (splenectomized). Splenectomy was performed in combination with surgical treatment of esophageal varices (14 cases), HCC (5 cases), or gastric cancer (1 case). The patients were referred for follow-up at our hospital. The mean duration of follow-up after splenectomy was 9.2 years. Thirty-two HCV-positive cirrhotic patients with an intact spleen served as controls and were followed up at our hospital. All controls had complications of esophageal varices, 17 of whom were treated for esophageal varices with endoscopic sclerotherapy and/or band ligation. Morphological diagnosis of post-hepatitic cirrhosis was performed in all splenectomies and 22 controls. The remaining 10 controls were diagnosed clinically based on typical clinical and laboratory findings of liver cirrhosis and characteristic liver findings at computed tomography and ultrasonography, because 6 patients showed complicated severe coagulopathy and 4 patients refused liver biopsy. No patients were diagnosed with chronic liver diseases, such as alcoholic hepatitis, autoimmune hepatitis, and chronic hepatitis B infection, or drug-induced liver disease. Patients with a history of alcohol abuse or diagnosed as HIV positive were excluded. None of our patients underwent anti-viral therapy using interferon during the period studied. Patient characteristics are shown in Table 1. Clinical features, severity of liver cirrhosis according to Child' s classification, association of HCC, HCV serotype, and liver function tests did not differ significantly between the two groups. Platelet counts were significantly higher in the splenectomized patients than in the controls. The study protocol was approved by the Ethics Committee of the Department of Internal Medicine, and informed consent was obtained from all patients.

Serum HCV RNA levels were measured to evaluate the effect of splenectomy on viral load. In addition, peripheral activity of NK cells, proportion of CD4 and CD8 subsets of T cells, and levels of serum β2-microglobulin and soluble interleukin 2 receptor (sIL2R) were examined.

Virology

The presence of HCV antibody was determined by the second and/or third generation of enzyme-linked immunosorbent assay (Ortho Diagnostic System Co., Ltd., Tokyo, Japan) and confirmed by recombinant immunoblot assay (Chiron RIBA-2 and/or RIBA-3). HCV serotype was examined by serotyping assay (SRL Laboratory Co., Tokyo, Japan) according to the method of Tsukiyama-Kohara *et al*[34]. Serotype 1 corresponded to types 1a and 1b, while serotype 2 corresponded to types 2a and 2b of the Simmonds classification[35]. Quantitative levels of HCV-RNA in serum samples were analyzed by combined reverse transcription PCR assay (Amplicor-HCV monitor; Nippon Roche, Tokyo, Japan) that could detect viral concentrations above 10^3 copies per mL[36]. If serum HCV-RNA was undetectable by this assay, we performed the Amplicor hepatitis C viral test twice, which is more sensitive and can detect as low as 10^2 copies per mL[37]. If HCV RNA was still undetectable, it was judged to indicate 'virus eradication'.

Table 1 Characteristics of cirrhotic patients with HCV infection

	Splenectomy (+)	Splenectomy (-)
Age (yr)	62.6±5.5	63.2±13.6
Gender (m:f)	8:12	13:19
HCV Serotype		
1	15	29
2	3	3
unknown[a]	2	0
Child's Classification		
A	11	18
B	6	8
C	3	6
Association of HCC	7/20	9/32
Total bilirubin (mg/dL)	1.2±0.5	1.2±0.7
Albumin (g/L)	35±6	37±4
γ-globulin (g/L)	23±0.8	20±5
AST (IU/L)	65.3±30.5	61.8±23.7
ALT (IU/L)	31.5±9.1	25.1±4.9
Platelets ($\times 10^4$ /μL)	15.2±4.5	8.1±2.7[b]
NK cell activity (%)	41.2±19.3	24.7±15.3[b]
CD4/CD8	1.2±0.4	1.6±0.6[c]
β2-MG (mg/L)	2.5±1.2	2.4±0.6[n.s]
sIL2R (U/mL)	917±445	842±190[n.s]

HCC: hepatocellular carcinoma; AST: aspartic aminotransferase; ALT: alanine aminotransferase; NK cells: natural killer cells; β2-MG: β2-microglobulin; sIL2R: soluble interleukin 2 receptor. a: undeterminable serotype; [b]$P<0.001$, [c]$P<0.05$ *vs* splenectomized patients; NS: no significant difference.

Immunological markers

NK cell activity was assessed against the k-562 cell line (Dainippon Pharmaceutical Co., Osaka, Japan) marked with ^{51}Cr using a cytotoxicity test for 3.5 h[38]. Blood samples taken from the cubital vein were collected into heparinized tubes. After centrifugation of the blood sample with a lymphocyte separation medium (Limphosepar I; Tokyo, Japan), the interface mononuclear cells were collected and suspended at a cell density of 1×10^6 /mL in RPMI-1640 medium (IBL; Gunma, Japan) and supplemented with 10% FBS (Cansera; Ontario, Canada). Peripheral blood monocytes (2×10^5 cells) were added to round-bottomed 96-well microplates containing ^{51}Cr-labeled target cells (1×10^4 cells) in 0.2 mL of RPMI-1640 medium supplemented with 10% FBS. The effector cell/target cell ratio was determined as 20. After centrifugation at 800 r/min for 5 min using an exclusive centrifuge for microplates, the cells were incubated for 3.5 h at 37℃ under 50 mL/L CO_2 in air. After incubation, the culture supernatant was harvested using PETΣ-96 (Sohken; Tokyo, Japan), and the radioactivity was determined using a gamma counter (1272 clini gamma, Wallac; Turuku, Finland). The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (experimental ^{51}Cr release - spontaneous ^{51}Cr release) /(maximal ^{51}Cr release-spontaneous ^{51}Cr release) × 100.

The proportion of peripheral CD4 and CD8 subsets

Table 2 Characteristics of cirrhotic patients after splenectomy

	Age	Sex	Years After splenectomy	Serotype	HCV RNA (k copies/mL)	Child's classification	association of HCC[1]
1	75	Female	19	1	23	C	no
2	60	Female	16	Unknown[2]	Negative	A	no
3	80	Female	15	1	140	A	no
4	70	Female	15	1	130	B	no
5	63	Male	14	1	2	B	yes
6	67	Female	12	1	460	A	no
7	62	Male	12	1	Negative	A	no
8	50	Male	12	1	Negative	A	no
9	73	Female	11	Unknown[2]	Negative	A	no
10	67	Female	11	2	<1	B	no
11	50	Male	10	1	480	C	no
12	60	Female	7	1	140	B	yes
13	56	Female	7	1	160	C	yes
14	38	Male	6	1	770	A	no
15	68	Male	5	1	210	B	yes
16	73	Female	2	1	510	A	no
17	66	Female	2	2	21	A	no
18	59	Female	2	1	420	A	yes
19	58	Male	1	2	14	B	yes
20	57	Male	1	1	380	A	yes

[1] Hepatocellular carcinoma; [2] undeterminable serotype.

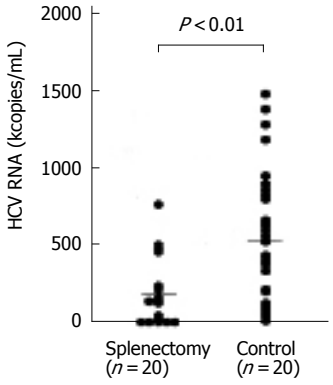

Figure 1 Serum HCV RNA titers in patients with liver cirrhosis. Splenectomized patients had significantly lower titers of HCV RNA than cirrhotic controls with an intact spleen.

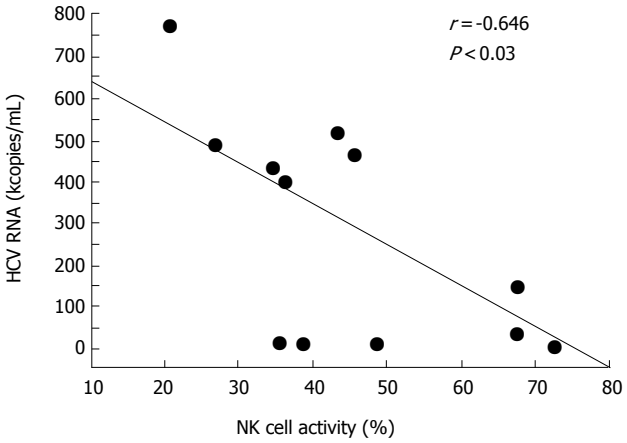

Figure 2 Relationship between HCV RNA titers and NK cell activity in non-serotype 2 splenectomized patients. There was a significantly negative correlation between HCV RNA titers and NK cell activity in non-serotype 2 splenectomized patients.

was assayed by flow cytometry. β2-microglobulin levels were measured by latex immunoaggregation assay (Eiken Co., Tokyo, Japan). sIL2R levels were determined using the cell-free IL-2R EIA kit.

Statistical analysis

Results were expressed as mean ± SD. Statistical analyses were carried out using the computer program StatView-J4.5 (Abacus Concepts, Inc. Berkeley, USA). Mean quantitative values were compared using Student's *t* test. Nonparametric data were compared using the Mann-Whitney U-test. All *p*-values were two-tailed. $P < 0.05$ was considered statistically significant.

RESULTS

HCV RNA titers (Figure 1)

Mean titers of HCV RNA were significantly lower in the splenectomized patients than in the cirrhotic controls ($186 \pm 225 \times 10^3$ copies/mL *vs* $541 \pm 417 \times 10^3$ copies/mL, $P < 0.01$). Among the serotype 1-related patients (15 splenectomized and 29 controls), the splenectomized patients showed a reduced viral load compared with the controls ($246 \pm 231 \times 10^3$ copies/mL *vs* $590 \pm 407 \times 10^3$ copies/mL, $P < 0.01$). Four (20.0%) of 20 splenectomized patients showed eradicated HCV in serum after 11 years of splenectomy in one patient, 12 years in two patients and 16 years in one patient. Two of the 4 patients had serotype 1-related HCV infection, while the serotype was undetermined in the other 2 patients (Table 2). No controls spontaneously eradicated HCV in their serum. Among the splenectomized patients with serotype 1, HCV RNA levels were lower, but not significantly lower in patients with a longer follow-up time (≥10 years) than in those with a shorter follow-up time (<9 years; $155 \pm 202 \times 10^3$ copies/mL *vs* $350 \pm 531 \times 10^3$ copies/mL, $P = 0.103$). No gender-related difference in virus load was observed in controls with serotype 1 (12 males: $649 \pm 469 \times 10^3$ copies/mL *vs* 17 females: $521 \pm 329 \times 10^3$ copies/ mL, $P = 0.43$).

Immunological markers (Table 1)

Peripheral activity of NK cells was significantly higher in the splenectomized patients than in the controls. In both splenectomized patients and controls, NK cell activity was lower in patients complicated by HCC than in those without HCC, although the difference was not significant [HCC (+): 33.6%±11.8%, HCC (-): 47.6%±18.1%, $P=0.16$]. NK cell activity in LC patients with an intact spleen was not significantly different from that in healthy subjects (data not shown).

The splenectomized patients showed a reduced proportion of CD4 cells, a similar proportion of CD8 cells and a significantly lower ratio of CD4/CD8 compared with those of the controls. Serum sIL2R and β2-microglobulin values were similar in both groups.

Relationship between HCV RNA levels and immunological markers

In the splenectomized patients without serotype 2-related infection, there was a significantly negative correlation between HCV RNA titers and NK cell activity (Figure 2). Such a relationship was not found in the controls. No relationships were found between HCV RNA levels and CD4/CD8 ratio, sIL2R, or β2-microglobulin.

DISCUSSION

This was the first retrospective study to examine the effect of splenectomy on the reduction of HCV in cirrhotic patients. We found that HCV RNA titers were significantly lower in cirrhotic patients after splenectomy than in cirrhotic controls with an intact spleen, indicating that splenectomy can reduce virus burden. The mechanisms through which splenectomy helps to reduce HCV remain to be determined, but a few possibilities have been suggested. One explanation for reduced viral load after splenectomy is that it might be immunologically based. The role of NK cells in controlling HCV replication remains obscure, but NK cells hold the potential to play a vital role in controlling HCV replication in hepatic cells using IFN-gamma-dependent mechanism[13]. Reports of the effect of splenectomy on the peripheral activity of NK cells are contradictory[27-31]. In the present study, HCV-positive cirrhotic patients splenectomy had the augmented activity of NK cells compared with those with an intact spleen. An inverse correlation between HCV RNA titers and NK cell activity was shown in the splenectomized patients, but not in the controls with an intact spleen. In addition, Ikuta *et al*[39] reported that liver mononuclear cells from splectomized mice produce a significantly larger amount of IFN-gamma than those from sham-operated mice. Taken together, increased NK cell activity post-splenectomy may reduce virus burden in HCV-positive cirrhotic patients.

The association of cirrhosis with HCC[21-23], cholestasis[40], and protein calorie malnutrition[20] is also important because these factors may influence the peripheral activity of NK cells in cirrhotic patients. Similar to previous reports, our data showed that cirrhotic patients complicated by HCC had a lower NK cell activity than those without HCC. The incidence of complication of HCC was slightly but not significantly higher in the splenectomized patients (35%) than in the controls (28%). Total bilirubin levels and severity of liver cirrhosis according to Child's classification were similar between the splenectomized patients and the controls. Accordingly, these biochemical and physiological factors did not cause increased activity of NK cells in patients after splenectomy.

Splenectomized patients with a longer follow-up time (≥10 years) had lower HCV RNA levels than those with a shorter follow-up time (9< years), although the difference was not significant. Further research should attempt to determine whether the lower HCV titers of patients with a longer follow-up time are due to the natural course of infection. To estimate the relevance of aging to virus load, we examined the changes in HCV levels over a mean period of 4.5 years in 20 patients with HCV-positive cirrhosis. The mean HCV RNA titers did not vary significantly during this period (baseline, 6.6±9.2 Meq/mL; after 4.5 years, 6.1±8.5 Meq/mL). Kato *et al*[41] reported that the amount of HCV RNA tends to increase as the duration of infection increases. These findings indicate that the virus load of splenectomized patients does not spontaneously reduce with age.

Interestingly, four splenectomized patients showed spontaneous eradication of HCV, whereas cirrhotic controls showed no such spontaneous clearance during the observation period. This is in contrast to the findings that spontaneous elimination of virus load rarely occurs in patients with persistent HCV infection and chronic liver disease[4-8]. To date, little attention has been focused on identifying the viral and host factors involved in the spontaneous disappearance of serum HCV RNA, because the time point of infection is unknown for many patients, which makes a prospective evaluation of long-term outcome of infection problematic. Evidence for factors related to the natural disappearance of HCV has been reported by Furuzaki *et al*[8]. HCV levels below 1.0 Meq/mL can contribute to natural clearance of the virus. Thus, reduced virus load caused by activated NK cells may have led to HCV eradication in four of the splenectomized patients.

The present findings suggest that splenectomy may diminish or remit virus burden in HCV-positive cirrhotic patients at least in part by increasing NK cell activity. However, simply because four HCV-eradicated patients underwent splenectomy before a method for HCV RNA assay was developed, it would be rash to conclude that splenectomy can lead to their clearance of HCV. Furthermore, influence of surgery for esophageal varices and HCC on virus load cannot be ruled out. A prospective study on the effect of splenectomy on HCV replication should be undertaken in order to clarify whether splenectomy may reduce virus burden and/or eradicate HCV in serum of HCV-positive cirrhosis.

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RAPID COMMUNICATION

Endoprosthesis implantation at the pharyngo-esophageal level: Problems, limitations and challenges

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Abstract

AIM: To present our experience with endoscopic placement of an esophageal endoprosthesis in 19 patients.

METHODS: A retrospective evaluation was made for the use of 19 stents positioned at the level of the cervical esophagus: 11 for malignant tumours (7 causing obstruction, 4 complicated by an esophago -tracheal or -cutaneous fistula), and 8 for an acquired benign tracheo-esophageal fistula due to prolonged intubation. The covered Ultraflex stent was used in all cases except two. These two patients had an esophagocutaneous fistula following laryngectomy and a Flamingo Wall stent was used.

RESULTS: Stent implantation was technically successful in all patients. Dysphagia score was improved from 3 to 2 in stenosis patients, while sealing of the fistula was achieved in all cases. The median hospital stay was 3 d for malignant tumour patients and 13.5 d for esophagocutaneous fistula patients. One Ultraflex stent and two Flamingo Wall stents were easily removed 33 d and 3 months respectively after implantation when the fistulas had totally occluded.

CONCLUSION: Endoprosthesis implantation for malignancy and/or fistula of malignant or benign origin at the level of the cervical esophagus is an easy, well tolerated, safe and effective procedure with no complications or mortality.

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Key words: Cervical endoprosthesis; Pharyngo-esophageal stenosis; Dysphagia; Esophageal carcinoma; Esophagotracheal fistula

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INTRODUCTION

The insertion of an endoprosthesis is one of the most acceptable means of palliative treatment of patients with obstructing esophageal lesions and/or an existing esophagorespiratory fistula. However, the proximity to the cricopharyngeal sphincter is traditionally regarded as a relative contraindication, because of the potential problems of persistent foreign body sensation, pain, odynophagia, compression of the trachea or proximal migration of the prosthesis[1-3].

Recently, this traditional view has begun to change, as witnessed by an augmented number of case reports or small series of data presentations[1,2,4-11]. In such patients, with the inability to swallow even their own saliva at times, palliative intubation aimed at relief of dysphagia, maintenance of nutrition and avoidance of respiratory complications is the primary treatment goal, while the close relationship between the cricopharynx and the cervical lesion continues to be a challenge for every endoscopist.

Herein, we present our experience with stent placement in the cervical esophagus at the level of hypopharynx and/or the upper esophageal sphincter.

MATERIALS AND METHODS

Patients

Over the last 10 years (1995-2004), 19 patients were referred to our department to be treated endoscopically for a lesion in the cervical esophagus. There were 11 patients with a malignancy and 8 patients who spontaneously developed tracheo-esophageal fistula [TEF] after prolonged tracheal intubation for mechanical ventilatory support in the ICU.

The eleven carcinoma patients (9 males and 2 females) with a median age of 70 years (range: 62 - 82 years) suffered from: laryngeal (3 cases), hypopharyngeal (2 cases) and esophageal inlet carcinoma (2 cases), all causing severe stenosis and obstruction, two cases of high esophagotracheal fistula, one due to radical thyroidectomy and concomitant radiotherapy and the other due to an inoperable hypopharyngeal carcinoma and two cases of esophagocutaneous fistula after total laryngectomy for carcinoma, performed postirradiation (Table 1).

The seven patients with obstructing-type cancer

Table 1 Data of carcinoma patients

	Age	Gender	Disease
1	64	Male	Laryngeal carcinoma causing obstruction
2	72	Male	Laryngeal carcinoma causing obstruction
3	75	Male	Laryngeal carcinoma causing obstruction
4	65	Male	Hypopharyngeal carcinoma causing obstruction
5	68	Male	Hypopharyngeal carcinoma causing obstruction
6	70	Female	Esophageal inlet carcinoma causing obstruction
7	80	Male	Esophageal inlet carcinoma causing obstruction
8	62	Female	Esophagotracheal fistula after thyroidectomy + radiotherapy
9	64	Male	Esophagotracheal fistula after hypopharyngeal carcinoma
10	82	Male	Esophagocutaneous fistulas after total laryngectomy + radiotherapy
11	72	Male	Esophagocutaneous fistulas after total laryngectomy + radiotherapy

Table 2 Data of benign disease patients

	Age	Gender	Underlined disease
1	21	Male	Multiple trauma
2	73	Female	Multiple trauma [in septic state]
3	76	Male	Cerebral hemorrhage
4	56	Male	Cerebral hemorrhage
5	47	Female	Post-operative complications [in septic state]
6	70	Female	Post-operative complications
7	72	Male	Post-operative complications [in septic state]
8	72	Male	Post-operative complications [in septic state]

presented with a median dysphagia score of 3 (unable to swallow liquids) ranging from 2 to 4. Under diazepam-induced conscious sedation they were subjected to a Savary bougie progressive dilatation of the stenosis for one to two sessions depending on the rigidity of the tumour. Dilatation was performed over a guide wire which was advanced into the stenosis through the endoscope up to the point of 12.8 mm in diameter, to facilitate the rapid expansion of the stent.

After dilatation the endoscope was advanced to the tumour site. The total length of the stenosis and the distance of the tumor upper orifice from the incisor teeth, were carefully recorded due to their great importance for the correct placement of the stent.

The remaining 4 patients had no need for dilatation, thus immediately following insertion of the endoscope and inspection of the tumourous fistulae, the exact distance of the most proximal end of the lesion from the incisors and the total length of the lesion were recorded as above.

Eight patients had benign TEF (5 males and 3 females with a median age of 71 years, range 21-76 years) due to multiple trauma (2 cases), extended cerebral hemorrhage (2 cases) and post-operative complications (4 cases) implicating cardio-respiratory insufficiency. All patients, previously subjected to a percutaneous endoscopic gastrostomy for feeding, had an overall median intubation time of 30 d (range 15 - 80 d) and had been subjected to percutaneous tracheostomy a median of 16 d (range, 5-62 d) before diagnosis of TEF. All were characterized by poor prognosis and 4 of them were in a septic state (Table 2).

The final diagnosis of TEF was made by esophagoscopy, during which the exact characteristics of the fistula, i.e. size and its relationship with the upper esophageal sphincter as well as the distance of the most proximal end of the lesion from the incisors, were carefully recorded.

Stent placement

A self-expandable, covered -proximal release type-Ultraflex stent [Microvasive, Boston Scientific Corp., Natick, Mass] with a proximal flare of 28 mm, a body diameter of 23 mm, and a length of 120 mm or 150 mm, was used in all cases except two patients with esophagocutaneous fistulae after laryngectomy. In these cases, a Flamingo Wall stent [Microvasive Endoscopy, Boston Scientific Corp., Natick, Mass] with a proximal diameter of 24 mm, distal diameter of 16 mm, and length of 120 mm, was considered the most suitable for fistula sealing.

All stents were placed over a guide wire and no fluoroscopy was used in any case. Patients with malignant lesions were treated under diazepam-induced conscious sedation; benign TEF patients were under mechanical ventilatory support due to the underlying illness and had no need for supplementary anesthesia. The whole procedure was performed at the bedside in the ICU.

The stent delivery catheter was passed over the pre-inserted guide wire and advanced so that the proximal end of the stent was at the estimated distance from the incisor teeth. Generally, all stents were gradually deployed in such a position so that at least 2 cm of the prosthesis was over both sides of the lesion. However, our landmark was the upper esophageal sphincter. The stent was thus deployed to achieve the minimal harmful sensation with maximum security regarding stent migration as well as early overlapping by tumour overgrowth, i.e. the upper end of all stents was just within the upper esophageal sphincter or at the hypopharynx.

After stent insertion, its proper position was controlled by endoscopy and under direct vision. In some cases x-ray imaging was additionally performed (Figures 1 and 2).

RESULTS

Prosthesis implantation was technically successful in all patients and there was no procedure-related mortality. No complications occurred and no patient experienced severe pain at the site of stent placement, lasting more than 24 h and needed narcotic analgesics. Dysphagia score was improved from a median value of 3 (range, 2-4) to

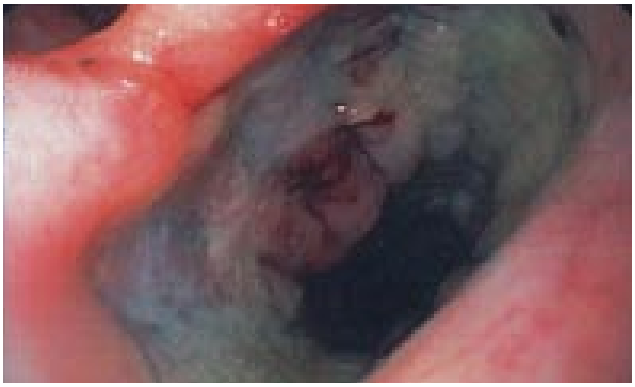

Figure 1 Endoscopic view of the soft funnel of the Ultraflex stent placed in the hypopharynx, posterior to the larynx. The stent was partially compressed in the antero-posterior direction at the level of arytenoid cartilages.

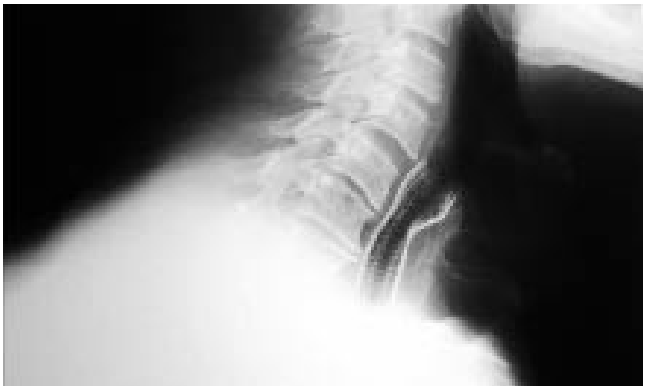

Figure 2 Plain radiograph of Ultraflex stent *in situ*, shows the fully expanded stent. Its proximal edge slightly protrudes in the hypopharynx (posterior endplate of C4).

a value of 2 (range,1-3) in esophageal stenosis patients. Fistula sealing was achieved in all cases, both benign and malignant (Table 3).

Regarding foreign body sensation, all conscious patients could well tolerate the stent placement in the first few days with no further difficulties. The two patients with esophagocutaneous fistula stented by Flamingo tubes experienced a foreign body sensation when the head/neck was bent since the proximal end of the stent was at the level of the mesopharynx, easily visible through the mouth opening (Figure 3). Both stents remained in place for three months until the fistula was totally closed, and were then removed by being grasped with retrieval forceps and pulled out without difficulty.

The median hospital stay was 3 d (range 2 - 4 d) for the 9 patients with stenosis and/or malignant esophagotracheal fistula. The other two patients with esophagocutaneous fistula remained hospitalised for 12 and 15 d, respectively due to cutaneous trauma debridement. Seven out of the eight TEF patients remained hospitalized in the ICU until their death, after 10 to 60 d, due to sepsis in 4, respiratory insufficiency in 2 and heart failure in one. The remaining patient, a 21-year-old multiple trauma victim, was weaned from a ventilator 33 d later and scheduled to be operated on for tracheoplasty. The Ultraflex stent was easily removed by simply grasping it with a pair of retrieval forceps under direct vision, just before the operation.

Table 3 Results after stent placement

Obstruction due to malignancy		**7 cases**
Stenting related mortality	0	
Improvement of dysphagia [score]	From 3 to 2	
Median hospital stay after stenting	3 d	
Malignant esophagotracheal fistula		**2 cases**
Stenting related mortality	0	
Sealing of fistula	2/2 (100%)	
Median hospital stay after stenting	3 d	
Malignant esophagocutaneous fistula		**2 cases**
Stenting related mortality	0	
Sealing of fistula	2/2 (100%)	
Median hospital stay after stenting	13.5 d	
Benign esophagotracheal fistula		**8 cases**
Stenting related mortality	0	
Sealing of fistula	8/8 (100%)	
Disease related mortality	7 cases	

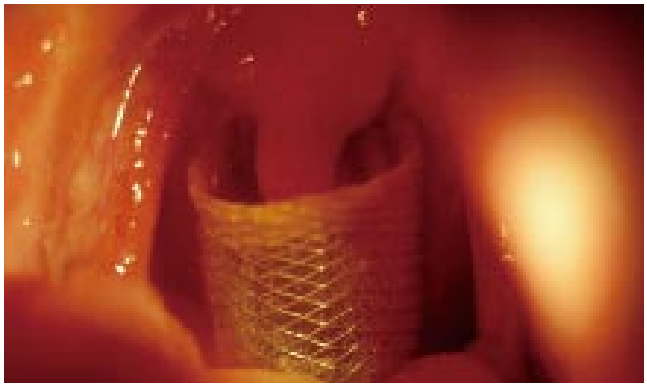

Figure 3 Direct view through the mouth opening of the upper part of the Flamingo stent placed temporarily at the cervical esophagus in the patient suffering from esophagocutaneous fistula after laryngectomy.

The 11 patients with malignancies were followed up every month. One patient with esophagocutaneus fistula died 7 months later, i.e. 4 months after fistula closure and stent removal. having developed total dysphagia due to recurrence of the disease. Unfortunately, this patient had totally refused a second stent; the second patient with esophagocutaneus fistula was alive 8 months after stenting. 5 months after fistula closure and stent removal, He is able to eat (dysphagia score 3) and no leakage has been reported; Seven patients remained alive for a median of 8 months (range 4 to18 months) and two patients were alive 10 months and 3 months after stenting. All these patients could eat semi-solid food and needed no other nutritional support.

DISCUSSION

The cervical esophagus is accepted as a segment between C6 at the pharyngoesophageal junction and the thoracic inlet at T1. It is endoscopically between 15 cm and 19cm from the incisor teeth and radiologically projects above the sternoclavicular joint[8,10]. At that level, any endoscopic procedure is more problematic even in the presence of a normal anatomical situation, since flexible endoscopy of the hypopharynx and upper esophageal sphincter is tech-

nically difficult, due to the reduced efficacy of insufflation and movements-related swallowing.

Generally, there is no report supporting placement of esophageal prosthesis for cervical lesions because of concerns about the increased risk of proximal migration of the stent into the hypopharynx and most importantly, the intolerable sensation of a foreign body[11,12]. However, there is no other acceptable means of palliating a terminal-stage tumor disease patient. This is because of the rapid decline of the patient's general condition upon the development of an esophagotracheal fistula due to aspiration pneumonia and malnutrition. On the other hand, as tumor stage is generally advanced and life expectancy is short, the major interest of any therapeutic procedure must be a rapid and successful palliation, ensuring acceptable quality of life, reducing the duration of hospital stay and cost.

There exists a general hesitation about applying stents in patients with benign diseases because of concerns regarding short-term complications and the absence of information regarding long term sequelae[3,13-15]. In our study, the patients with TEF of benign origin were all critically ill with a short term expectation of life if left untreated.

We question the risk of stent migration. In benign cases the risk is reported to be as high as 24%[15], which is probably related to a smaller mucosal surface area with less inward force anchoring the device. We experienced no migration in our cases, which might be attributed to the upper conical configuration of the stent, the external pressure induced by the cuff of the endotracheal tube, the constriction of the upper part of the stent by the upper esophageal sphincter, and mainly the absence of head-neck movements as well as swallow movements, due to the deep sedation of the patients. Moreover, the small number of patients could explain the absence of this complication in our series.

In the malignant cases, the risk of cervical stenting relates to the possibility of proximal migration, which shares the danger of sudden upper respiratory tract occlusion[3,8,10,16]. We experienced no migration in our patients, since the tumour masses were hard and protrusive and occluded the esophagus, which kept the stent in place, while in the two laryngectomy cases, it was impossible for the stent to impair breathing.

The second main concern for stenting at the level of cervical esophagus is the theoretical probability of a foreign body sensation[3,10-12]. Although such a sensation was experienced by some of our tumour-bearing patients, this was minimal and well tolerated. Most likely, by the time when a prosthesis becomes necessary, such patients especially those after laryngestomy with a Flamingo stent no longer have normal sensation, probably due to local infiltration of the nerves innervating the hypopharynx, cricopharyngeal sphincter and upper esophagus resulting in hypo/anesthesia. This propensity to infiltrate local neural structures is reflected by the frequent occurrence of unilateral or bilateral vocal cord paralysis. In addition, previous radiotherapy or surgery also impairs normal sensation. The two patients who were placed Flamingo stents for sealing the esophagocutaneous fistula after laryngectomy experienced a foreign body sensation every time they moved their heads forward. However, in these cases the stent was high at the level of the mesopharynx and easily visible even though the mouth was opened.

The procedure of stenting itself is totally uneventful. The use of local anesthesia and conscious sedation, in conjunction with a slim endoscope, can facilitate the procedure, which is time consuming only in the case of a very rigid and narrow malignant stricture requiring a guidewire to be advanced blindly through the stricture for bougie dilatation. Otherwise, the total endoscopic procedure takes only a few minutes, that is, the time needed for passing the endoscope through the lesion, making the appropriate measurements of total length of the lesion and distances of its proximal margin from upper esophageal sphincter and from incisor teeth, and advancing a guidewire through the endoscope into the stomach. The endoscope is then withdrawn and the stent is advanced 'blindly' over the guidewire, using only the numerical marks on the stent's sheath (centimeters from its proximal edge) for correct positioning. The use of general anesthesia does not facilitate the endoscopic maneuvering. However, insertion of the scope under direct inspection of the esophageal opening, by means of a laryngoscope as for tracheal intubation, is easier, but is not a reason to give general anesthesia to an otherwise conscious patient.

Thus, the eight mechanically ventilated TEF patients were under general anesthesia, while the remaining malignant lesion patients were simply given midazolam for conscious sedation. We experienced no problem with the latter group, but we could not give any favor to the former. However, this fact may be partially related to the benign nature of the disease in this group of patients.

With regard to the technical problems of proper positioning, the theoretical point of difficulty is the proximity of the mesopharynx and epiglottis. Profili *et al*[10] and Conio *et al*[17] have advised peroral administration of iodinated contrast medium for exclusive fluoroscopy guidance throughout the procedure. However, we found it was not useful and adds excessive difficulties, either because of the inability of a sedated patient to swallow or simply because of the increased time required to complete the procedure, or just because the procedure should be done on the ICU bed. We consider that the main difficulty is the accurate deployment of the proximal end of the stent close to the cricopharynx, because of the retraction of the expandable stent on deployment. The radiopaque markers used for stent placement under fluoroscopic assistance are not always reliable, because they are intended to indicate the position of a fully expanded stent. The problem was overridden when the proximally released type of Ultraflex prosthesis was used, allowing more accurate placement because we carefully measured the distances of the orifice of the upper esophageal sphincter and the proximal edge of the lesion from the incisor teeth, thus enabling us to know exactly where the proximal end of the stent should begin to deploy.

Additionally, the use of the Ultraflex stent has the advantages of being less rigid, thus reducing pain during movements of the head and neck, and has smooth edges, making it atraumatic when positioned in the hypopharynx,

despite continuous soliciting during swallowing. This prosthesis can exert a constant, gentle radial force on the esophageal wall, but withstand angulation forces better than the Song and Gianturco stents, as well as the Wallstent which is stiffer and thus less suitable for lesions in this area[12,17].

A further advantage is the rapid expansion to the full diameter, enabling all patients to ingest well-chewed food two days after intervention. The rapid expansion also results in tight fixation to the esophageal wall without any tendency to dislocate and an immediate and complete sealing. In the case of tracheoesophageal fistula, it has successful rate of 73%-100%[5,16,18-20], referring both to malignant and benign fistulae. Moreover, its funnel-shape facilitates the collection of saliva and maintains proper positioning.

For the esophagocutaneus fistula patients we preferred the Flamingo Wall stent due to a number of distinct characteristics: conical shape with proximal flaring and lange braiding angle in the upper part and small in the distal part of the stent. When swallow movements and oesophageal peristalsis propel the stent downwards, its upper end becomes trapped in the laryngoplasty area, whereas the lower end becomes stretched, thus resisting distal migration. Moreover, its polyethylene cover is on the inside of the stent, thus increasing the possibility of anchoring the metal mesh to the esophageal mucosa, since there is no stricture to hold it in place[12,21]. To the best of our knowledge, there are no comparative studies on types of stents except two which relate to the distal esophagus, suggesting that all stents offer the same degree of palliation while Ultraflex and Flamingo stents are both equally less atraumatic than the Gianturco stent[21, 22].

Stent placement in the setting of chemoradiotherapy may be associated with life-threatening complications such as esophageal perforation or bleeding. Kinsman *et al*[23] reported that the complication rate is 36.4% and the mortality rate is 23% in patients receiving radiation and/or chemotherapy, compared with 2.5% and 0% of those without prior therapy. Sumiyoshi *et al*[24] have recorded 6 sudden massive hemorrhages in a group of 22 patients. Additionally, it is likely that T4 cancers are susceptible to pressure necrosis from stent with a consequent increase in the risk of perforation into adjacent structures[24]. Fortunately, we did not experience such complications in our patients. This could be partially explained by the fact that by using the Ultraflex stent, excessive dilatation of the malignant stricture is avoided.

Finally, the quality of life becomes an overriding issue in patients with inoperable cancer, therefore the ability to swallow their saliva and maintain oral intake is important to most patients with esophageal tumours[25]. Since the most realistic goal of any palliative therapy is maximal relief of symptoms with minimal risk, the insertion of a stent with no or little risk of dilation and a minimum of post-procedure complications are the optimum. Good symptom relief of dysphagia and successful occlusion of fistulae are clearly possible by the use of a stent as shown in our malignant and benign cases and more importantly, many patients with malignant strictures or fistulae are able to swallow their saliva after stent placement.

In conclusion, the results of the present series support the thesis that the presence of a lesion within 2 cm of the cricopharyngeal muscle should no longer be considered a contraindication for the palliative or temporary use of an endoprosthesis.

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RAPID COMMUNICATION

Local regulator adrenomedullin contributes to the circulatory disturbance in cirrhotic rats

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Abstract

AIM: To investigate whether adrenomedullin, a potent vasodilator peptide, plays a role in the circulatory disturbance in cirrhosis.

METHODS: Cirrhosis was induced in rats by weekly gavage of carbon tetrachloride. Hemodynamic studies were performed *in vivo* using radioactive microspheres and *in vitro* using isolated aortic rings. The adrenomedullin concentrations were measured by radioimmunoassay.

RESULTS: Acute administration of adrenomedullin to the control rats reduced the systemic arterial pressure along with an increase of serum levels of the stable metabolite of nitric oxide (NOx), in a dose-dependent manner. Chronic infusion of adrenomedullin reduced the vascular resistance and increased the blood flow in the systemic and splanchnic circulation. Intravenous administration of anti-adrenomedullin antibody did not affect any hemodynamic parameters in the cirrhotic rats, whereas this antibody ameliorated the blunted contractile response to phenylephrine, α-adrenergic receptor agonist, in the aortic rings of the cirrhotic rats. The adrenomedullin concentrations in the aorta were higher in the cirrhotic rats than in the controls, and correlated with the mean arterial pressure in the cirrhotic rats. Moreover, adrenomedullin blunted the contractile response to phenylephrine in both of the control aorta and cirrhotic aorta, but not in the presence of NG-nitro-L-arginine methyl ester, an NO synthase inhibitor.

CONCLUSION: Adrenomedullin overproduced in the vascular wall may contribute to the circulatory disturbance in cirrhosis as a local regulator of the vascular tonus rather than a circulating hormone.

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Key words: Adrenomedullin; Liver cirrhosis; Vasodilation; Circulatory disturbance; Vascular tonus; Circulating hormone

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INTRODUCTION

Arterial hypotension, high cardiac output, low vascular resistance, and hyporeactivity to vasoconstrictors are hemodynamic features in human and experimental liver cirrhosis[1-3]. These circulatory disturbances may be attributed to arterial vasodilation that results from overproduction or reduced degradation of vasodilator substances[1-3]. Several circulating vasodilator peptides including substance P, calcitonin gene-related peptide, and glucagon are increased in the cirrhotic patients[4-6]. Moreover, many studies have suggested that the vasodilator substances produced in the vascular wall such as nitric oxide (NO) and carbon monoxide, an end product of the haeme oxygenase pathway, may be coordinately associated with arterial vasodilation in cirrhosis[7-9]. Therefore, the high levels of the circulating vasodilator peptides and the increased vascular production of vasodilators can contribute to the pathogenesis of arterial vasodilation leading to the circulatory disturbance in liver cirrhosis.

Adrenomedullin (AM) is the potent hypotensive peptide discovered in the human pheochromocytoma, and is considered to cause a potent vasodilation via synthesis of NO in the vascular endothelial cells as well as an increase of the intracellular adenosine 3',5'-cyclic monophosphate in the vascular smooth muscle cells[10-11]. AM is abundant in the adrenal medulla, but is widely distributed in the human and rat organs including the vascular tissue[12,13]. Several clinical studies have

demonstrated that the circulating AM levels are increased along with progression of the liver disease and correlate with the hemodynamic parameters in cirrhosis[14-17]. Moreover, the gene expression of AM in the vascular tissue was more enhanced in the cirrhotic rats than in the controls[18]. These findings raise the possibility that AM may be involved in the circulatory disturbance in cirrhosis. However, whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis remains to be established. In this study, the role of the circulating AM in the hemodynamic derangement in cirrhosis was investigated in the presence of exogenous AM and/or anti-AM antibody using radioactive microspheres. Moreover, the AM concentrations in the aorta were evaluated in relationship to the systemic blood pressure, and the effect of AM on the vascular tonus was investigated in the presence of exogenous AM and/or anti-AM antibody using isolated aortic rings. This study aimed to investigate whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis.

MATERIALS AND METHODS

Animal preparation

The experiments were performed on male Sprague-Dawley rats. Liver cirrhosis was induced by weekly intragastric administration of carbon tetrachloride[19]. The rats weighing about 150 g were given phenobarbital (35 mg/dL) in the drinking water. After 2 weeks when the rats were about 250 g, the initial dose (0.04 mL) of intragastric carbon tetrachloride was begun. Body weight was monitored and the dose of intragastric carbon tetrachloride was adjusted according to the change of body weight. After 8-10 doses of carbon tetrachloride, micronodular liver cirrhosis was induced in the most rats and the half of cirrhotic rats developed ascites. The control rats were treated with phenobarbital alone. All animals received humane care and all experiments were performed according to the guidelines of the Committee for the Care and Use of Laboratory Animals in Nara Medical University.

Hemodynamic studies

Hemodynamic studies were performed by the same operator to reduce the operator-dependent variability. Ketamine (100 mg/kg, i.m.) was used as an anesthetic drug, because it closely resembled the conscious state in terms of hemodynamics[20]. The left femoral artery, right jugular vein, femoral vein, and portal vein were cannulated with PE-50 catheters. The left ventricle was catheterized with another PE-50 tube. Each catheter used was filled with heparinized saline. The cardiac output and regional blood flow were measured using radioactive microspheres[21]. A reference sample was obtained from the femoral artery for 75 seconds at a rate of 1 mL/min using continuous withdrawal pump (CFV2100; Nihon Kohden, Tokyo, Japan). Approximately 60 000 microspheres labeled with ^{57}Co (15.5 ± 0.1 μm diameter, specific activity: 610 MBq/g; New England Nuclear, Boston, MA, USA) were injected into the left ventricle 15 seconds after the start of blood withdrawal. The cardiac output was calculated as follows: The cardiac output (mL/min) = injected radioactivity (cpm) × reference blood flow (mL/min) / reference blood radioactivity (cpm). The cardiac index was expressed per 100 g of body weight. The abdominal organs were cut into small pieces and placed in counting tubes. The radioactivity of each organ was determined with a gamma counter. For calculation of the regional blood flow, the injected radioactivity was replaced by radioactivity of each organ in the previous equation. The portal venous inflow was calculated as the sum of the blood flows to the stomach, spleen, small and large intestines, pancreas, and mesentery. The vascular resistance was calculated from the ratio between the perfusion pressure and the blood flow in each vascular territory. The hemodynamics were investigated as follows: 1) Control rats were infused with either vehicle (n = 6) or human AM (0.1, 0.3, 1.0 nmol/kg/min for 10 min, n = 6, respectively) via a femoral vein catheter. Another six control rats were given human AM at a dose of 0.3 nmol/kg/min for 10 min after an intravenous injection of anti-AM antibody (500 μg/kg). The mean arterial pressure and serum levels of NO× (NO_2^- + NO_3^-), a stable metabolite of NO, were determined before and after the infusion. 2) To compare the magnitude of depressor response of the exogenous AM, cirrhotic rats were also infused either vehicle (n = 6) or human AM (0.1, 0.3, 1.0 nmol/kg/min for 10 min, n = 6, respectively) and the mean arterial pressure was determined before and after the infusion. 3) Control rats were chronically infused with either vehicle (n = 8) or human AM (1.0 μg/h, n = 8) for 14 d using a mini-osmotic pump (alzet model 2002, Alza, CA, USA). The pumps were connected to the left jugular vein cannulated with PE-60 and placed in the subcutaneous tissue. The human AM dose was determined according to a previous study[22]. On d 14 of chronic infusion, hemodynamic studies were performed using radioactive microspheres. 4) Cirrhotic rats with ascites were repeatedly injected with either anti-AM antibody (500 μg/kg, n = 8) or vehicle (n = 8) via the tail vein on the day of, and 3 and 6 d after the development of ascites. Hemodynamic studies were performed on the day after the final injection.

Isolated aortic ring studies

On the day of the experiment, the thoracic aorta was removed from control rats and cirrhotic animals with ascites and cut into 3-mm rings. The rings were suspended between two triangular stainless steel stirrups in a 20-mL jacketed organ chamber containing modified Krebs-Henseleit solution (118 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 11.1 mmol/L glucose, 27.2 mmol/L $NaHCO_3$, 0.03 mmol/L Na_2 ethyle nediaminetetraacetic acid, 1.8 mmol/L $CaCl_2$) at 37 ℃ and bubbled with 950 mL/L O_2 and 50 mL/L CO_2. The lower stirrup was anchored and the upper stirrup was attached to a force-displacement transducer (TB-652T; Nihon Kohden) to record the isometric force. All rings were

stretched to generate a resting tension of 2 g, which was optimal for contractions of the aortic rings in response to phenylephrine, an α-adrenergic receptor agonist. After 1 h of equilibration, the presence of functional endothelium was determined by the addition of acetylcholine (10 μmol/L). All rings were rinsed and allowed to equilibrate for an additional 1 h in the presence of indomethacin (10 μmol/L) to prevent the influence of the endogenous prostanoids. The aortic rings of the control rats or cirrhotic animals with ascites ($n = 8$, respectively) were incubated for 30 min with anti-AM antibody (1 mg/L) or vehicle. Another control and cirrhotic rings were incubated with either human AM (100 nmol/L) or vehicle in the presence or absence of NG-nitro-L-arginine methyl ester (L-NAME) (30 μmol/L) ($n = 8$, respectively). Then, the cumulative dose-response curves to phenylephrine (1 nmol/L to 10 μmol/L) were evaluated. On completion, the rings were dried and weighed. The force of contraction was expressed as mg of contraction per mg of dried tissue. From each dose-response curve, the maximum response (Rmax) and phenylephrine concentration required for 50% of the maximum response (EC_{50}) were calculated with a nonlinear regression method using computerized curve-fitting software (StatView 5.0 program, Abacus Concept Inc., Berkeley, CA, USA), and were used to compare the contractility and reactivity of phenylephrine-induced contraction, respectively.

Measurements

The AM concentrations in the aorta were measured in the control rats and the cirrhotic animals with/without ascites ($n = 10$, respectively). The aorta was homogenized for 1 min in 10 volumes of 1 mol/L acetic acid and immediately heated at 100 ℃ for 10 min. The homogenates were centrifuged at 15 000 g for 10 minutes at 4 ℃. The supernatants were frozen until analyzed. The AM concentrations were measured by radioimmunoassay[18]. The protein concentrations were determined by Bradford's method[23]. Serum NOx levels were measured as previously described[24].

Chemicals

The human AM and anti-AM antibody were kindly supplied from Diagnostic Science Division, Shionogi & Co., Ltd. (Settsu, Japan). The anti-AM antibody which specifically binds to the C-terminal structure of AM and neutralize the effect of AM, belongs to immunoglobulin G1 subclass and equally cross-reacts with the rat AM [1-50], but not with calcitonin gene-related peptide or amylin[25]. The mouse IgG, KCl, acetylcholine, L-NAME, indomethacin, and phenylephrine were purchased from Sigma Chemical (St. Louis, MO, USA).

Statistic analysis

All analyses were performed with StatView 5.0 program (Abacus Concept Inc.). Comparisons were made using the two-tailed Student's t test for quantitative variables. All data are expressed as mean ± SE. $P < 0.05$ was considered statistically significant.

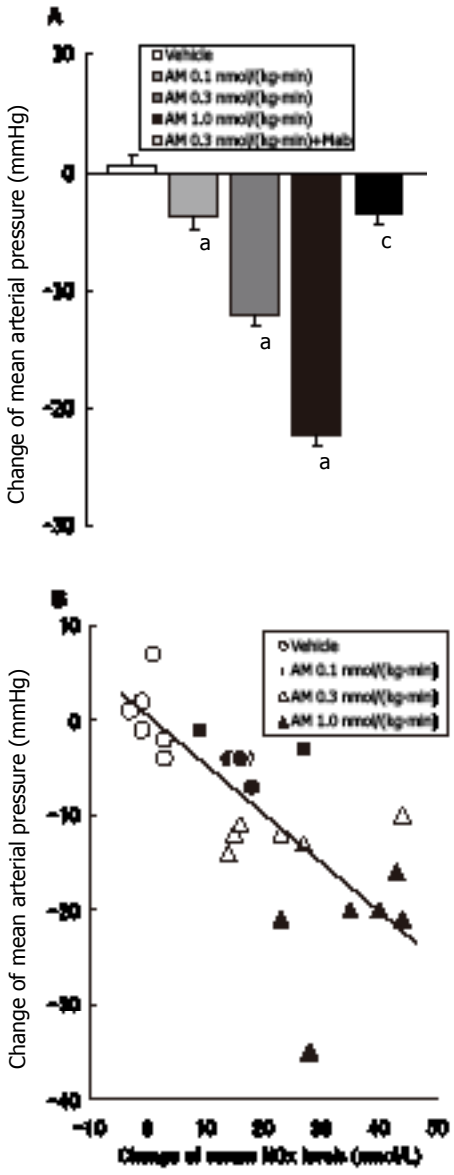

Figure 1 Changes of the mean arterial pressure and serum NOx levels by acute administration of exogenous adrenomedullin. **A**: Arterial pressure; $^{a}P < 0.05$ *vs* vehicle; $^{c}P < 0.05$ *vs* AM 0.3 nmol/(kg·min); **B**: serum NOx levels. mean ± SE. $r = -0.72$, $P < 0.05$, $n = 6$.

RESULTS

Acute administration of exogenous AM

Acute administration of human AM reduced the systemic arterial pressure in the control rats in a dose-dependent manner (Figure 1A, the changes of the mean arterial pressure, vehicle: 0.5 ± 1.6 kPa, AM 0.1 nmol/(kg·min): -3.8 ± 0.8 kPa, $P < 0.05$ *vs* vehicle, AM 0.3 nmol/(kg·min): -12.0 ± 0.6 kPa, $P < 0.05$ *vs* vehicle, AM 1.0 nmol/(kg·min): -22.2 ± 2.7 kPa, $P < 0.05$ *vs* vehicle, respectively). The changes of the mean arterial pressure by AM infusion (0.3 nmol /(kg·min)) were abolished by the pre-treatment with anti-AM antibody (-3.5 ± 0.6 kPa, $P < 0.05$). Moreover, AM infusion increased serum NOx levels in a dose-dependent manner, and the changes of serum NOx levels

Table 1 Hemodynamic effects of chronic administration of adrenomdullin in control rats

	Vehicle ($n=8$)	AM ($n=8$)	P value
Mean arterial pressure (mmHg)	132±3	122±4	<0.05
Cardiac index (mL/min·100g bw)	26.0±2.1	34.1±2.9	<0.05
Systemic vascular resistance (mmHg·min·100g bw/mL)	5.3±0.4	3.8±0.4	<0.05
Portal pressure (mmHg)	6.9±0.5	7.0±0.5	NS
Portal venous inflow (mL/min·100g bw)	3.5±0.4	5.0±0.6	<0.05
Portal venous system resistance (mmHg·min·100g bw/mL)	2.1±0.3	1.6±0.3	NS
Splanchnic arterial resistance (mmHg·min·100g bw/mL)	38.5±4.1	25.7±4.0	<0.05

Control rats were chronically infused with either vehicle or human adrenomedullin (AM) (1.0 mg/h) for 14 d using mini-osmotic pump. On day 14 of chronic infusion, hemodynamic study was performed using radioactive microspheres. Values are presented as mean±SE of 8 separate experiments.

Table 2 Effects of anti-adrenomedullin antibody on hemodynamics and aortic ring contraction of cirrhotic rats.

	Vehicle	Anti-AM antibody
Hemodynamic effects		
Mean arterial pressure (mmHg)	117 ± 3	121 ± 4
Cardiac index (mL/min·100g bw)	39.5 ± 2.9	38.2 ± 3.8
Systemic vascular resistance (mmHg·min·100g bw/mL)	3.1±0.4	3.4±0.4
Portal pressure (mmHg)	13.0 ± 0.4	12.8 ± 0.5
Portal venous inflow (mL/min·100g bw)	6.8 ± 0.8	5.6 ± 1.0
Portal venous system resistance (mmHg·min·100g bw/mL)	2.2 ± 0.3	2.6 ± 0.4
Aortic ring contraction		
Rmax (mg/mg tissue)	998 ± 96	1499 ± 137a
EC50 (nmol/L)	85.7 ± 11.8	53.8 ± 6.2

Cirrhotic rats with ascites were repeatedly injected either vehicle or anti-adrenomedullin (anti-AM) antibody [500 mg/(kg·times)] via tail vein on the day of occurrence of ascites, 3 and 6 d after. Hemodynamic study was performed on the next day of the final injection. Values are presented as mean±SE of 8 separate experiments. The contraction of aortic rings to phenylephrine was evaluated in the presence of vehicle or anti-AM antibody. Values are presented as mean±SE of 8 rings. Rmax: maximal contraction to phenylephrine, EC_{50}: phenylephrine concentration required for 50% of Rmax, $^aP<0.05$ *vs* vehicle.

by AM infusion negatively correlated with the changes of the mean arterial pressure (Figure 1B, $r=-0.72$, $P<0.05$). Exogenous AM reduced the systemic arterial pressure in the cirrhotic rats, as well (vehicle: -0.5±1.8 kPa, AM 0.1 nmol/(kg·min): 0.3±1.9 kPa, AM 0.3 nmol/(kg·min): -4.0±0.6 kPa, AM 1.0 nmol/(kg·min): -13.2±1.7 kPa, $P<0.05$ *vs* vehicle, respectively), but the magnitude of depressor response in the systemic arterial pressure was lower in the cirrhotic rats than in the controls.

Chronic administration of exogenous AM

In agreement with the results of acute administration, chronic administration of exogenous AM caused systemic hypotension as compared with vehicle infusion (Table 1). Chronic infusion of AM increased the cardiac index and reduced the systemic vascular resistance as compared with vehicle infusion. Moreover, chronic AM infusion increased the portal venous inflow and reduced the splanchnic arterial resistance as compared with vehicle infusion. The portal pressure and portal venous system resistance were unchanged by chronic AM infusion.

Effects of anti-AM antibody on hemodynamics and vascular tonus in cirrhotic rats

To evaluate whether the circulating endogenous AM is associated with the circulatory disturbance in cirrhosis, the effects of anti-AM antibody on the hemodynamics were investigated in cirrhotic rats with ascites (Table 2A). Despite the repeated administration of anti-AM antibody that neutralizes the circulating AM, the systemic and splanchnic circulations of the cirrhotic rats were both unchanged. To evaluate whether the endogenous AM in the vascular tissue plays a role in the vascular tonus in the cirrhotic rats, the effects of anti-AM antibody on the phenylephrine-induced contractile response of the control and cirrhotic aortas were evaluated. In the cirrhotic aorta, the anti-AM antibody enhanced the contractility of the phenylephrine-induced contraction without affecting the reactivity as compared with vehicle-treatment (Table 2B). On the other hand, this antibody did affect the contractile response of the control aortas as compared with vehicle.

AM concentrations in the aorta

The AM concentrations in the aorta were higher in the cirrhotic rats than in the controls (Figure 2A, 21.9±2.3 *vs* 12.9±1.2 fmol/mg, $P<0.05$). The cirrhotic rats with

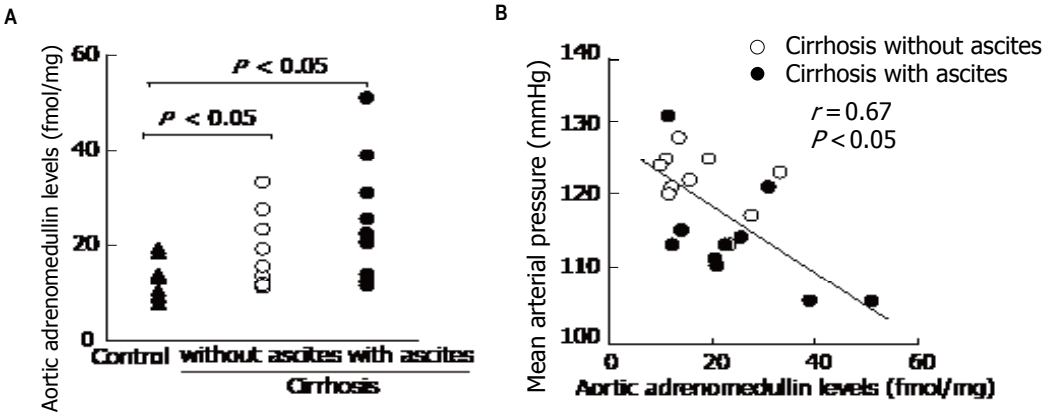

Figure 2 Adrenomedullin concentrations in the aorta in the control and cirrhotic rats (**A**) and their relation with the mean arterial pressure (**B**). ▲ : Control rats, ○ : Cirrhotic rats without ascites, ●: Cirrhotic rats with ascites. mean ± SE. $n = 10$.

Table 3 Contractile response of control and cirrhotic aortic rings to adrenomedullin and/or N^G-nitro-L-arginine methyl ester

	Control		Liver cirrhosis	
	Rmax (mg/mg tissue)	EC_{50} (nmol/L)	Rmax (mg/mg tissue)	EC_{50} (nmol/L)
Vehicle	1860±152	68.8±9.4	998±96[a]	85.7±11.8
AM	1330±118[a]	82.7±12.3	698±94[c]	91.0±12.8
L-NAME	2362±182[a]	61.8±7.1	2274±148[c]	72.1±6.7
L-NAME+AM	2242±91[a]	55.7±9.5	2092±219[c]	53.8±6.2

The contraction of aortic rings to phenylephrine was evaluated in the presence of vehicle or anti-AM antibody. Values are presented as mean±SE of 8 rings. Rmax: maximal contraction to phenylephrine, EC^{50}: phenylephrine concentration required for 50% of Rmax, [a]$P < 0.05$ *vs* vehicle-treated control aorta, [c]$P < 0.05$ *vs* vehicle-treated cirrhotic aorta. AM, adrenomedullin; L-NAME, N^G-nitro-L-arginine methyl ester.

ascites showed the highest aorta AM concentration (24.8 ± 4.0 fmol/mg) which was approximately two-fold increased as compared with that in the controls. Moreover, the AM concentrations in the aorta negatively correlated with the mean arterial pressure in the cirrhotic rats ($r = -0.67$, $P < 0.05$, Figure 2B).

Interaction of AM with NO in phenylephrine-induced contraction of the aortic rings

To investigate the interaction between AM and NO on the vascular tonus, the effect of AM on phenylephrine-induced contraction of the aortic rings was examined in the presence or absence of L-NAME (Table 3). AM-treatment reduced the contractility of the aorta as compared with vehicle-treatment in both of the control and cirrhotic aortas (Rmax: control; 1330 ± 118 *vs* 1860 ± 152 mg/mg tissue, $P < 0.05$, cirrhosis; 698 ± 94 *vs* 998 ± 96 mg/mg tissue, $P < 0.05$), whereas the reactivity showed no difference between AM-treatment and vehicle-treatment (EC_{50}: control; 82.7 ± 12.3 *vs* 68.8 ± 9.4 nmol/L, cirrhosis; 91.0 ± 12.8 *vs* 85.7 ± 11.8 nmol/L). L-NAME potentiated the contractility of both the control and cirrhotic aorta as compared with vehicle without affecting the reactivity (Rmax: control; 2362 ± 182 mg/mg tissue, $P < 0.05$, cirrhosis; 2274 ± 148 mg/mg tissue, $P < 0.05$, EC_{50}: control; 61.8 ± 7.1 nmol/L, cirrhosis; 72.1 ± 6.7 nmol/L). When the aortic rings were pre-treated with L-NAME, AM had no effects on the contractile response of both the control and cirrhotic rings (Rmax: control; 2242 ± 91 mg/mg tissue, cirrhosis; 2092 ± 219 mg/mg tissue, EC_{50}: control; 55.7 ± 9.5 nmol/L, cirrhosis; 53.8 ± 6.2 nmol/L).

DISCUSSION

Arterial vasodilation leading to a low systemic vascular resistance is the most outstanding hemodynamic alteration in the human and murine liver cirrhosis[1-3]. This vasodilation has been suggested to be a major pathogenic mechanism for hyperdynamic circulation characterized by arterial hypotension, hypervolemia, and high cardiac output[1-3]. Although the precise mechanism of the vasodilation in liver cirrhosis remains unknown, the overproduction or reduced degradation of endogenous vasodilators may play a major role. AM, a potent vasodilator peptide, is overproduced by various stimuli including endotoxin, cytokines, vasoactive substances and/or shear stress[26,27], which are enhanced in liver cirrhosis[28,29]. Moreover, the circulating AM levels and the gene expression of AM in the vascular tissues are both elevated in the human and/or murine liver cirrhosis[14-18]. These findings indicate that AM may be implicated in the circulatory disturbance in liver cirrhosis. The aim of this study was to investigate whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis.

Acute administration of exogenous AM to control rats reduced the systemic arterial pressure along with an increase of serum NOx levels in a dose dependent manner, and

the changes of serum NOx levels correlated with the changes of the mean arterial pressure. Moreover, chronic infusion of AM (1.0 μg/h for 14 d) which keeps plasma human AM concentrations within the physiological limit[22] reduced the vascular resistance and increased the blood flow in both of the systemic and splanchnic circulations. These results, together with the existence of AM receptors in the vascular endothelial cells[11], suggest that AM may cause arterial vasodilation via NO synthesis in the vascular wall and lead to a hemodynamic alteration resembling liver cirrhosis.

Whether the circulating endogenous AM is associated with the circulatory disturbance in liver cirrhosis remains to be established. In this study, we used the anti-AM antibody to abolish the effect of the circulating endogenous AM. This antibody possesses an extremely potent neutralizing potency against AM[25], which may allow us to elucidate the role of the endogenous AM in the circulatory disturbance in cirrhosis. Five hundred μg/kg of the anti-AM antibody abolished hypotension following AM infusion at the dose of 0.3 nmol/kg/min for 10 min. Our previous study showed that plasma concentrations of AM in the cirrhotic rats with ascites were 19.2 ± 5.4 fmol/mL[18]. Therefore, the dose of anti-AM used in this study corresponds to that to neutralize the 200-fold of the circulating AM levels of the cirrhotic rats, indicating the enough dose to neutralize the effect of the circulating AM. However, this neutralizing antibody did not affect any hemodynamic parameters in the cirrhotic rats. Moreover, most of the circulating AM is reportedly occupied by glycine-extended AM, an inactive intermediate form of AM, and reflects the process of AM production in the tissue[30]. Therefore circulating endogenous AM may not play a significant role in the circulatory disturbance of liver cirrhosis.

There are no significant difference among the AM levels in plasma samples obtained from the hepatic vein, renal vein, pulmonary artery and femoral artery in cirrhotic patients[15]. The lack of significant arterio-venous difference in the AM levels in various vascular territories suggests that the increased plasma AM levels in cirrhotic patients do not result from an increased production in a specific organ. In this study, the AM concentrations in the aorta were more enhanced in cirrhotic rats than in the controls, and correlated with the mean arterial pressure in the cirrhotic rats. It is, therefore, possible that the endogenous AM contributes to the circulatory disturbance in cirrhosis as a paracrine and/or autocrine regulator of the vascular tonus rather than a circulating hormone. To investigate the role of the endogenous AM in the vascular tonus of cirrhotic rats, we performed isolated aortic ring studies. The cirrhotic aorta showed a blunted contractile response to phenylephrine as compared with the controls. The anti-AM antibody ameliorated the blunted contractile response in the cirrhotic aorta, but did not affect the contraction of the control aorta. This finding, together with the increased AM concentrations in the cirrhotic aorta, indicates that the endogenous AM in the aorta may play a role in the vascular tonus in liver cirrhosis.

It is widely recognized that NO plays a major role in the blunted vascular tonus in cirrhosis[31,32]. Moreover, AM and NO stimulate the synthesis and secretion of each other[11,33]. We, therefore, investigated the interaction between AM and NO in the contractile response of the cirrhotic aorta. In both of the control and cirrhotic aortas, AM reduced the contractile response, but not in the presence of NO synthase inhibitor. Prostanoids are unlikely to explain the blunted contractile response caused by AM, because sufficient indomethacin was used to inhibit cyclooxgenase. These findings, together with an increase in serum NOx levels by the exogenous AM infusion, indicate that AM overproduced in the vascular wall of cirrhotic rats may regulate the vascular tonus via NO synthesis in a paracrine and/or autocrine manner. In liver cirrhosis, an increase in hepatic vascular resistance is the initial phenomenon leading to portal hypertension. This is primarily due to the structural distortion of the hepatic microcirculation caused by cirrhosis, but is also associated with a deficient NO production in the liver, which results in an increased hepatic vascular resistance in contrast to an increased portal inflow via a vasodilation in pre-hepatic (splanchnic) vessels[34]. Considering the interaction between AM and NO, AM may be associated with portal hypertension through an imbalance of the NO production in the hepatic and pre-hepatic vessels.

Interestingly, the magnitude of hemodynamic alteration caused by AM infusion to the control rats was less than that in the cirrhotic animals with ascites, despite the similar hypotension in both groups. It has been considered that the circulatory disturbance in cirrhosis is initially caused by arterial vasodilation and thereafter promoted by an increase in the blood volume resulting from the impaired water and sodium excretion by activation of the endogenous vasoconstrictive systems[1-3]. Because the chronic infusion of AM suppresses the renin activity and aldosterone concentration[22,35], the lack of activation of the vasoconstrictor system may result in a less hemodynamic alteration in the AM-infused rats as compared with the cirrhotic animals. This finding provides further support for the concept that activation of the vasoconstrictive system as well as arterial vasodilation is essential for the circulatory disturbance in liver cirrhosis.

In conclusion, the AM concentrations in the aorta were elevated and negatively correlated with the systemic arterial pressure in the cirrhotic rats. Anti-AM antibody ameliorated the blunted contractile response of the cirrhotic aorta, whereas neutralization of circulating AM by anti-AM antibody did not affect the hemodynamic parameters in cirrhosis. These findings indicate that increased AM production in vascular tissue may contribute to the circulatory disturbance in cirrhosis, acting as a local regulator of the vascular tonus rather than a circulating hormone.

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RAPID COMMUNICATION

Circadian variation in expression of G_1 phase cyclins D_1 and E and cyclin-dependent kinase inhibitors p16 and p21 in human bowel mucosa

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Abstract

AIM: To evaluate whether the cellular proliferation rate in the large bowel epithelial cells is characterized by circadian rhythm.

METHODS: Between January 2003 and December 2004, twenty patients who were diagnosed as suffering from primary, resectable, non-metastatic adenocarcinoma of the lower rectum, infiltrating the sphincter mechanism, underwent abdominoperineal resection, total mesorectal excision and permanent left iliac colostomy. In formalin-fixed and paraffin-embedded biopsy specimens obtained from the colostomy mucosa every six hours (00:00, 06:00, 12:00, 18:00 and 24:00), we studied the expression of G_1 phase cyclins (D_1 and E) as well as the expression of the G_1 phase cyclin-dependent kinase (CDK) inhibitors p16 and p21 as indicators of cell cycle progression in colonic epithelial cells using immunohistochemical methods.

RESULTS: The expression of both cyclins showed a similar circadian fashion obtaining their lowest and highest values at 00:00 and 18:00, respectively ($P < 0.001$). A circadian rhythm in the expression of CDK inhibitor proteins p16 and p21 was also observed, with the lowest levels obtained at 12:00 and 18:00 ($P < 0.001$), respectively. When the complexes cyclins D_1-p21 and E-p21 were examined, the expression of the cyclins was adversely correlated to the p21 expression throughout the day. When the complexes the cyclins D_1-p16 and E-p16 were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high levels of p16 expression at 18:00.

CONCLUSION: Colonic epithelial cells seem to enter the G_1 phase of the cell cycle during afternoon (between 12:00 and 18:00) with the highest rates obtained at 18:00. From a clinical point of view, the present results suggest that G_1-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity.

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Key words: G1 phase proteins; CDK inhibitors; Cell proliferation; Circadian rhythm

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INTRODUCTION

Eukaryotic cell division occurs in four phases of the cell cycle. The cells are prepared for DNA replication in G_1-phase. DNA is replicated during the S phase, a gap (G_2) period which allows preparation for mitosis before chromosome segregation and cytokinesis in M phase (mitosis). During development, differentiation, or growth factor withdrawal, cells can enter an inactive period (G_0-phase) before returning to G_1-phase[1].

Cyclins represent important regulators of cell cycle process. There are at least 15 distinct cyclin genes in the human genome that fall into three categories, and each category regulates specific passage through the cell cycle[2,3]. Passage through G_1 to S phase is regulated by cyclins C,

regulated by cyclins A and B$_{1-2}$[4-7]. Their levels in cell cytoplasm increase or decrease depending on the stage of cell cycle[2, 3, 8, 9].Elevated nuclear expression of cyclins favour the progression from one phase to the next while their low expression decelerates this progression[2-4, 7].

Cell cycle progression is mediated by the activation of a family of protein kinases, known as cyclin-dependent kinases (CDKs). CDKs constitute a large family of proteins, which act in a variety of key regulatory pathways, including control over the cell cycle and gene transcription[2, 9]. They are also divided in G$_1$-phase CDK (CDK4), S-phase CDK (CDK2) and M-phase CDK (CDK1)[8, 9]. Their levels in the cells remain fairly stable, but each must bind to the appropriate cyclin (whose levels fluctuate) in order to be activated[3, 8]. Cyclin D$_1$ (an early G$_1$-phase cyclin) mainly binds to and activates CDK4 and CDK6[8, 10] while cyclin E (a late G$_1$-phase cyclin) binds to and activates CDK2[8, 10].

CDK inhibitors inhibit the passage through the various phases of the cell cycle[11, 12]. The CDK inhibitors p16 and p21 protein families mediate regulation of cyclin/CDK activity[11, 12]. p16 family contains four members and inhibits CDK4 and CDK6, forming binary complexes with CDK4 *in vitro*[11, 12]. p21 family contains three members and interacts with both cyclin D and E complexes in G$_1$-phase and preferentially inhibits CDK2 activity[10].

In mammals, physiological (e.g. cardiac rhythm) or biochemical (e.g. hormone levels) processes vary in a regular and predictable periodic manner, with respect to the time of the day, which is called endogenous circadian rhythm[13, 14]. At the cellular level, each cell goes through the cell cycle in an orderly and controlled fashion, where the multiple steps associated with each phase should be successfully completed before progressing to the next phase[15]. At the tissue level, experimental[10, 16, 17] and clinical[18-26] studies suggest that a greater proportion of cycling cells in a specific organ, enters S-phase and mitosis at specific times of the day.

The present study was designed to evaluate whether the cellular proliferation activity in the large bowel epithelial cells shows variation over the 24 hours of a day and if this variation is characterized by circadian rhythm. As indicators for cellular proliferation activity we examined the quantitative expression of cyclins D$_1$ and E, as well as the inhibitor proteins p16 and p21 in biopsy specimens taken from the normal bowel mucosa of a permanent colostomy at six-hour intervals.

MATERIALS AND METHODS

Patients

Between January 2003 and December 2004, eighty-six patients suffering from colon and rectal cancer were surgically treated in our department. Among them, there were twenty patients (twelve men and eight women, median age 67 years, interquartile range 55-78 years) who were diagnosed as suffering from adenocarcinoma of the lower rectum infiltrating the sphincter mechanism. In all patients, the preoperative and intraoperative staging work-up revealed primary, resectable, non-metastatic lower rectal cancer. In order to achieve an oncological procedure, all patients underwent abdominoperineal resection, total mesorectal excision and permanent left iliac colostomy. All patients were operated on electively and neither neoadjuvant nor adjuvant chemo-radiotherapy was administered during the period of the study.

Biopsies

Biopsy specimens of bowel mucosa were obtained from the site of colostomy of these twenty patients undergone an abdominoperineal resection. Biopsies were collected at the time when the bowel retained its normal function after the operation and the colostomy was fully functional. Patients received the usual in-hospital low fibre diet. In order to avoid disturbance of cell proliferation, no enemas, bowel preparations and paraffin oil were used[18, 25]. All patients followed their usual sleep schedule (sleeping between 22:00 and 07:00) without receiving any sedatives[18, 19, 22, 25]. Their sleep pattern was interrupted only at the time when the colostomy specimens were taken. Specimens of 2-4 mm^2 were collected every 6 hours from the colostomy mucosa, approximately 3 cm lower to the colostomy orifice, using biopsy miniforceps. The examinations were performed at 00:00, 06:00, 12:00, 18:00 and 24:00. The biopsies were fixed in 10% buffered formalin and embedded in paraffin wax using conventional techniques.

Immunohistochemistry

Immunostainings for cyclins and CDK inhibitors were performed using mouse monoclonal antibody for cyclin D$_1$, rabbit polyclonal antibody for cyclin E, mouse monoclonal antibody for p16 protein and mouse monoclonal antibody for p21protein (Santa Cruz Biochemicals, Santa Cruz, California, USA) with a Vectastain Elite ABC-peroxidase kit (Vector Laboratories, Peterborough, United Kingdom) and a liquid DAB substrate-chromogen system (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. An additional step of antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was performed before antibody incubation for p21. The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). The percentage of positively stained cells in immunohistochemistry experiments was obtained by counting epithelial cells in each case by two independent observers.

Statistical analysis

All graphics were constructed using Microsoft Excel for Windows XT Professional. Statistical differences between the groups were determined by the Student's *t*-test. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the STATA statistical package (StataQuest Version 4.0, College Station, Texas, USA, 1995).

RESULTS

The median and interquartile range (IR) values of the percentage of the positively stained cells in immunohistochemistry for every single protein examined throughout the day of the present study, are presented in Table 1. The observed differences in the expression of the studied proteins between 00:00 and 24:00, could be explained by the fact that more bowel mucosa epithelial cells entered the

Table 1 Percentage of stained cells in immunohistochemistry for cyclin D1, cyclin E, protein p16 and protein p21 expression in bowel mucosa specimens

TIME	Cyclin D1 (Median + IR)	Cyclin E (Median + IR)	p16 (Median + IR)	p21 (Median + IR)
00:00	6 (4 - 8)	4 (2.75 - 5)	13 (11 - 15.25)	14 (10.75 - 16.25)
06:00	7 (5 - 8)	5.5 (5 - 7)	10 (8 - 11.25)	10 (8 - 11)
12:00	8 (6 - 9)	8.5 (7.75 - 10)	8 (6.75 - 8.25)	8 (6.75 - 8)
18:00	13 (11 - 15)	13.5 (11.75 - 16)	13.5 (9 - 15.25)	6 (5 - 7.25)
24:00	8 (6.75 - 10)	8 (6 - 9.25)	15 (12.75 - 16)	14 (11 - 15)

IR: Interguartile range.

proliferative activity as time passed.

The interindividual values for cyclin D_1 expression varied between 3% and 19%. Its expression gradually increased between 00:00 and 18:00 and gradually decreased between 18:00 and 24:00, obtaining the lowest and highest values at 00:00 and 18:00, respectively. The differences between the highest values of cyclin D_1 expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for cyclin E expression varied between 1% and 17%. Its expression gradually increased between 00:00 and 18:00 and then gradually decreased, obtaining its lowest and highest values at 00:00 and 18:00, respectively. Similarly to cyclin D_1, the differences between the values of cyclin E expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p16 expression varied between 4% and 19%. Its expression gradually decreased between 00:00 and 12:00 and gradually increased between 12:00 and 24:00, obtaining its lowest and highest values at 12:00 and 24:00, respectively. The differences between the low values of p16 expression at 12:00, as compared to the higher values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p21 expression varied between 6% and 14%. Its expression gradually decreased between 00:00 and 18:00 and gradually increased between 18:00 and 24:00, obtaining its lowest values at 18:00 and its highest values at 00:00 and 24:00. The differences between the lowest values of p21 expression at 18:00, as compared to the values at 00:00, 06:00 and 24:00, were highly statistically significant ($P < 0.001$).

The present study concluded that expression of both cyclins showed circadian rhythm in a similar fashion. The higher levels of both proteins were obtained between 12:00 and 24:00 (highest at 18.00), while the lower levels were observed between 00:00 and 12:00 (lowest at 00:00).

A circadian rhythm in the expression of inhibitor proteins p16 and p21 was also observed. The lower levels of p16 expression were obtained between 06:00 and 12:00 (lowest at 12:00), while the lower levels of p21 expression were obtained between 12:00 and 18:00 (lowest at 18:00).

When the complexes of cyclins D_1 - p21 (Figure 1A) and E - p21 (Figure 1B) were examined, the present the expression of both cyclins was adversely correlated to the p21 expression throughout the day. The highest levels of cyclins were correlated to the lowest levels of p21.

When the complexes of cyclins D_1 - p16 (Figure 1C) and E - p16 (Figure 1D) were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high expression levels of p16 at 18:00.

DISCUSSION

How the circadian variation in proliferation at the tissue level relates to the control of the cell cycle, is a subject of continuous study[22]. The predictable association between certain cell cycle proteins and defined events during the cell cycle may be used to study the timing of cell cycle phases in normal tissues[22]. In malignant tissue, the expression level of cyclins and their inhibitors may have important prognostic and therapeutic implications, especially in the cell cycle phase-dependent toxicity of anticancer agents[20, 21].

Bucchi *et al*[18] are the first to investigate the presence or absence of rhythm in normal bowel mucosa proliferation. In their study, rectal biopsies were obtained every 2 or 3 h for 24 h using standard biopsy forceps during flexible sigmoidoscopy from 16 volunteers under fasting and fed conditions. Incorporation of [^{3}H] thymidine was measured in each specimen. Both fed and fasting subjects showed circadian variation in DNA synthesis in rectal mucosa that peaked at 07:00. Although thymidine incorporation fell during fasting, the circadian rhythm remained intact.

Marra *et al*[19] investigated the proliferation rhythm within the rectal crypt epithelium using the ^{3}H-thymidine autoradiographic method and calculated the ratio (labeling index) of the S-phase cells to total cells in the crypt. By taking biopsies every 4 h using standard biopsy forceps via rectoscopy from 23 normally fed subjects, they found a circadian rhythm in the labeling index with a peak at 01:28 in the morning. The base of the crypt and the upper 40% (which contains mainly differentiated cells) did not show circadian variation in the labeling index.

Brandi *et al*[25] studied the circadian variations of rectal cell proliferation in five patients by taking biopsies via proctoscopy from apparently normal mucosa 10 cm from the anal verge every 6h in a 24h period. Labeling index was evaluated as the percentage of labeled cells with respect to the whole cell population in the crypt. The results of the study suggest that rectal cell proliferation fluctuates during the day with the lower rates noted between 22:00 and 02:00.

By measuring the ratio of the S-phase cells to the whole cell population with the [^{3}H] thymidine technique, all previously mentioned studies[18, 19, 25] have demonstrated a rhythmicity in the rectal mucosa proliferation. Due to potential limitations of the [^{3}H] thymidine technique [27], most recent studies on the cellular proliferation rhythm in different tissues, are focused on the immunohistochemically quantitative expression of phase specific proteins. In the present study we examined the quantitative expression of the G_1-phase specific cyclins D_1 and E and the CDK

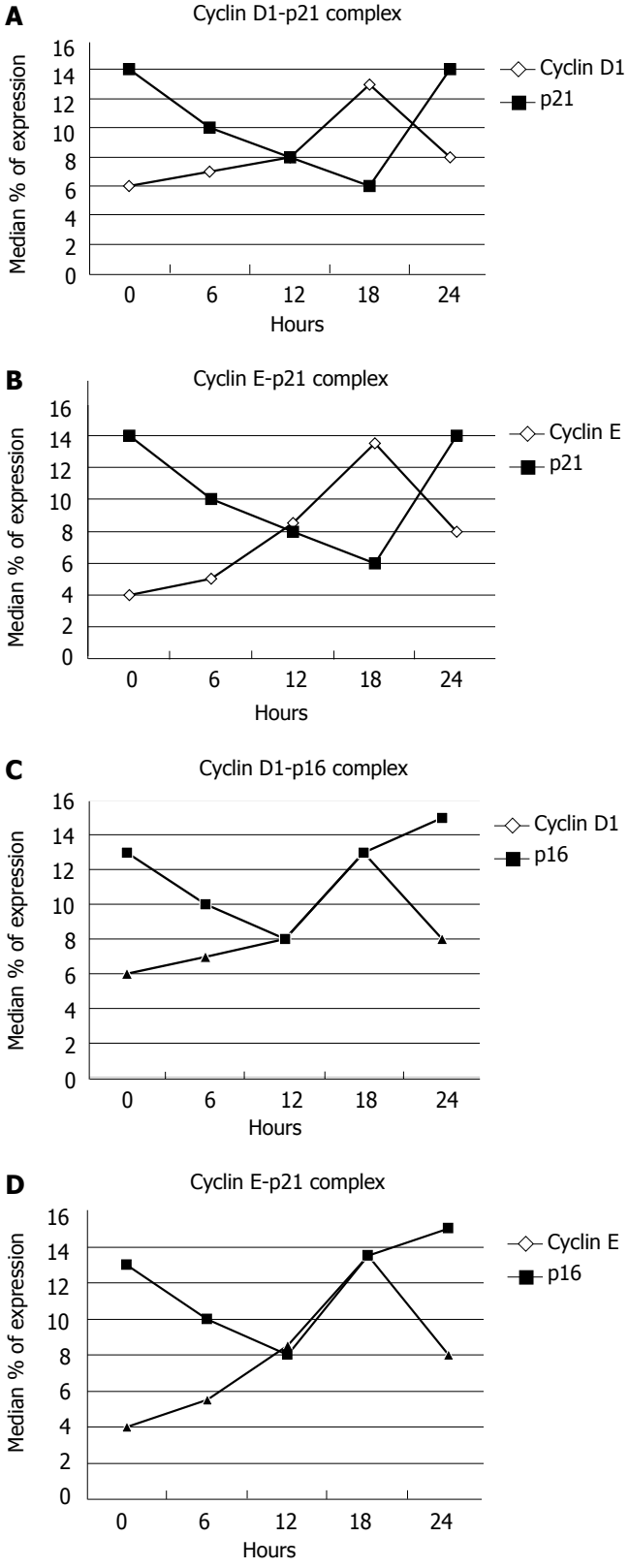

Figure 1 Graphical presentation of simultaneous quantitative expression of cyclin D1 and p21 (**A**), cyclin E and p21 (**B**), cyclin D1 and p16 (**C**), and cyclin E and p16 (**D**) in colostomy specimens sampled every 6 hours for 24 hours.

inhibitors p16 and p21 in normal large bowel mucosa.

Quantitative cyclin D_1 expression has been observed in normal bowel mucosa[28], dysplastic and non-dysplastic adenomatous polyps of the small and large bowel[18, 23, 29]. Its over-expression is related to adenocarcinoma of the colon and rectum (independently of the differentiation or Dukes' stage)[23], pancreatic[30], esophageal[30], endometrial[30], head and neck cancers[31].

Normal cells maintain strict control of cyclin E activity, while its deregulation plays a fundamental role in carcinogenesis. Cyclin E activities mainly consist of passage of cells through the restriction point "R" for cells entering the division from the resting state to G_1-phase[32]. Cyclin E functions not only as a S-phase entry regulator, but also plays a direct role in the initiation of DNA replication, by inducing S-phase specific genes[33, 34]. It is expressed higher than in many human tumors[35, 36] and the accumulation of cyclin E is considered a marker for the transition from adenoma to adenocarcinoma[37, 38].

p16 is a tumor suppressor gene and regulates cell proliferation by inhibiting CDK4 and CDK6 activities. Transient expression of p16 leads to inhibition of DNA synthesis by hypophosphorylating the retinoblastoma (Rb) gene protein[39-42]. The p16/Rb tumor suppressor pathway is frequently defective in many human tumors either by inactivating p16 or Rb or by over-expressing cyclin D_1 or CDK4. In cases of colorectal cancer, only a low frequency of p16 mutation has been found[39, 43]. Dai *et al*[44] hold that p16 expression begins in the earliest detectable stages of human colonic neoplasia and exerts a continuous constraint of tumor growth. A recent experimental study[45] showed that interaction of p16 expression and CDK4 may become a new prognostic marker in colorectal cancer. Tada *et al* [46] studied the possible role of p16 in the development of colonic neoplasms and found that p16 is overexpressed in 98% of adenocarcinomas and that colorectal cancer with reduced p16 expression is more aggressive in lymphatic infiltration.

The p21 protein family can interact with both cyclin and CDK subunits. Members of the p21 family interact with cyclins D and E during the G_1-phase of the cell cycle, preferentially inhibiting CDK2 activity and promoting assembly of cyclin D/CDK 4 complex *in vivo* and *in vitro*[47]. p21 constitutes the first molecule which is considered as a wild-type activated factor (WAF1) due to its upregulation by the tumor suppressor protein p53 and also as a cell-derived inhibitor of DNA synthesis[48]. Expression of p21 has been detected *in vivo* by immunohistochemistry in cells of upper crypts and lower villus. The above mentioned areas are associated with enterocyte differentiation[48] which is associated with a withdrawal from the cell cycle and the transcriptional activation of p21 either dependently or independently of the tumor suppressor p53[48-50].

The results of the present study demonstrated that the expression of both cyclins showed a similar circadian fashion, with the higher levels obtained between 12:00 and 24:00 (highest at 18.00) and the lower levels between 00:00 and 12:00 (lowest at 00:00). These findings partly support the theory for coordinating and cascading activity between them during the G_1 phase of the cell cycle [10]. As cyclin D_1 represents an early G_1 phase cyclin, while cyclin E constitutes a late G_1 phase cyclin, simultaneous increase and decrease of their expression during the same periods of time, require further investigation. A future study focusing on their expression at shorter intervals may disclose more accurately their fluctuation during the day. Circadian rhythm was also observed in the expression of

both inhibitor proteins, with the lowest values obtained at 12:00 and 18:00 for p16 and p21 expression, respectively. By comparing cyclins D_1-p21 and E-p21 complexes, the lowest expression levels of p21 and the highest expression levels of both cyclins were observed at 18:00. When the complexes of cyclins D_1-p16 and E-p16 were examined, the inhibitory action of p16 protein, successfully arrested the cell cycle during the night and early in the morning. Why high expression levels of p16 correlate to the highest expression levels of both cyclins at 18:00 remains unclear.

In conclusion, the expression of all examined parameters (which are involved in the progression from the G_1- to S-phase of the cell cycle) is characterized by circadian rhythm. Colonic epithelial cells seem to enter the G_1 phase of the cell cycle during afternoon between 12:00 and 18:00, because during that period the higher expression levels of cyclins D_1 and E correlate to the lower expression levels of the expression of CDK inhibitor proteins p16 and p21. From a clinical point of view, the present results suggest that G_1-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity[2, 3, 51-53]. Further studies on the accurate circadian rhythm in anatomically intact human colonic epithelium and malignant tissues are required.

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RAPID COMMUNICATION

Portal vein thrombosis: Prevalence, patient characteristics and lifetime risk: A population study based on 23 796 consecutive autopsies

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Abstract

AIM: To assess the lifetime cumulative incidence of portal venous thrombosis (PVT) in the general population.

METHODS: Between 1970 and 1982, 23 796 autopsies, representing 84% of all in-hospital deaths in the Malmö city population, were performed, using a standardised protocol including examination of the portal vein. PVT patients were characterised and the PVT prevalence at autopsy, an expression of life-time cumulative incidence, assessed in high-risk disease categories and expressed in terms of odds ratios and 95% CI.

RESULTS: The population prevalence of PVT was 1.0%. Of the 254 patients with PVT 28% had cirrhosis, 23% primary and 44% secondary hepatobiliary malignancy, 10% major abdominal infectious or inflammatory disease and 3% had a myeloproliferative disorder. Patients with both cirrhosis and hepatic carcinoma had the highest PVT risk, OR 17.1 (95% CI 11.1-26.4). In 14% no cause was found; only a minority of them had developed portal-hypertension-related complications.

CONCLUSION: In this population-based study, PVT was found to be more common than indicated by previous clinical series. The markedly excess risk in cirrhosis and hepatic carcinoma should warrant an increased awareness in these patients for whom prospective studies of directed intervention might be considered.

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Key words: Epidemiology; Venous thrombosis; Portal hypertension; Cirrhosis; Gastrointestinal cancer

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INTRODUCTION

Extrahepatic portal vein thrombosis (PVT) may be caused by a variety of conditions including cirrhosis, cancer and abdominal infectious and inflammatory processes. In the wake of thrombus organisation and vessel involution follows development of tortuous collaterals, so called cavernous transformation[1]. Symptom development is often insidious and related to the progression of portal hypertension. The concept of PVT as a rare disease is mainly based on clinical series and case reports[2-7]. Estimates of frequency and of distribution of etiological fractions vary widely between studies[2, 4,5,6, 8,9,10,11]. To some extent this may be explained by lack of precision in these small series, but a contribution of case selection and ascertainment bias should also be considered.

For several decades, the city of Malmö in southern Sweden has been a centre for epidemiological research, including clinical and autopsy-based studies of cardiovascular diseases[12,13,14,15]. Between 1970 and 1982, close to 24 000 autopsies, comprising 84% of all in-hospital deaths in the city, were performed. All procedures followed a standardised protocol including examination of the portal vein and its major tributaries. In consequence with the course in PVT, the prevalence at autopsy can be regarded as a proxy for the cumulative incidence during lifetime to develop PVT. The size of the cohort together with the high autopsy rate thus provides a unique opportunity to yield a robust estimate of risk in the general population. Further aims have been to evaluate likely causes to PVT and clinical characteristics related to portal hypertension, and to estimate the absolute risk of PVT in high-risk disease categories as characterised by autopsy findings.

MATERIALS AND METHODS

Study cohort

Malmö is a city with a single referral centre for post-

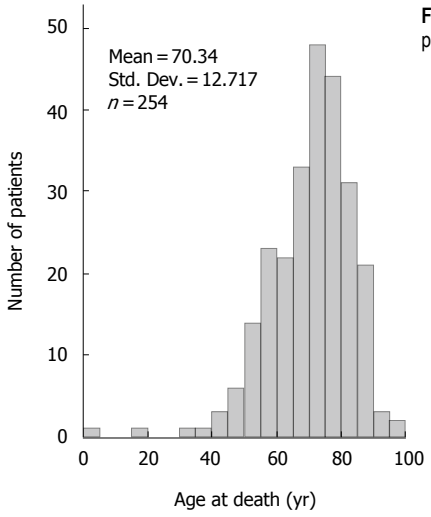

Figure 1 Age distribution of patients at death.

mortem examinations, the Department of Pathology at Malmö University Hospital. Between January 1, 1970 and December 31, 1982, when the city population declined from 264 000 to 230 000 inhabitants, 35 784 deaths occurred in the Malmö population. Among the 28 196 deaths occurring among in-patients at the three hospitals, which served the Malmö population during this period, a total of 23 796 clinical autopsies were performed, corresponding to an autopsy rate of 84%.

All autopsies were performed using a standardised protocol and carried out or supervised by senior pathologists. All findings were classified and coded according to the Standardised Nomenclature of Pathology (SNOP), as defined by the College of American Pathologists in 1965. The death certificates were issued by the pathologist. Based on the clinical picture and on autopsy findings, an underlying cause of death and up to six contributing causes were determined and classified using the ICD-8 code.

Classification of PVT patients in relation to potential causes

All cases with SNOP code 48-80-3700/3703, denoting *portal vein thrombosis* (*PVT*), were identified and validated individually. The following disease categories in the literature regarded as major causes of PVT were identified[5]:

-*cirrhosis,* where classification was based on SNOP codes 4850 - 4857 and/or cirrhosis as underlying or contributing death cause (ICD 8 code 571.00 - 571.99). PVT patients with cirrhosis were further classified according to presence or absence of *primary hepatic cancer* (hepatocellular carcinoma or intrahepatic cholangiocarcinoma);

-*primary hepatobiliary cancer,* comprising primary hepatic cancer (with or without cirrhosis), extrahepatic cholangiocarcinoma and gall bladder carcinoma;

-*secondary malignancy of the hepatobiliary region* (metastatic malignancy to the liver, biliary tract, gall bladder, duodenum or pancreas), further classified according to primary tumour location as expressed by SNOP code;

-*myeloproliferative disorders*: non-specific myeloproliferative disorder (SNOP code 7690); agnogenic myeloid metaplasia (7691); myelofibrosis (7692); histiocytic medullary reticulosis (7698); megablastic erythropoesis (7706); neutrophilic (7711) and eosinophilic (7713) leukocytosis; leukaemoid reaction (7714); erythrocytosis (7715); reticulocytosis (7716); lymphocytosis (7718); thrombocytosis (7719); erythrophagocytosis (7763); erythroid (7771), granulocytic (7772), reticular cell (7777) and megakaryocytic (7778) hyperplasia; polycythaemia vera rubra (7779); and chronic myeloic leukaemia (9867);

-*major abdominal infections and inflammations,* including liver abscess, abdominal abscess, purulent peritonitis, acute necrotising pancreatitis and ulcerative colitis.

In consequence with the possibility PVT being the result of more than one factor, a patient might be included in more than one of these categories. PVT patients in whom none of the above causes could be established were identified and analysed separately.

Classification of risk groups for PVT

PVT prevalence and risk, expressed as odds ratio, were assessed in relation to the following disease categories: *cirrhosis* (with and without primary hepatic cancer), *primary hepatobiliary cancer* (with and without cirrhosis), *secondary hepatic malignancy* (secondary malignancy of the hepatobiliary region not being readily definable in terms of SNOP codes) and *myeloproliferative disorders*. Presence of secondary hepatic malignancy was based on SNOP code 56-8006. Among these, patients with metastatic disease from pancreatic, gastric and colorectal carcinoma were further identified and similarly analysed. For the other disease categories, patients were classified using the definitions stated in the previous section.

Definition of clinical complications in PVT patients

In patients with PVT, the prevalence at autopsy of the following complications typically related to portal hypertension was ascertained and analysed in relation to disease categories: ascites, oesophageal varices and gastrointestinal bleeding.

Statistical analysis

Distributions of age at death were expressed in terms of means and variance and groups defined by presence or absence of potential causal factor compared with one-way analysis of variance (ANOVA). Proportions were compared using two-sided Fisher's exact test for univariate analyses. The PVT risks in relation to respective disease category were also expressed in terms of odds ratios with computation of 95% confidence intervals.

RESULTS

PVT prevalence and patient demographics

PVT was found at autopsy in 254 (1.1 %) of the 23 796 patients; in 125 (1.0 %) out of 12 157 men and 129 (1.1 %) of the 11 639 women ($P = 0.57$). The age distribution is depicted in Figure 1. With the exception of one patient, who was less than 1 year old, the age ranged between 17 and 96 years. Mean (95 % CI) age was 68 (66-70) years in

Table 1 Prevalence in patients with portal vein thrombosis (PVT) of conditions known to be major causes: Age- and gender distribution within categories

Patient category	*n* (%) of PVT patients	*n* (%) within risk category	Age (yr) Median	Mean (95% CI)	Female gender (%)
Cirrhosis [1]	72 (28)		70	68.5 (65.8 - 71.3)	28 (39)
with primary hepatic cancer		26 (36)			
without primary hepatic cancer		46 (64)			
Primary hepatobiliary cancer [1]	59 (23)		72	72.1 (69.4 - 74.8)	26 (44)
hepatic carcinoma [2]		38 (64)			
extrahepatic biliary / gall bladder carcinoma		21 (36)			
Secondary malignancy of the hepatobiliary region [1]	111 (44)		70	69.0 (66.7 - 71.2)	59 (52)
from pancreatic carcinoma		47 (42)			
gastric carcinoma		20 (18)			
colorectal carcinoma		11 (10)			
lung cancer		7 (6)			
malignant lymphoma		5 (4)			
mammary adenocarcinoma		4 (4)			
other primary cancer [3]		17 (15)[1]			
Myeloproliferative disorders [1]	7 (3)		72	65.4 (44.2 - 86.6)	5 (71)
Major abdominal infection / inflammation [1,4]	25 (10)		73	71.9 (66.5 - 77.3)	15 (60)
No cause identified	36 (14)		73	70.8 (65.9 - 75.7)	21 (57)
All patients with PVT	254 (100)		72	69.9 (68.3 - 71.4)	129 (51)

[1] categories not mutually exclusive. [2] hepatocellular carcinoma (26), intrahepatic cholangiocarcinoma (6), mixed (4), anaplastic (1) and unspecified (1) carcinoma. [3] malignant melanoma (3), female genital tract cancer (3), uroepithelial cancer (2), urinary bladder carcinoma (2), prostatic carcinoma (2), oesophageal cancer (1), duodenal cancer (1), renal cancer (1), adrenal cancer (1), sarcoma (1). [4] liver abscess (13), peritonitis (8), acute necrotising pancreatitis (4), abdominal abscess (2), ulcerative colitis (1) (more than one alternative possible).

Table 2 Prevalence and relative risk (odds ratio) of PVT in relation to disease condition with major risk of PVT

Patient category	*n* (%)	PVT (%)	O.R. (95% C.I.)[1]	*P* value	O.R. (95% C.I.)	*P* value
Cirrhosis	1193 (5.0)	72 (6.0)	7.9 (6.0 - 10.5)	< 0.001		
with primary hepatic cancer	182	26 (14.3)	17.1 (11.1 - 26.4)	< 0.001	3.5 (2.1 - 5.8)	< 0.001
without primary hepatic cancer	1011	46 (4.5)	5.2 (3.7 - 7.2)	< 0.001	1[2]	
Primary hepatobiliary cancer	698 (2.9)	59 (8.5)	10.8 (8.0 - 14.7)	< 0.001		
hepatic carcinoma	392	38 (9.7)	11.5 (8.0 - 16.5)	< 0.001		
with cirrhosis	182	26 (14.3)	17.1 (11.1 - 26.4)	< 0.001	2.8 (1.3 - 5.6)	0.004
without cirrhosis	210	12 (5.7)	5.8 (3.2 - 10.6)	< 0.001	1[2]	
extrahepatic biliary / gall bladder carcinoma	313	21 (6.7)	7.2 (4.5 - 11.4)	< 0.001		
Secondary hepatic malignancy						
from all tumours	3446 (14.5)	113 (3.3)	4.9 (3.8 - 6.2)	< 0.001		
from pancreatic carcinoma	312	36 (11.5)	13.9 (9.6 - 20.2)	< 0.001	5.2 (3.4 - 7.8)[3]	< 0.001[3]
gastric carcinoma	316	18 (5.7)	5.9 (3.6 - 9.7)	< 0.001	1.9 (1.2 - 3.2)[3]	0.019[3]
colorectal carcinoma	637	13 (2.0)	2.0 (1.1 - 3.5)	0.028	0.5 (0.3 - 1.0)[3]	0.063[3]
Myeloproliferative disorders	231 (1.0)	7 (3.0)	3.0 (1.4 - 6.3)	0.012		
All patients	23796 (100)	254 (1.0)				

[1] *vs.* all patients with absence of respective condition. [2] reference category. [3] *vs.* patients with liver metastatic disease from other cancer forms.

men and 71 (69 - 74) years in women. Median age at death was 72 years; 70 years in men and 74 years in women.

Distribution of potential causative factors in PVT patients

Seventy-two (28 %) of the PVT patients had cirrhosis, and one third of those had also primary hepatic cancer (Table 1). In all 59 PVT patients (23 %) had primary hepatobiliary cancer and 111 (44 %) had secondary malignancy of the hepatobiliary region. Major abdominal infectious or inflammatory disease was present in 25 (10 %), 7 (3 %) had myeloproliferative disorders, whereas in 36 (14 %) of the patients with PVT, none of the above causal factors could be identified. The age distribution in these categories was similar (Table 1). Sixty-one per cent of the PVT patients with cirrhosis were men ($P = 0.018$), otherwise there were no gender differences.

PVT prevalence and risk in relation to presence of disease

Cirrhosis was present in 5 % (1193 / 23796) of the cohort and related to a 7.9 times increased odds for PVT (95 % CI: 6.0-10.5) (Table 2). Patients who also had primary hepatic cancer had a 3.5 times higher odds for PVT (95 % CI 2.1-5.8) than cancer-free cirrhosis patients, with a PVT prevalence of more than 14 %. Secondary hepatic malignancy was associated with 4.9 times increased odds (95 %

Table 3 Portal hypertension-related complications in categories of PVT based on likely etiology

Patient category	*n*	*n*	Complication					
			Ascites		Oesophageal varices		Gastrointestinal bleeding	
			n (%)	*P* value[2]	*n* (%)	*P* value[2]	*n* (%)	*P* value[2]
Cirrhosis[1]	72		45 (62)	<0.001	42 (58)	<0.001	34 (47)	<0.001
with primary hepatic cancer		26	21 (81)		13 (50)		7 (27)	
without primary hepatic cancer		46	24 (52)		29 (63)		27 (59)	
Primary hepatobiliary cancer[1]	59		34 (58)	0.016	15 (25)	0.18	15 (25)	0.72
without cirrhosis		33	13 (39)		2 (6)		8 (24)	
Secondary hepatobiliary malignancy[1]	111		46 (41)	0.61	4 (4)	<0.001	15 (14)	0.002
Myeloproliferative disorders[1]	7		4 (57)	0.47	2 (29)	0.62	2 (29)	0.67
Major abdominal infection / inflammation[1]	25		12 (48)	0.67	1 (4)	0.06	5 (20)	0.81
No cause identified	36		6 (17)	<0.001	2 (6)	0.036	3 (8)	0.02
All patients with PVT	254		110 (43)		48 (19)		59 (25)	

[1] Categories not mutually exclusive; [2] in comparison with all PVT patients not belonging to category.

CI 3.8 - 6.2). Within this group, pancreatic cancer patients had a 6.7 times higher odds for PVT (95 % CI: 3.4 - 13.1) than patients with liver metastatic disease from colorectal cancer.

Complications related to portal hypertension

Among the patients with PVT, the highest rates of complications related to portal hypertension were found in the subgroup with concomitant cirrhosis: 62 % had developed ascites, 58 % had oesophageal varices and 47% terminal gastrointestinal bleeding (Table 3). Corresponding figures in all PVT patients were 43 %, 19 % and 25 % ($P<0.001$ for all three comparisons), whereas of the 36 patients where no cause of PVT could be identified at autopsy only a minority had these signs of portal hypertension: ascites was only present in 6 (17 %; $P<0.001$), oesophageal varices in 2 (6 %; $P=0.036$) and only 3 (8 %; $P=0.020$) had developed gastrointestinal bleeding.

DISCUSSION

Our finding of an overall risk in the general population of 1 % to develop PVT during lifetime does not corroborate the concept of PVT being a rare disease. The figure might even be an underestimation, since it cannot be ruled out that in some cases of partial thrombosis earlier in life a spontaneous resolution may have occurred. It should therefore be relevant to view these patients with renewed interest.

Among the major causes of PVT in adults, cirrhosis is generally named the most common, followed by neoplasia[5-6]. In this study the order of magnitude was reversed, with cancer in two thirds of the patients. By nature of being an autopsy study it is a selection of the sickest patients, and in some of these the thrombosis may be a late event in advanced cancer disease. Discrepancies in proportion of cirrhosis patients compared with previously published case series might also reflect patient selection and ascertainment bias resulting from an increased diagnostic activity in high-risk categories, but one should also consider the impact of disease duration. An intriguing finding was the heterogeneity of risk within the group of metastatic malignancy, with a significantly higher risk with pancreatic cancer in comparison with other major gastrointestinal adenocarcinomas.

When assessing the risk for a patient with a certain condition to develop an infrequent complication like PVT, knowledge from case series on the distribution of causes to that complication may be helpful and is sometimes the only available information. It may be misguiding, though. In this study, secondary malignancies accounted for a twice as large fraction of PVT cases as primary hepatobiliary cancer, but the actual risk of PVT in patients with secondary malignancies was less than half of that associated with primary hepatic cancer. In patients with both cirrhosis and hepatic cancer the risks appeared to be additive resulting in a prevalence of 14%. Whether increased diagnostic and therapeutic activities would be effective to reduce this rate is to be considered for further studies. A recent study, though, indicates that even hepatocellular cancer patients, who have developed portal vein tumour thrombosis, may benefit from an active treatment if timely diagnosed with respect to hepatic function[16].

In one sixth of the PVT patients no explanation could be found despite the thorough examination. This group differed from the other patients by a low frequency of complications typical to portal hypertension. A cross-sectional study like the present has obvious limitations for assessment of previous diseases, and this group may harbour some cases of juvenile PVT, though recent longitudinal studies indicate that PVT is an infrequent complication to umbilical vein sepsis, and in the western world the risk of this complication to umbilical vein catheterisation has declined considerably over time[5,17-18]. Similarly, it cannot be ruled out that the thrombosis in the odd case might have been caused by surgical trauma not evident from the recent patient history. With increasing possibilities to detect various trombophilic disorders the proportion of non-explainable, so called idiopathic PVT has gradually become smaller[5,10,19-22]. The present study does not allow an investigation of markers of the haemostatic system, but it is not unreasonable that the

PVT in some of these cases may be an expression of a hypercoagulability state.

Autopsy rates have declined considerably also in Malmö, following changes in legislation, and it is unlikely that a similar study will ever be performed again. With regards to the setting in time, some limitations should be discussed. Apart from aspects of the design previously viewed, the potential effects on the epidemiology of an increasing use of antithrombotic therapies should be considered. The study does not allow an assessment of actual medications, but even today the majority of these conditions would not warrant primary or secondary anticoagulant prophylaxis according to guidelines[23].

From this first population-based study we conclude that PVT is more common than previously indicated by clinical series. The greatly increased risk in patients with cirrhosis and hepatic carcinoma should warrant an increased awareness in these patients. While anticoagulation therapy might be considered for certain patient categories, this remains to be evaluated in prospective studies.

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RAPID COMMUNICATION

Effects of hypobaric hypoxia on adenine nucleotide pools, adenine nucleotide transporter activity and protein expression in rat liver

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Abstract

AIM: To explore the effect of hypobaric hypoxia on mitochondrial energy metabolism in rat liver.

METHODS: Adult male Wistar rats were exposed to a hypobaric chamber simulating 5000 m high altitude for 23 h every day for 0 (H0), 1 (H1), 5 (H5), 15 (H15) and 30 d (H30) respectively. Rats were sacrificed by decapitation and liver was removed. Liver mitochondria were isolated by differential centrifugation program. The size of adenine nucleotide pool (ATP, ADP, and AMP) in tissue and mitochondria was separated and measured by high performance liquid chromatography (HPLC). The adenine nucleotide transporter (ANT) activity was determined by isotopic technique. The ANT total protein level was determined by Western blot.

RESULTS: Compared with H0 group, intra-mitochondrial ATP content decreased in all hypoxia groups. However, the H5 group reached the lowest point (70.6%) ($P < 0.01$) when compared to the control group. Intra-mitochondrial ADP and AMP level showed similar change in all hypoxia groups and were significantly lower than that in H0 group. In addition, extra-mitochondrial ATP and ADP content decreased significantly in all hypoxia groups. Furthermore, extra-mitochondrial AMP in groups H5, H15 and H30 was significantly lower than that in H0 group, whereas H1 group had no marked change compared to the control situation. The activity of ANT in hypoxia groups decreased significantly, which was the lowest in H5 group (55.7%) ($P < 0.01$) when compared to H0 group. ANT activity in H30 group was higher than in H15 group, but still lower than that in H0 group. ANT protein level in H5, H15, H30 groups, compared with H0 group decreased significantly, which in H5 group was the lowest, being 27.1% of that in H0 group ($P < 0.01$). ANT protein level in H30 group was higher than in H15 group, but still lower than in H0 group.

CONCLUSION: Hypobaric hypoxia decreases the mitochondrial ATP content in rat liver, while mitochondrial ATP level recovers during long-term hypoxia exposure. The lower level of extra-mitochondrial ATP may be related to the decrease of ANT activity during hypoxia exposure.

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Key words: Adenine nucleotide pool; Hypoxia; Liver; Mitochondria

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INTRODUCTION

The liver is the largest metabolic organ in the body. It performs a number of important and complex biological functions that are essential for survival. It also plays important roles in metabolism of carbohydrates, proteins, lipids, drugs, as well as in bile formation and secretion. Energy metabolism is closely related to these normal functions of liver. Mitochondria are the "energy factory" of cells. The adenine nucleotide transporter (ANT) is the most integral protein in inner mitochondrial membrane and consists of two identical subunits of 32 KD[1]. It catalyzes the transporter of cytosolic ADP and mitochondrial ATP in the process of phosphorylation[1]. Consequently, ANT is an important link between the cytosolic energy consumption and mitochondrial energy process yield[1,2]. Hypoxia could influence mitochondrial oxygenation respiration function[3,4] and F0-F1 ATPase activity of rat brain[4]. However, little is known about the relationship between the effects of hypobaric hypoxia on mitochondrial energy metabolism changes and ANT function in rat liver exposed to hypoxia. We therefore used

HPLC, isotopic assay and Western blot to examine the inner- and extra-mitochondria adenine nucleotide pool, ANT activity and its total protein level of rat liver exposed to hypobaric hypoxia.

MATERIALS AND METHODS

Chemicals

[2,8-^{3}H] ADP was obtained from Perkin-Elmer. Atractyloside (ATR), adenosine-5'-diphosphoric acid (ADP), albumin bovine serum (BSA), nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolye-phosphate (BCIP) and mouse anti-goat IgG-alkaline phosphatase (IgG-AP) were supplied by Sigma. Goat anti-human ANT polyclonal antibody was purchased from Santa Cruz.

Animals and treatments

Adult male Wistar rats (150-200 g) were used in the experiments. The rats were exposed to a hypobaric chamber simulating 5000 m high altitude for 23 h every day for 0 (H0), 1 (H1), 5 (H5), 15 (H15) and 30 d (H30) respectively. Animals were fed with laboratory chow and tap water *ad libitum*. Rats were sacrificed by decapitation at seal level (H0 group) and hypobaric chamber simulating 4000 m high altitude (H1, H5, H15, H30 groups). After decapitation the liver tissues were immediately excised, and part of it was frozen in liquid nitrogen, and then transferred to -70℃ for storage until use. The remaining liver tissues were rapidly placed into ice-cold isolation medium [0.25 mol/L sucrose, 10 mmol/L 4-(2-hydroxymethyl)-1-piperazine ethane sulfonic acid (HEPES), 1 mmol/L ethylene diamine tetraacetic acid (EDTA), pH 7.4][5]. The tissues were chopped finely with scissors while being washed three times with ice-cold isolation medium and then manually homogenized by 15 up and down strokes with Teflon glass pestle. The liver mitochondria were isolated by centrifugation as established in our laboratory[3,4]. The protein content in the mitochondria suspension was assayed by Lowry's method using BSA as the standard.

Assay of mitochondria adenine nucleotide pool

Adenine nucleotides were separated and quantitated by HPLC[4,6]. Briefly, 500 mmol/L ice-cold $HClO_4$ was added to 200 μL liver mitochondria suspension and after 5 min incubation at 4 ℃, the liquid was centrifuged (12 000 r/min, 20 min) at 4 ℃ and the supernatant was saved and neutralized with 1 mol/L K_2CO_3 to pH 6.5-7.0. The liquid was centrifuged (12000 r/min, 10 min) at 4 ℃ again. The samples were maintained frozen at -70 ℃ until defrosted and analysed by HPLC equipped with high pressure pumps (Waters, USA), fit with 4.6 mm× 250 mm hypersil 18 (5 μm pore size, from Sigma). The samples were applied in a total volume of 20 μL. The results were shown as nmol adenine nucleotides/mg mitochondrial protein.

Assay of liver tissue adenine nucleotide pool[5,6]

The liver tissues stored at -70 ℃ were rapidly transferred into ice-cold 0.5 mol/L $HClO_4$, chopped finely with scissors, and then manually homogenized by 10 up and down strokes with Teflon glass pestle. The homogenate was placed at 4℃ for 5 min, centrifuged (12 000 r/min, 20 min) at 4 ℃. The supernatant was saved and neutralized with 1 mol/L K_2CO_3 to pH 6.5-7.0. The liquid was centrifuged (12 000 r/min, 10 min) at 4 ℃ again and maintained frozen at -70 ℃ until supernatant was analyzed by HPLC as described above. The results were expressed as nmol/mg tissue.

^{3}H- ADP label and liquid scintillation

The activity of ANT was determined at 4℃ by isotopic technique[1]. ^{3}H- ADP label was stopped by atractyloside, a specific inhibitor of ANT. Fifty microliter mitochondrial suspension solution was diluted by 150 μL ice-cold isolation medium. Twenty microliter ^{3}H- ADP (specific activity 33.9 Ci/mmol ADP) 0.3 μmol/L were added. After 10 s incubation at 4 ℃, the reaction was inhibited by 50 μL 3.2 nmol/L ATR, then centrifuged (12 000 r/min, 20 min) at 4 ℃. The precipitation was dissolved with 1 ml ice-cold isolation medium, centrifuged (12 000 r/min, 20 min) at 4℃ again for 20 min and the process was repeated three times. The pellet was digested at 70 ℃ with 7 mol/L $HClO_4$ 200 μL, 8.8 mol/L H_2O_2 400 μL for 40 min. The 200 μL sample was dissolved in 1 ml scintillator (5 g PPO was dissolved by 700 mL dimethylbenzene and 300 mL anhydro-ethanol) and measured in liquid scintillation counter. ANT activity was calculated from the radioactivities of H^3-ADP [count per min (cpm)]. Nonspecific binding of H^3-ADP to mitochondria was determined by incubation of mitochondrial samples with 50 μL 3.2 nmol/L ATR prior to addition of 0.3 μmol/L ^{3}H- ADP. The results were expressed as pmol ADP/min per milligram mitochondrial protein.

Western blot

The rat liver mitochondrial ANT protein level was determined by Western blot[1]. The mitochondria sample was solubilized in sample buffer and supplemented with 5 μL β-mercaptoethanol. Following that, it was heated for 10 min at 100 ℃ and supplemented with 5 μL β-mercaptoethanol again. Electrophoresis was performed on a 12% polyacrylamide slab gel with Tris/glycine running buffer. The staking gel contained 5% polyacrylamide. The lanes were loaded with 30 μL aliquots of solubilized mitochondria (10 μg). After separation the protein bands were stained with Commassie blue for 4 h or transferred to polyvinylidene difluoride (PVDF) sheet for 90 min. The PVDF sheet was washed twice with Tris-buffered saline (TBS) containing 0.1% Tween-20 (0.1% TBST), pH 7.4 at room temperature. ANT polyclonal antibody at a 1:1000 dilution was used. Incubation was performed in 0.1% TBST, pH 7.4 for at least 12 h at 4℃. The sheet was washed six times with 0.1% TBST at room temperature. Mouse anti-goat IgG coupled with alkaline phosphatase at a 1:1000 dilution was used as secondary antibody. Incubation was performed in 0.1% TBST, pH 7.4 for 1 h at room temperature. The sheet was washed six times with 0.1% TBST again. Antigen was visualized by luminescence (NBT/BCIP). Signals were quantified with Smartview (Furi

Table 1 Changes of total adenine nucleotide pool in liver tissue during hypoxia exposure (nmol/mg tissue, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	9.32±1.63	9.02±0.87	4.00±0.26
H1	6	7.14±1.41	5.53±0.71[b]	2.40±0.29[b]
H5	6	4.90±0.74[b]	4.57±0.48[b]	2.41±0.17[b]
H15	6	6.08±1.57[a]	5.40±1.31[b]	3.11±0.25[b]
H30	6	6.58±1.19[a]	6.21±1.36[b]	3.27±0.30[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group.

Table 2 Changes of intra-mitochondrial adenine nucleotide pool of rat liver during hypoxia exposure (nmol/mg protein, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	6.84±1.57	11.34±1.97	5.47±0.54
H1	6	3.68±0.42[b]	4.52±1.07[b]	3.86±0.20[b]
H5	6	2.72±1.09[b]	4.76±1.56[b]	4.03±0.25[b]
H15	6	3.94±0.68[b]	5.07±0.10[b]	4.50±0.35[b]
H30	6	3.77±0.67[b]	8.70±2.11[b]	4.72±0.60[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group.

Science and Technology Co., Ltd., Shanghai, China).

Statistical analysis

Results were expressed as mean ± SD. Significant difference was determined by one-way ANOVA followed by LSD test between different groups. Statistical analyses were performed using SPSS 12.0 software.

RESULTS

Changes of adenine nucleotide pool in rat liver tissue during hypoxia exposure

Compared with H0 group, liver tissue ATP and ADP content decreased significantly in all hypoxia groups ($P<0.01$). Tissue AMP in groups H5, H15, and H30 was significantly lower than in H0 group ($P<0.05$ and $P<0.01$), while H1 group had no marked change compared to H0 group (Table 1).

Changes of intra-mitochondrial adenine nucleotide pool of rat liver during hypoxia exposure

Compared with H0 group, intra-mitochondrial ATP content decreased in all hypoxia groups, which in H1 group was 70.6% of that in H0 group ($P<0.01$) reaching the lowest point. Intra-mitochondrial ADP and AMP levels showed the same change and were significantly lower in H0 group than that in all hypoxia groups ($P<0.01$) (Table 2).

Changes of adenine nucleotide pool in extra-mitochondria of rat liver during hypoxia exposure

Compared with H0 group, extra-mitochondrial ATP and ADP content decreased significantly in all hypoxia groups ($P<0.05$ and $P<0.01$). Extra-mitochondrial AMP in groups H5, H15, and H30 was significantly lower than

Table 3 Changes of adenine nucleotide pool in extra-mitochondria of rat liver during hypoxia exposure (nmol/mg tissue, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	8.64±1.58	8.08±0.93	3.45±0.22
H1	6	6.77±1.40	5.07±0.74[b]	2.01±0.27[b]
H5	6	4.63±0.82[b]	4.09±0.60[b]	2.01±0.15[b]
H15	6	5.68±1.60[a]	4.90±1.34[b]	2.67±0.27[b]
H30	6	6.21±2.01[a]	5.37±1.53[b]	2.80±0.33[a]

[c]

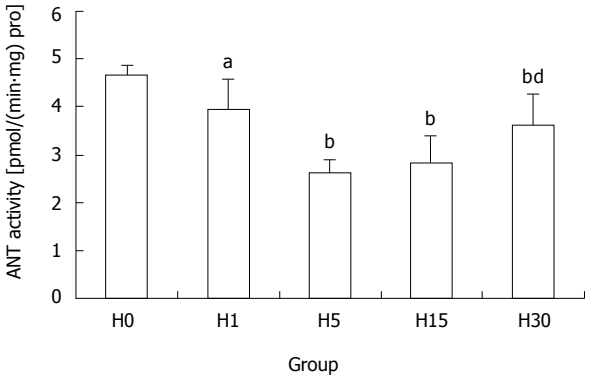

Figure 1 Change of mitochondrial ANT activity of rat liver during hypoxia exposure (mean±SD). [a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group; [d]$P < 0.01$ *vs* H15 group.

that in H0 group ($P<0.01$), while in H1 group it had no marked change compared with H0 group (Table 3).

Change of mitochondrial ANT activity of rat liver during hypoxia exposure

Compared with H0 group, the activity of ANT decreased significantly in all hypoxia groups, which in H5 group was 55.7% of that in H0 group ($P<0.01$), being the lowest. Activity in H30 group was higher than that in H15 group ($P<0.01$), but was still lower than in H0 group ($P<0.01$) (Figure 1).

Effect of hypoxia on mitochondrial ANT protein expression

ANT protein expression in H5, H15, and H30 groups, compared with H0 group, decreased significantly, which in H5 group was the lowest point (27.1%) ($P<0.01$). The expression in H30 group was higher than that in H15 group ($P<0.01$), but was still lower than that in H0 group ($P<0.01$) (Figures 2 and 3).

DISCUSSION

ATP is the direct energy for cell usage. Mitochondrial ATP level is influenced by two factors. First, mitochondrial ATP is produced by oxidative phosphorylation. Mitochondria oxidative respiration and phosphorylation states are the main factors that affect the ATP level. Second, mitochondrial ATP provides energy for cytoplasm as well as for its own demand such as the synthesis of mitochondrial DNA, RNA and proteins, etc. Our previous work showed that during hypoxia exposure, mitochondrial

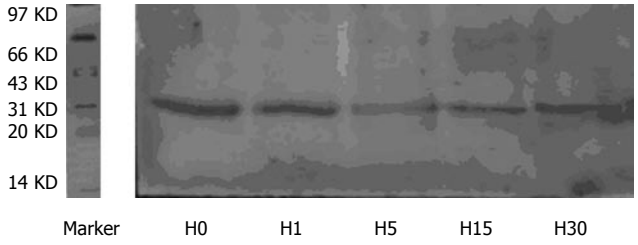

Figure 2 Western blot results of mitochondrial ANT protein in rat liver exposed to hypobaric hypoxia.

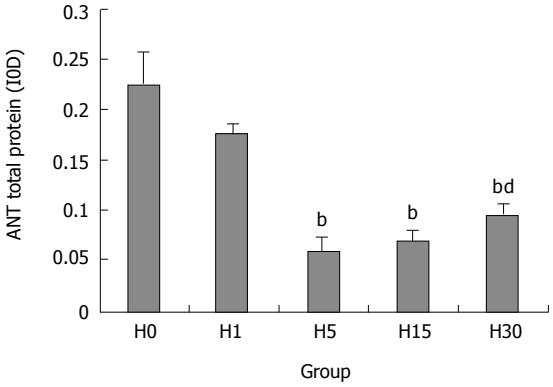

Figure 3 The effect of hypoxia on mitochondrial ANT total protein level (mean± SD). $^{b}P < 0.01$ *vs* H0 group; $^{d}P < 0.01$ *vs* H15 group.

ATP content and F_0-F_1 ATPase activity of rat brain decreased significantly compared with control [4,7]. This indicates that hypoxia could influence ATP production and then the mitochondrial ATP level. However, there were no reports about the hypobaric hypoxia effect on rat liver mitochondrial adenine nucleotide pool.

The current results showed that ATP level in rat liver mitochondria reduced during hypoxia exposure, which in H1 group was the lowest point, 70.6% of control ($P<0.01$). The decrease of mitochondrial ATP content may be related to the following factors. First, mitochondrial oxidative respiratory function was inhibited. Our previous study showed that hypobaric hypoxia inhibited the oxidative respiratory function of mitochondria in rat brain[3,4,6]. Our results (data not shown) also revealed that hypoxia significantly decreased the mitochondrial three state oxygen consumption and respiratory control rate in rat liver, while significantly increased the rat liver mitochondrial four state oxygen consumption. Second, hypoxia decreased the mitochondrial membrane potential (MMP). Our results (data not shown) demonstrated that hypoxia inhibited the MMP of rat hepatocytes. However, MMP is the motive power of mitochondrial ATP synthesis. Thirdly, hypoxia lowered the mitochondrial F_0-F_1 ATPase activity[4,8]. Fourthly, the lower mitochondrial ATP content may be also related to the reduction of intra-mitochondrial ADP concentration[1], intra-mitochondrial Ca^{2+} content[9] and extra-mitochondrial adenine nucleotide pool[10, 11] .

ANT is the most integral protein in inner mitochondrial membrane and consists of two identical subunits of 32 KD[1]. It is a key energy link between the mitochondria and cytoplasm since it catalyses the transmembrane exchange between ATP synthesized by the F1-Fo ATP synthase inside mitochondria and ADP generated by the metabolism in cytoplasm[1,6]. The ADP/ATP exchange follows the Michaelis-Menten kinetics and ANT activity is moderate, 1500-2000 molecules per min[12]. Schonfeld et al reported that matrix adenine nucleotides and the ANT protein content are associated with the changes of the ANT activity in rat heart mitochondria[1]. Rulfs et al also reported that matrix adenine nucleotide concentration influenced the ANT activity in rabbit liver mitochondria[13]. However, little is known about the effect of hypobaric hypoxia on mitochondrial ANT activity in rat liver.

Our study showed that the activity of ANT after hypoxia exposure decreased significantly. The ANT activity was the lowest point, 55.7% of control after hypoxia exposure for 5 d ($P<0.01$), while after hypoxia exposure for 30 d it was higher than after 15 d exposure ($P<0.01$), but was still lower than control. This indicates that hypoxia could inhibit the mitochondrial ANT activity in rat liver. The decrease of ANT activity may be related to the following factors. First, the ANT protein level and content was the main factor. Our results showed that ANT protein expression decreased significantly in H5, H15, H30 groups, which in H5 group was 27.1% of that in H0 group. Second, MMP also influenced the ANT activity[14]. Passarella *et al* reported that helium neon laser increased the rate of ADP/ATP exchange through increasing the MMP in rat liver[15]. In the presence of an MMP of about 100 mV positive inside, the rates of the [^{14}C] ATP_{out}/ADP_{in} exchanges were stimulated[3]. All these indicate that MMP is one of the most important factors that affect the ANT activity. The mechanism that decrease of MMP reduced ANT activity is not clear. Thirdly, the change of ANT conformation also influenced its activity. ANT has two conformational states, cytosolic conformation (c-confromation) and matrix conformation (m-conformation)[16,17]. ANT is not a pore, which opens or closes simply as a response to stimuli. Conformational changes have to occur to release nucleotides to the matrix (and the reverse) without creating leakage in the membrane. Fourthly, it was reported that the size of mitochondrial adenine nucleotide pool influenced the ANT activity. Previous studies showed that the postnatal increase in the matrix adenine nucleotides concentration contributed to the increase of ANT activity in rat liver[13] and heart[1].

The decrease of extra-mitochondrial ATP level influences the mitochondrial carrier family including ANT synthesis and transport. Extra-mitochondrial ATP level is mainly determined by the ANT activity. However, the lower ANT protein level has an identical role in influencing ANT activity during hypoxia. So the ANT activity-ANT protein level- ATP content form the vicious cycle and aggravate the dysfunction of cell energy metabolism during hypoxia exposure.

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CASE REPORT

Liver cell adenoma: A case report with clonal analysis and literature review

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Abstract

We report a case of liver cell adenoma (LCA) in a 33-year-old female patient with special respect to its clonality status, pathogenic factors and differential diagnosis. The case was examined by histopathology, immunohistochemistry and a clonality assay based on X-chromosomal inactivation mosaicism in female somatic tissues and polymorphism at androgen receptor focus. The clinicopathological features of the reported cases from China and other countries were compared. The lesion was spherical, sizing 2 cm in its maximal dimension. Histologically, it was composed of cells arranged in cords, most of which were two-cell-thick and separated by sinusoids. Focal fatty change and excessive glycogen storage were observed. The tumor cells were round or polygonal in shape, resembling the surrounding parenchymal cells. Mitosis was not found. No portal tract, central vein or ductule was found within the lesion. The tumor tissue showed a positive reaction for cytokeratin (CK) 18, but not for CK19, vimentin, estrogen and progesterone receptors. Monoclonality was demonstrated for the lesion, confirming the diagnosis of an LCA. Clonality analysis is helpful for its distinction from focal nodular hyperplasia.

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Key words: Liver Cell adenoma; Clonality; Literature review

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INTRODUCTION

Liver cell adenoma (LCA) is a rare benign liver tumor. It is considered a hepatocellular neoplasm, while some authors hold that LCA originated from liver progenitor cell[1]. A female predominance was noticed for the development of LCA based on the reports from Western countries, with a majority of cases associated with a long-term use of oral contraceptives or other steroids. Other diseases should be excluded before establishing the diagnosis of an LCA. It is indeed a difficult task to distinguish LCA from focal nodular hyperplasia (FNH) when the morphologic features are not prominent and a central scar is absent. Then some molecular approaches, including clonality analysis, may be helpful. In this article, a case of LCA in a female Chinese patient is presented, with its clonality status demonstrated.

CASE REPORT

A hepatic mass was found in the right lobe of a 33-year-old woman during her routine medical check-up. She was then admitted to Tangdu Hospital in Xi'an in January 28, 2003. She had never used oral contraceptives, and she had no history of alcohol abuse or hepatitis. No record of HCC or any hereditary disease was found among her family members. The parameters of routine clinical biochemistry, including values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GTP) and concentrations of α-fetoprotein (AFP) and plasma proteins, were all within normal ranges. The laboratory tests failed to show any positive signal in her serum for hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody.

Ultrasonography revealed a solid mass in the posterior part of the right lobe of liver. Computed tomography (CT) scanning showed reduction of density for the lesion sizing 2.0 cm in diameter (Figure 1), indicating malignant potential. Laparotomy was then performed on February 1, 2003. Size of the liver appeared normal, with a mass found in the right lobe. Partial hepatectomy was then performed. Appearance, color and texture of the surrounding liver

were normal, without any indication of cirrhosis, pronounced fibrosis or cholestasis.

Histological and immunohistochemical procedures

The sample was fixed in 40 g/L formaldehyde solution, embedded in paraffin. Sections of 4 μm in thickness were prepared and stained by hematoxylin and eosin (HE), Masson trichrome methods and periodic acid-Schiff (PAS) reaction. Immunostaining was carried out using a streptavidin-labeled peroxidase (S-P) kit (KIT9730) as described previously[2]. The primary antibodies used in this study included those against cytokeratin (CK) 18, CK19, vimentin, CD34, estrogen receptor (ER), progesterone receptor (PR), AFP, S-100 protein, HBsAg, hepatitis B core antigen (HBcAg), as well as an anti-HCV antibody. All of the reagents for immunostaining were supplied by Maxim Biotechnology Corporation Limited, Fuzhou, China.

Clonal analysis

Sections of 10 μm in thickness were prepared, deparaffinized, rehydrated and HE stained. Neoplastic tissues were dissected using a syringe needle from 4 different tumor areas, sizing 0.5 cm × 0.5 cm for each. Normal liver tissue was also isolated from the surrounding parenchyma at 3 different sites of the same size and analyzed in a parallel way as reference samples. Genomic DNA was extracted using a QIAamp kit (Qiagen, Mannheim, Germany). Polymorphism was examined at the androgen receptor (AR) and phosphoglycerokinase (PGK) loci as described previously[3,4], with the former gene proved polymorphic at the CAG short-tandem repeat (STR) located in exon 1. Loss of X-chromosomal inactivation mosaicism was demonstrated by pretreatment of DNA with methylation-sensitive restriction enzyme *Hba* I and amplification via nested PCR. The CAG STR-polymorphic alleles were resolved on a 100 g/L denaturing polyacrylamide gel at 120 V for 4 h. A reduction of at least 50% in density of either band, as compared to that obtained using the sample not treated with *Hba* I, is regarded as loss of X-chromosomal inactivation mosaicism [5].

RESULTS

A partial resection liver specimen, sizing 4.0 cm × 3.0 cm × 2.0 cm, presented with a spherical mass of 2.0 cm in its maximal dimention. The mass was beneath the hepatic capsule, yellow brown in color and soft in its texture, without any necrotic focus or fibrotic scar in its cut surface. There was a clear border, but not a fibrotic septum between the lesion and surrounding liver tissue that appeared red brown and apparently normal. Microscopically, the lesion was composed of cells arranged in two-cell-thick cords, with the cell cords separated by sinusoids (Figure 2A). Focal fatty change and excessive glycogen storage were present (Figure 2A and 2B). The tumor cells were round or polygonal, apparently resembling the surrounding liver parenchyma cells in size and shape. Mitosis was not found. There was no portal tract or hepatic venule in the tumor. Ductule or scattered ductular cells (the so-called "oval cells"[6,7] or "liver progenitor cells"[1]) were absent, and immunostaining for the ductular cell markers, including CK19 and S-100 protein[7,8], failed to show any positive cell within the lesion. The tumor cells were positive for CK18 (Figure 2C), but negative for AFP, vimentin and p53 protein. They did not show any positive signal for ER or PR. Both neoplastic and the adjacent parenchymal tissues were negative for HBsAg, HBcAg and HCV antigen.

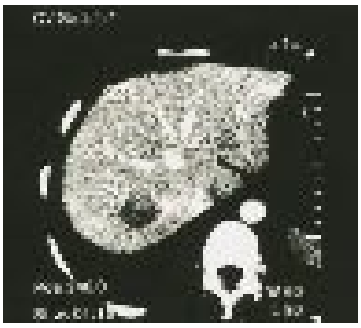

Figure 1 CT scanning showing a lesion with reduction of density in the posterior part of the right lobe of liver.

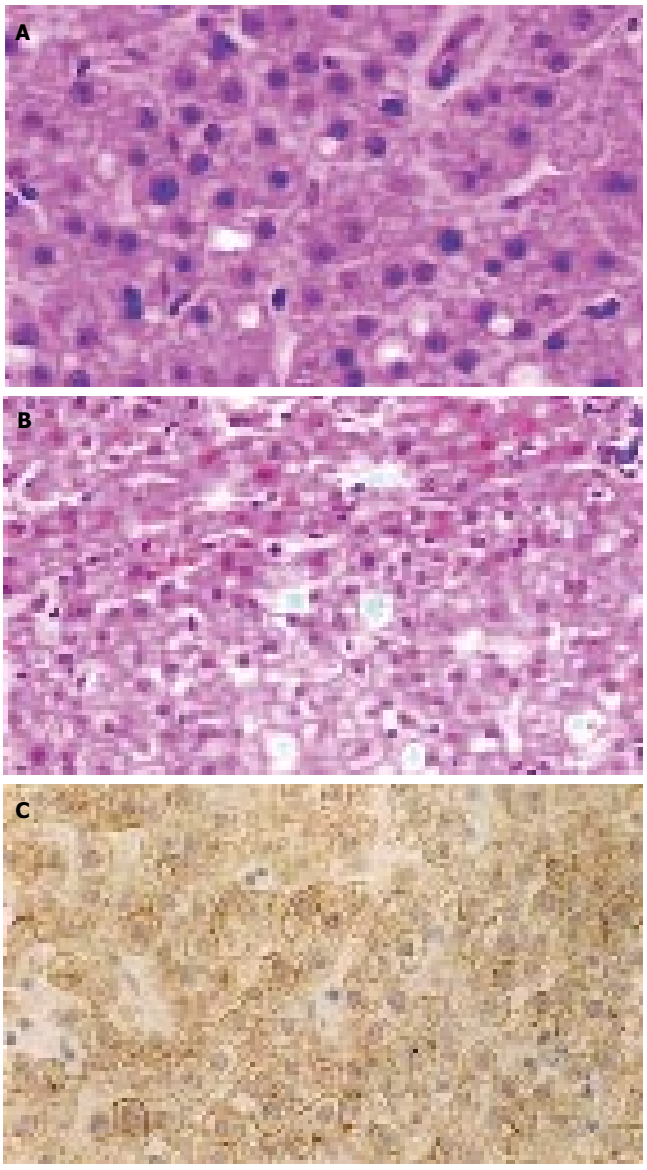

Figure 2 Liver cell adenoma (× 400). **A:** Similar to normal hepatocytes, arranged in two-cell-thick cords separated by hepatic sinusoids (HE); **B:** Showing the border between LCA (lower) and surrounding liver parenchyma (upper) (HE); **C:** CK18 immunoreactivity (S-P).

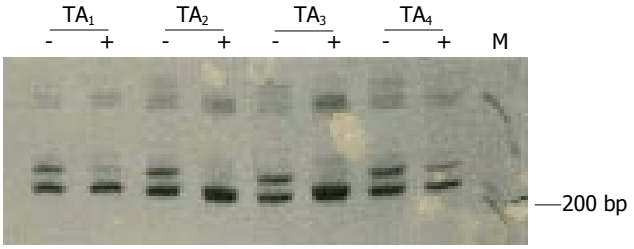

Figure 3 Clonal analysis on tissues from 4 tumor areas (TAs). The pretreatment with *Hha* I results in loss of the upper band for TA_2 and TA_3, and intensity reduction by factors of 71.8% and 57.1% for TA_1 and TA_4, respectively. +, with H*ha* I pretreatment; -, without *Hha* I treatment.

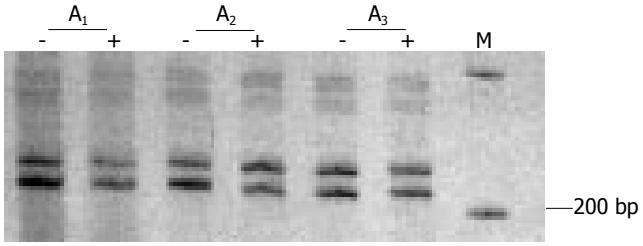

Figure 4 Data of clonality test of three areas of liver parenchymal tissues (A_1-A_3). Neither of the bands on gel shows marked intensity change for the samples pretreated with Hha I (+) compared to those not treated (-).

Clonality status of the lesion was determined by an assay based on the CAG-STR polymorphism at exon 1 of AR gene. Pretreatment with *Hha* I resulted in pronounced reduction or loss of the upper band for all of the tissue samples from 4 different areas of the lesion (Figure 3), demonstrating loss of the X-chromosomal inactivation mosaicism. The adjacent liver parenchyma, however, did not show the change (Figure 4). The data proved the monoclonal, neoplastic nature of the lesion, confirming the diagnosis of LCA. The patient has survived for 30 mo after the operation without any indication of recurrence.

DISCUSSION

LCA is a rare benign hepatic neoplasm, accounting for less than 2% of all hepatic tumors[9]. A pronounced female predominance was noted mainly by authors from Western countries. It often occurs in women of 20 to 40 years during their child-bearing period[10,11], being closely associated with long-term use of steroids, mainly oral contraceptives[12-14]. In fact, it was rarely reported before the introduction of oral contraceptives in 1960s. Edmondson *et al*[15] found only two cases in 48 900 necropsies performed in Los Angeles General Hospital during the period from 1918 to 1954. In 1973, Baum *et al*[16] pointed out the possible link between the use of oral contraceptives and LCA development. Data from several reports has confirmed the etiologic association. Leese *et al*[17] reported 24 cases of HA, 16 (66.7%) of them with a history of using oral contraceptives. Tumor regression was observed in some cases after withdrawal of the hormones[18,19], and then the tumor remained silent or grew slowly for many years, or even progressed to HCC[20], albeit infrequently. Complete remission was observed in an LCA patient, who had used oral contraceptives for 8 years, after the hormone withdrawal for 9 months[21].

Through literature review, the clinicopathological data of 127 cases of LCA reported from Chinese patients were collected and compared to those of 130 patients from Western countries, with the male/female ratios being 1.8/1 and 1/2.9, respectively. The ages of the Chinese patients ranged from 2 to 73 years, with their mean and average values estimated to be 31.0 and 35.8 years, respectively. For patients from Western countries, the ages ranged from 11 months to 82 years, with their mean and average values 30.0 and 31.6 years, respectively. Only 3.9% (5/127) of Chinese patients had a history of using oral contraceptives, the percentage being much lower than that for the patients from Western countries (54/130, 41.5%). Difference is evident, therefore, between the LCAs occurring in China and in Western countries in their etiologic associations, indicating possibly different pathogenic pathways. This, at least partly, provides an explanation for the distinct gender distribution pattern in Chinese patients.

Other factors are also linked to LCA development, including glycogen storage diseases [22] and administration of danazol[23], phenobarbital[24] and androgenic/anabolic steroids[9,25-33]. Among the 130 cases from Western countries, 4 (3.1%) were found to have glycogen-storage diseases, but only one (0.8%), in the Chinese group, was with the association. For some LCA cases, there seemed no identifiable pathogenic factor. The majority of the cases from the Chinese group fell into this category. Among the 127 Chinese patients, 13 (10.2%) were seropositive for HBsAg, and 8 of them were with chronic hepatitis[34-37]. Among the patients from Western countries, however, only one (0.8%) was shown to be an HBsAg-carrier. In consideration of the high prevalence (up to 10%[38]) of HBsAg-carrier state in China, the data does not provide support for the role of persistent HBV infection in development of the solitary LCA.

Most of LCAs grow slowly and are asymptomatic or cause only mild symptoms, while rupture and hemorrhage may occur in some tumors (15%-33%[39]), and malignant transformation was also observed in a minority of the cases (5/39, 12.8%[20]). Ultrasonography, CT scanning and magnetic resonance imaging (MRI) are useful for detecting hepatic occupation and determining its location and size. Imaging approaches may be helpful for identifying LCA from other hepatic lesions, and a peritumorous halo, demonstrated by CT scanning, was considered indicative of LCA[40,41]. These features, however, are not specific[40], and the accurate preoperative diagnosis of LCA, particularly the distinction from HCC, is frequently problematic. This is even more serious as a surgical consideration in China where incidence of HCC is overwhelmingly high compared to that of LCA. For the pathologists, well differentiated HCC and FNH are among the hepatic lesions that should be excluded before making a diagnosis of LCA.

HCC is diagnosed usually at ages between 40 and 60 years, with a pronounced male predominance. In China, including Hong Kong and Taiwan, about 80% of HCCs were found in patients with chronic hepatitis B and cirrhosis or advanced liver fibrosis. An elevated level of circulating AFP is indicative of HCC, but this change may

not be evident in patients with an early-stage, often well differentiated, HCC. Liver-cell plates more than three cells thick, acinar structures, increased nuclear/cytoplasmic ratio, prominent nucleoli, mitoses, increased cytoplasmic basophilia, loss of the reticulin fibers, absence of Kupffer cells, presence of vascular invasion or immunoreactivity for AFP, all indicate HCC. However, none of these features can be relied upon with certainty, as some are also seen in the hepatic lesions with high-grade SCC, including the premalignant nodules of altered hepatocytes[2], adenomatous hyperplasia[42-46] and LCA. The most helpful parameters, in our consideration, are thickness of the liver-cell plates (more than three cells), cell density (an increase of two folds compared to the surrounding liver parenchyma) and vascular invasion[38]. It should be noted that male, cirrhosis, and chronic HBV infection strongly indicate that a hepatic neoplasm is malignant. Conversely, female and a history of oral contraceptive use support the diagnosis of LCA. Exceptional difficulties may be encountered in some of the LCAs associated with long-term use of anabolic steroids or metabolic disorders. Some of them may show pronounced architectural disturbance and cellular atypia, which make their distinction impossible from well-differentiated HCCs based on histological grounds alone. Malignant transformation is proposed for such cases[13,20,47,48], but they often show more favorable clinical courses, or even regression after withdrawal of the steroid[21]. Such lesions may represent the borderline hepatocellular neoplasm, and their behaviors should be determined by careful postoperative observations.

FNH occurs most commonly in young women, having similar etiological associations and clinical manifestations to LCA[49-51]. It is a localized lesion, frequently solitary, within an otherwise normal or nearly normal liver. The lesion is similar to cirrhosis by its histology, and a central stellate fibrous region containing large vessels can often be found. Its development has been attributed to the vascular malformation[52,53]. Usually, FNH is readily distinguished from LCA by its central scar, multinodularity and presence of proliferating bile ductules in the fibrous septa. It may become a diagnostic problem, however, when the central fibrous region is not evident. Data from different laboratories have demonstrated the polyclonal cell composition and indicated non-neoplastic nature for the lesion[54,55]. In contrast, an LCA was shown to be monoclonal[56], and our data confirm the conclusion that LCA is a neoplastic lesion. The clonality assays, therefore, are helpful for the differential diagnosis between LCA and some FNH lesions without an identifiable central scar.

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CASE REPORT

Adult intussusception caused by cystic lymphangioma of the colon: A rare case report

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Abstract

We experienced a case of intussusception caused by cystic lymphangioma of the colon in a 32 years old female who was admitted to our hospital for the chief complaint of bloody stool. In the colonoscopic examination, cystic mass with stalk which had smooth mucosal surface was noted at the descending colon. Abdominal ultrasonography and computed tomography revealed left colon intussusception with a multilocular cystic tumor as a leading point. Emergent operation was performed. On the histopathologic examination, the cystically dilated spaces lined by endothelium and septated by fibrous septa were present. The pathological diagnosis was cystic lymphangioma of the colon. Although intussusception due to lymphangioma in an adult are rare, it should be taken into consideration that it is possible diagnosis.

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Key words: Cystic lymphangioma of the colon; Adult intussusception

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INTRODUCTION

Lymphangioma is a benign tumor consisting of several expanded lymphatics, each lymphatics is surrounded by benign endothelial cells and it could be developed in any parts of the body. However, it is developed primarily in the neck, the axillary area, the scapula and other areas where lymphatic organs are abundant[1,2]. Lymphangioma is hardly developed in the digestive organs and the development of the colon is very rare. It was reported for the first time by Chisholm and Hillkowitz in 1932 and recently, with the increased application of colonoscopy and endoscopic ultrasonography, these cases are on the rise[4,5]. We report a case who was diagnosed as intussusception due to cystic lymphangioma by abdominal ultrasound, computed tomography and colonoscopy.

CASE REPORT

A 32-year-old female was admitted to our hospital due to bloody stool for 3 d. In her past, family and social history, specific findings were not detected. At the time of visit, blood pressure of 100/60 mmHg, pulse rate of 76 per min and body temperature of 36.7 ℃ was recorded. In physical examination, the acute ill appearance was detected and she were conscious. The conjunctiva was pale and the head-neck lymph node was not palpated. In abdominal examination, the liver or the spleen was not palpated and the bowel sound was normal. But, left lower quadrant pain was detected, however, the rebound tenderness was not detected. In laboratory findings, in peripheral blood test, the number of leukocyte was 16 140/mm^3, hemoglobin was 128 g/L, and platelet was 317 000/mm^3. In biochemical test was all normal. CEA, CA 125 and CA 19-9 showed normal limits.

In colonoscopic examination, a cystic mass with a stalk of the descending colon was detected (Figure 1). The mass was covered with the normal mucosa with smooth surface and palpated with the tissue biopsy forceps, it was cystic that changed readily. On the next day, the patient reported severe abdominal pain and thus abdominal ultrasonography and abdominal computed tomography were performed. In abdominal ultrasonography, a cystic mass divided by several septa was detected and the situation was that intussusception by this mass (Figure 2). In abdominal computed tompgraphy, a mass was detected and in the proximal area, intussusception was detected (Figure 3). The patient underwent emergent surgery and the partial resection of the descending colon was performed. The resected specimen was a mass 2.5 cm× 3.5 cm× 5.0 cm in size and similarly to ultrasonographic finding. The specimen was a bag shape, its inside was divided into several fibrous septa and filled with clear serous liquid (Figure 4). In histological finding, the cyst was located below the mucosal mem-

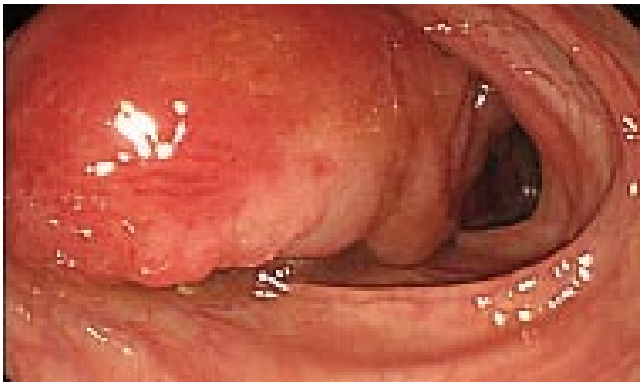

Figure 1 Colonoscopic examination shows a 2.5 cm x 2.5 cm x 5.0 cm sized round, smooth surfaced mass covered by normal mucosa in the descending colon. This mass is pedunclated cystic nature, and compressible pillow sign positive.

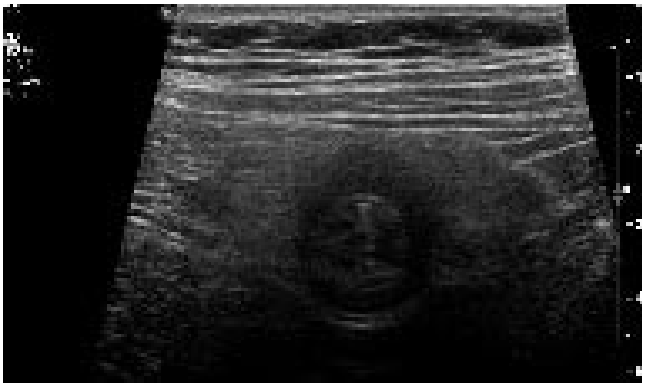

Figure 2 Ultrasonography of the mass shows a multilocular cystic lesion with septa.

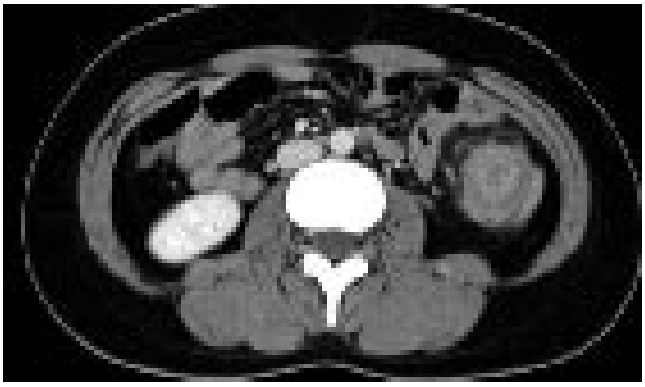

Figure 3 Computed tomography shows the cystic lesion in the descending colon, occupying the whole lumen, and also shows multicentric target sign and sausage-shaped inhomogeneous soft-tissue mass.

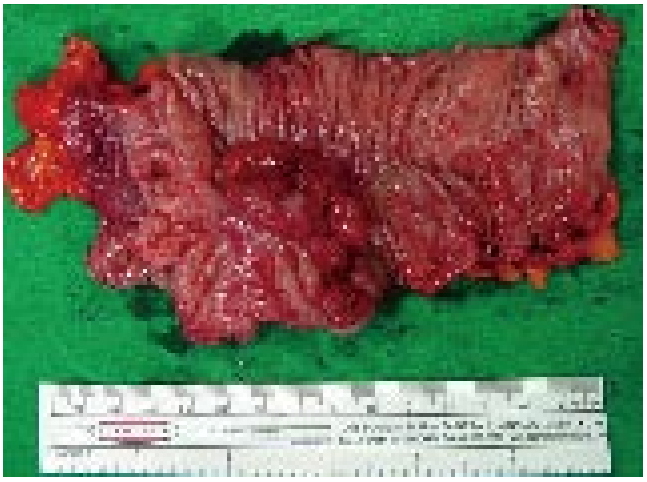

Figure 4 Macroscopic finding of the resected specimen reveals 2.5 cm x 3.5 cm x 5.0 cm sized multiseptated submucosal cystic tumor, which changed shape easily. The tumor shows fluctuation and serous clear fluid was aspirated by needle puncture.

brane, the inside was surrounded by normal epithelial cells and divided by several fibrous septa (Figure 5). Based on the above findings, she was diagnosed as intussusception caused by cystic lymphangioma.

DISCUSSION

Intussusception in adults is developed relatively infrequently. They are equivalent to 1% of colonic obstructive patients and they are equivalent to 5 - 10% of total intussusception[1,2]. Different from in children, they occur secondarily by definite lesions. For the diagnosis of such intussusception in adults, computed tomography has been reported to be the most useful radiological method[1]. This case is a patient that intussusception is caused by the cystic lymphangioma in the descending colon. Lymphangioma within the abdominal cavity is rare, and it occupies less than 5% of the entire lymphangioma[3]. Most reported cases are the cases detected accidentally during colonoscopy or barium enema and definitely diagnosed histologically by the resection of the lesion[5, 7]. Pathophysiologically, according to its shape, it is classified into 3 types: (1) simple capillary, (2) cavernous and (3) cystic. As in our case, cystic cases are most frequent. Recently, with the increase of the application of colonoscopy, the reported cases of the lymphangioma of the colon are increased [4,5]. The findings suggesting lymphangioma are the characteristics that are the round and soft surface, a wide base area, pink color and semi-transparent. In response to the peristaltic movement or the position of patient, its shape changes and it has the characteristic that the diameter and the location change upon pressing by forceps. In our case also, it was stalked cystic and the smooth surface of mass was covered with the normal mucosa of large intestine, and palpated with the biopsy forceps, it showed the pattern of readily changing its shape. The disease should be differentiated from other mass lesions, such as lipoma, cavernous angioma, leimyoma and enteric duplication cyst. However, endoscopic findings are very diverse, subjective and it is not of help to the diagnosis. So, the confirmation of its structure by endoscopic ultrasonography is important for the differential diagnosis[6]. In ultrasonography, it is detected as a mass limited to the submucosal area, separated well from adjacent tissues, and divided by several septa and for the cases showing such findings, it could be definitely diagnosed without further tests, and for the asymptomatic cases, the follow-up observation without treatment is sufficient[7]. Occasionally, nonspecific symptoms, in other

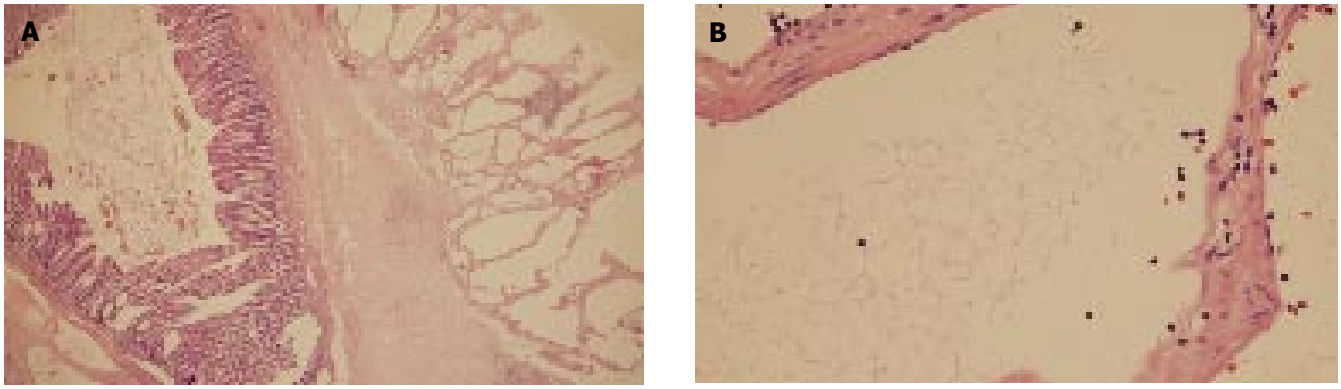

Figure 5 (**A**) Cystically dilated spaces are covered with single layer of endothelial cells and separated by fibrous septa are present in the submucosa. The overlying colonic mucosa is normal (H & E stain, x100). (**B**) Atypical cell are not noted, and endothelium is lined by benign cuboidal cells (H-E, original magnification, x400).

words, hemorrhage or abdominal pain may be reported, it may induce protein loss enteropathy, and intussusception may be developed by the mass[8]. Our case is also the example that the patient was admitted for bloody stool and developed intussusception on the next day. In such cases, if symptomatic or with complications, it should be removed by surgery or endoscopic resection. The indications of endoscopic polyp resection are staked masses or partially staked masses less than 2 cm in size[6]. However, in the cases of masses with a large diameter, surgical resection is required. It has been reported that generally, in the patient with intussusception, there are a large volume of hemorrhage, or protein loss enteropathy, a large mass in size, and asymptomatic patients showed a mass in a small size[9]. We experienced a case of intussusception in a 32-year-old female patient admitted for bloody stool, and it is an important causality although rare, therefore, the case is reported here together with a review of the literature.

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CASE REPORT

Spontaneous rupture of splenic hamartoma in a patient with hepatitis C virus-related cirrhosis and portal hypertension: A case report and review of the literature

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Abstract

Spontaneous rupture is a rare complication of splenic hamartoma. A review of the literature revealed only four such cases. To the best of our knowledge, this is the first report of spontaneous rupture of splenic hamartoma associated with liver cirrhosis and portal hypertension. A 53-year-old woman, who was followed up for aortic dissection and hepatitis C virus (HCV)-related liver cirrhosis, was referred with sudden left chest and shoulder pain. An abdominal ultrasound showed intraabdominal bleeding, and computed tomography indicated rupture of a splenic tumor. Emergent splenectomy was carried out. The postoperative course was uneventful, and the patient was discharged on the 13th postoperative day. Pathology revealed the tumor to be a ruptured splenic hamartoma. The non-tumorous splenic parenchyma revealed congestive changes. We consider that the presence of liver cirrhosis and portal hypertension are risk factors for spontaneous rupture of the splenic hamartoma.

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Key words: Splenic hamartoma; Spontaneous rupture; Hepatitis C virus; Cirrhosis; Portal hypertension

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INTRODUCTION

Splenic hamartoma is a rare benign tumor, and is usually asymptomatic. Its spontaneous rupture is an uncommon, but life-threatening, complication. A review of the literature revealed only four such cases [1-4]. Furthermore, there has been little discussion of underlying disease and no previous reports of a ruptured splenic hamartoma in a patient with chronic liver disease. Here we report a case of spontaneous rupture of splenic hamartoma associated with hepatitis C virus (HCV)-related cirrhosis and portal hypertension.

CASE REPORT

A 53-year-old woman was referred to our emergency care center with sudden left chest and shoulder pain without a history of trauma. She was being followed up for aortic dissection (DeBakey typeⅢb) and HCV-related liver cirrhosis. The aortic dissection had been stable for seven years and the pseudo-lumen was also patent. Portal hypertension and moderate splenomegaly was associated with the liver cirrhosis. During the clinical examination, the patient lost consciousness and her blood pressure dropped below 60 mmHg, though rapid infusion of saline prompted a swift recovery of consciousness and blood pressure. Initially, ischemic heart disease was suspected, but a chest X-ray and electrocardiogram showed no abnormal findings. Laboratory data were: hemoglobin, 8.2 g/dL; platelets, $5.9 \times 10^4/m^3$; total bilirubin, 1.2 mg/dL; asparate aminotransferase, 63 IU/L; alanine aminotransferase, 53 IU/L, albumin, 2.8 g/dL; prothrombin time, 74% to control. An abdominal ultrasound revealed fluid collection suggestive of intraabdominal bleeding. Computed tomography indicated rupture of a splenic tumor, which had been not detected by the previous screening for the aortic dissection (Figure 1). The patient was transferred to our intensive care unit, where conservative treatment was started, beginning with a blood transfusion. Interventional treatment was not initially selected because of the aortic dissection. However, her blood pressure dropped again, necessitating an emergency operation.

At laparotomy, there was a massive hemorrhage with recovered blood weighing 2 880 g. Active bleeding continued from the ruptured spleen, and a splenectomy was performed. The spleen weighed 520 g and measured 15 × 10

Table 1 Literature review of spontaneous rupture of splenic hamartoma

Author	Year	Age/gender	Spleen weight/size	No. of tumors	Tumor size	Underlying disease
Morgenstern *et al* [1]	1984	73 yr/F	117 g/11×7×4.5 cm	1	2.5 cm	no
Ferguson *et al* [2]	1993	48 yr/F	459 g/15.5×8.0×4.5 cm	1	5.4 cm	no
Yoshizawa *et al* [3]	1999	5 mo/F	230 g/6×9×6 cm	1	5.0 cm	no
Ballardini *et al* [4]	2004	60 yr/M	NA	1	NA	lung cancer (chemotherapy)
Present case	2005	53 yr/F	520 g/15×10×4 cm	4	4.5 cm	liver cirrhosis, portal hypertension

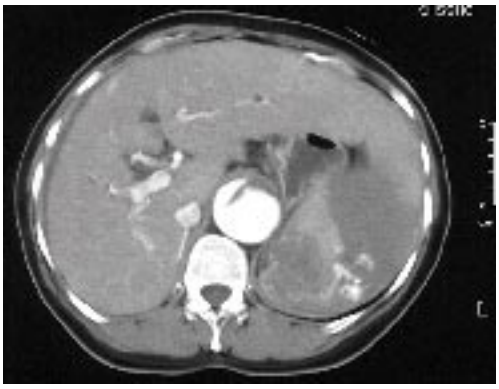

Figure 1 Enhanced computed tomography showed intraabdominal fluid collection near the spleen, and contrast material outflow from the splenic tumor.

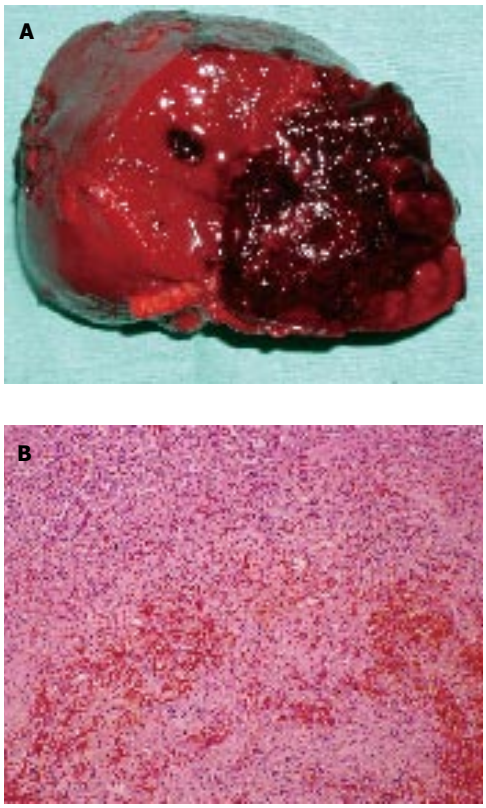

Figure 2 **A:** Macroscopic findings for the ruptured splenic hamartoma. The hamartoma is well demarcated from the surrounding parenchyma and significant hemorrhage is shown; **B:** Microscopy showed a red pulp with no lymphatic structures, or trabeculae of the spleen. (hematoxylin-eosin, original magnification, ×200).

cm with subcapsular hemorrhage. Macroscopically, there were four nodules on the cut surface of the specimen. The nodule near the upper pole was the largest, 4.5 × 3.0 cm in diameter, and ruptured, causing intraabdominal hemorrhage (Figure 2A). Microscopically, the nodules were not encapsulated and composed of sinusoidal spaces and cords of Billroth as in normal red pulp without lymphatic follicles or fibrous trabeculae (Figure 2B). The vessel spaces linked by plump endothelial cells were filled with a mixed population of erythrocytes and lymphoreticular cells. Pathology revealed the tumor to be a ruptured splenic hamartoma, of the red pulp type. The non-tumorous splenic parenchyma showed proliferation of splenic sinuses and cords with extramedullary hematopoiesis, indicating congestive changes. These findings corresponded to the splenomegaly caused by portal hypertension. The postoperative course was uneventful, and the patient was discharged on the 13th postoperative day.

DISCUSSION

Splenic hamartoma is a rare benign tumor composed of all the normal constituents of splenic parenchyma arranged in a disorganized fashion. The incidence has been reported as 0.024-0.13% from autopsy cases [5, 6]. Clinical symptoms include mass effect due to splenomegaly, pancytopenia, hematological disorders, and spontaneous rupture [5-7]. The average spleen weight and size of all reported hamartomas were about 600 g and 5.1 cm, respectively [6, 7].

Spontaneous rupture of splenic hamartoma is rare. The cause of rupture has not been specified in any of the small number of cases reported to date (Table 1). The cases discussed in these reports showed mild to moderate splenomegaly, however, the weight of the spleen (117-459 g) was below the average, and the size of the hamartomas (2.5 cm-5.4 cm in diameter) did not far exceed the average. There were also no underlying diseases, except for one case, in which the patient had lung cancer. These cases indicate that the weight of the spleen, the size of the hamartoma, or any underlying diseases, is not closely related to rupture of the hamartoma. In contrast to these cases, our case was characterized by moderate splenomegaly in association with hepatitis C virus-related cirrhosis. The patient had no other risks of spontaneous

splenic rupture, such as a history of trauma.

Whether or not hepatitis C virus-related cirrhosis contributed to the rupture of the splenic hamartoma in our case is unclear. Spontaneous rupture of the spleen is a rare complication of liver cirrhosis [8-14]. In these reported cases, portal hypertension associated with liver cirrhosis has been suspected of playing a role in splenic rupture. In our case, portal hypertension and splenomegaly due to cirrhosis was apparent, and microscopy revealed congestive changes in the non-tumorous splenic parenchyma. Since the hamartoma nodules are not encapsulated and composed of sinusoidal spaces and cords of Billroth as in normal red pulp, it is quite likely that the hamartoma itself was affected by portal hypertension. Although an association of splenic hamartoma with liver cirrhosis and portal hypertension has been demonstrated in several studies [1, 15-17], to the best of our knowledge, this is the first report of spontaneous rupture of splenic hamartoma associated with liver cirrhosis and portal hypertension.

Recent progress in imaging enables us to detect splenic tumors, and elective surgery is sometimes conducted [18-20]. Operative indications for splenic hamartoma include the possibility of malignant disease and the risk of spontaneous rupture. If splenic hamartoma is suspected by image findings, elective splenectomy should be taken into consideration to make a definitive diagnosis and to prevent rupture [21]. We consider that the presence of liver cirrhosis and portal hypertension are risk factors for spontaneous rupture of the splenic hamartoma.

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CASE REPORT

A case of primary biliary cirrhosis complicated by Behçet's disease and palmoplantar pustulosis

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Abstract

A 46-year-old woman was diagnosed with palmoplantar pustulosis (PPP) at the Department of Dermatology, Fukushima Medical University Hospital in 2000, and was treated with ointment. However, because liver dysfunction developed in 2003, she was referred to our department, where primary biliary cirrhosis (PBC) was also diagnosed on the basis of clinical findings. One year later, at the age of 49, she developed manifestations of Behçet's disease (BD), including erythema nodosum in the lower extremities. Because she had a history of uveitis, recurrent oral ulceration was present, and the HLA typing was positive for B51, BD was additionally diagnosed. Liver function normalized within three months of the start of treatment with ursodesoxycholic acid (UDCA). This is the first case of PBC associated with BD and PPP.

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Key words: Primary biliary cirrhosis; Behçet's disease; Palmoplantar pustulosis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease associated with a variety of other conditions, including Sjögren's syndrome, Hashimoto's thyroiditis, scleroderma or other components of CREST syndrome, and inflammatory arthritis [1,2], but its association with Behçet's disease (BD) is very rare, and so is its association with palmoplantar pustulosis (PPP) [3]. We report the first case of PBC associated with BD and PPP, and discuss the relationship among these three diseases in terms of the environmental factors, such as infectious agents, described in the literature.

CASE REPORT

A 37-year-old woman suffered from uveitis in 1991. In 2000, she was diagnosed with palmoplantar pustulosis (PPP) at the Department of Dermatology, Fukushima Medical University Hospital, and was treated with ointment. However, liver dysfunction developed, and she was referred to our department in March 2003 at the age of 49 years.

At our first medical examination, she complained of no symptoms involving the oral mucosa or extremities except the lesion of pustulosis. Blood pressure was normal, and there was no history of diabetes mellitus or hyperlipidemia. Laboratory data showed liver dysfunction with elevated levels of AST (149 IU/L), ALT (340 IU/L), ALP (428 IU/L; normal, 125-335 IU/l), GGT (228 IU/L; normal, 6-30 IU/l), T-Bil (0.7 mg/dL) and IgM (130 mg/dL; normal, 52-270 mg/dL). The titer of anti-mitochondrial autoantibodies (AMA) was 1:20, and the index of AMA-M2 antibody was 32 (normal, <7). Results of hepatitis B and C serologic tests were negative. After one month, subarachnoid hemorrhage occurred owing to the bursting of a cerebral artery aneurysm. An emergency brain operation was performed, revealing four cerebral artery aneurysms including the ruptured one (Figure 1), which were clipped successfully. Her hospital course was otherwise uneventful, and her slight cerebral signs resolved within days of the operation, and she was discharged from the hospital, neurologically intact. Liver biopsy was, therefore, postponed. PBC associated with PPP was diagnosed on the basis of clinical findings based on 'Criteria for Diagnosis of PBC in Japan' by the Study Group for Autoimmune Hepatitis, a subdivision of the Research Group for Intractable Hepatitis, sponsored by the Ministry of Health and Welfare of Japan [4]. Liver function normalized within three months of the start of treatment with ursodesoxycholic acid (UDCA) (600 mg/d).

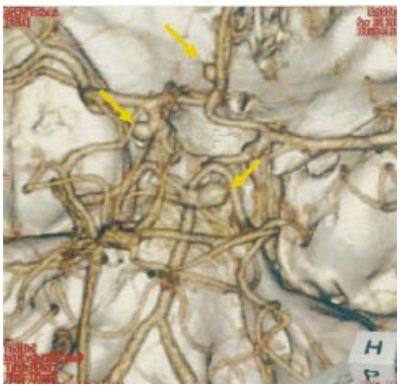

Figure 1 Computed tomographic angiography shows aneurysms of the left. internal carotid-posterior communicating artery, left. anterior communicating artery, and right. posterior cerebral artery (arrows).

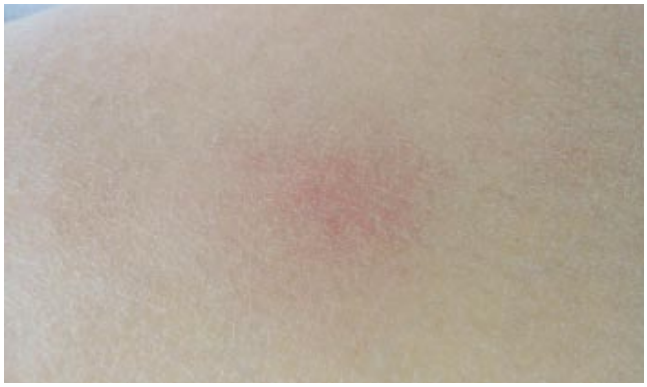

Figure 2 Typical eruption of erythema nodosum shows erythematous nodules on the anterior aspect of the leg.

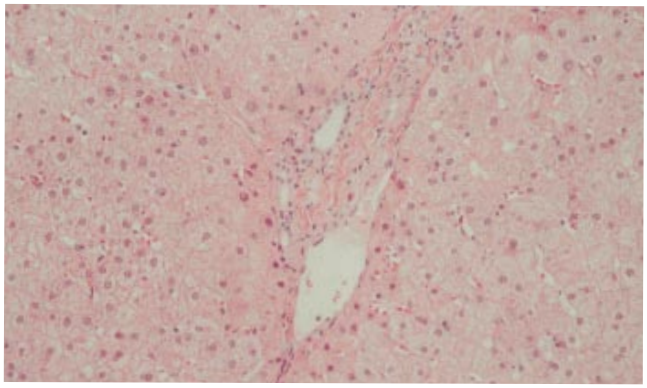

Figure 3 Liver biopsy specimen shows slightly enlarged portal tracts with no evidence of chronic nonsuppurative destructive cholangitis. Hematoxylin and eosin, original magnification ×100.

Erythema appeared in the lower extremities in July 2004 (Figure 2). Oral aphthous ulcers were sometimes noted, too. Lymphadenopathy was not recognized. No abnormal findings were noted in the chest or abdomen. Laboratory data at the time were as follows: increased level of CRP, 3.1mg/dL (normal, <0.3) and ESR, 73mm/h (normal, 3-15); WBC, 9 400/mm^3, (normal, 2800-8800). Liver function and renal function were normal. HLA typing was positive for A2, A30 (19), B54 (22), B51 (5), Cw1 and DR9. Because HLA B51 was positive, a skin biopsy of the erythema was performed. Histological examination showed erythema nodosum characterized by a neutrophilic inflammatory infiltrate involving the septa of the subcutaneous tissue. Incomplete type Behçet's disease (BD) was diagnosed on the basis of recurrent oral aphthous ulcers, erythema nodosum, and past history of uveitis according to the diagnostic criteria from the Behçet's Disease Research Committee of Japan (1987 revision)[4]. A non-steroidal anti-inflammatory drug was administered orally as palliative treatment, and erythema nodosum resolved.

Liver biopsy was performed in January 2005, but histological examination did not show typical chronic nonsuppurative destructive cholangitis (CNSDC) (Figure 3) because liver function was improved by 2-year UDCA treatment.

DISCUSSION

BD is a well-known multisystem inflammatory disorder of unknown etiology that is characterized by oral and genital ulcers, uveitis, and a variety of other manifestations, such as erythema nodosum, polyarthritis, thrombophebitis, and ulceration of the intestinal mucosa [6]. Involvement of the liver is less common; however, systemic amyloidosis and Budd-Chiari syndrome have been documented [7, 8]. Susceptibility to BD is associated with the HLA B51 allele.

There have been only a few cases of PBC associated with BD [9]. In the present case, the clinical manifestations of BD were oral aphtha, erythema nodosum and uveitis, and there was no genital ulcer or intestinal involvement. However, it is possible that the intracranial aneurysms were related to BD. Cerebral artery aneurysms in patients with BD are uncommon, but there are 14 cases reported in the literature [10]. These cases are quite similar to ours in which the aneurysms are multiple. Furthermore, in our case there was no risk factor that would produce a cerebral vessel event: hypertension, hyperlipidemia, or diabetes mellitus. Some BD case reports describe the efficacy of steroid therapy for multiple nonruptured cerebral artery aneurysms [11-13]. Kerr *et al*, however, report a patient with BD who developed multiple aneurysms and subarachnoid hemorrahge in rapid succession under steroid therapy [14]. In our patient, follow-up brain computed tomographic angiography was performed after surgical treatment, but no aneurysms were detected.

PBC is an autoimmune liver disease characterized by the occurrence of AMA. Clinically, PBC is associated with a large variety of other diseases, such as arthropathy, CREST syndrome, and autoimmune thyroiditis [15]. To our knowledge, however, no report has ever described the simultaneous development of three autoimmunological diseases, PBC, BD, and PPP.

This is the first case report of PBC complicated by BD and PPP, which is a localized type of psoriasis. Although psoriasis is characterized by proliferation of the epidermis, the immune system plays a prominent role in its development [16]. Interaction between keratinocytes and T cells is involved in the pathogenesis, just as interaction between biliary epithelial cells and T cells is in the pathogenesis of PBC. In BD, lymphocytic infiltration is observed around the arteries. Moreover, some workers suggested that some microbial antigen is closely associated with the development of these three diseases [17-19].

Memory T cells specific to these bacterial antigens may be activated by antigens on the arteries, skin or liver. We believe that patients with PBC associated with BD and PPP hold the key to clarifying the nature of these diseases.

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CASE REPORT

Chest wall metastasis from unknown primary site of hepatocellular carcinoma

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Abstract

Previous reports of a solitary metastatic hepatocellular carcinoma have been rare. Because this tumor has a different treatment modality and prognosis, an accurate differential diagnosis is essential. Here we report a rare case of a solitary chest wall metastasis from unknown primary site of hepatocellular carcinoma. It involves a 51-year-old man who was admitted to our hospital because of a palpable left upper chest wall mass. The mass was resected and pathologic examination confirmed a diagnosis of metastatic hepatocellular carcinoma. Despite our investigation, no evidence was found that indicated the primary origin of the hepatocellular carcinoma. Four months later, the patient was admitted again because of spinal cord compression at the third and fourth thoracic vertebrae. Emergent decompressive laminectomy was performed and microscopic features revealed the same pathology as the initial chest wall mass resected 4 months earlier. After one year, a follow-up abdominal computed tomography (CT) still revealed no evidence of primary hepatocellular carcinoma.

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Key words: Chest wall; Hepatocellular carcinoma; Metastasis

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INTRODUCTION

The most common causes of a chest wall mass in adults are infection or tumor,[1] and the majority of the malignant masses are hematological in origin. Malignant chest wall masses arising from a primary focus in the lung (metastatic lung cancer) or the liver (metastatic hepatocellular carcinoma) are rare.[2] Primary hepatocellular carcinoma often metastasizes out of the liver. Metastasis typically occurs in patients with advanced stages of the disease, and the most common site is the lungs, followed by the abdominal wall, lymph nodes, bone, adrenal gland, pancreas, kidney and spleen.[3,4] Katyal *et al* [5] categorized the intrahepatic stage of the tumor according to TNM staging, and reported that extrahepatic metastasis of small hepatocellular carcinomas (confined to stage one) was rare. References indicates that hepatocellular carcinomas found outside the liver without a primary focus are usually due to carcinogenesis of ectopic liver. Cases of hepatoid adenocarcinoma have previously been reported, but a solitary extrahepatic hepatocellular carcinoma that has metastasized to the chest wall has rarely been reported. Because these types of tumors have different treatment modalities and prognoses, an accurate differential diagnosis is essential. Here, the authors have diagnosed a solitary metastatic hepatocellular carcinoma of the chest wall, with an unknown primary focus, and have been closely following the patient for one year since the diagnosis.

CASE REPORT

A 51-year-old male visited the hospital because of a left upper chest wall mass that developed during the prior 8 months. Ten years ago he was diagnosed with chronic viral hepatitis B but was lost to follow-up in the intervening period. The patient had not received any treatment since the mass was first discovered, and the mass progressively grew in size until the patient sought treatment at the hospital. The patient was a heavy drinker and smoker, with a drinking history of two bottles of beer per week for 30 years, and a smoking history of 15 pack years. There was no significant family history. The patient's initial vital signs were stable and he appeared relatively well, and was alert and responsive. On physical examination, a hard, palpable mass about 10 cm × 12 cm in size was noted on the left upper anterior chest wall. Palmar erythema and spider angioma on the anterior chest wall were noted, but there were no other specific complaints. Initial laboratory findings were

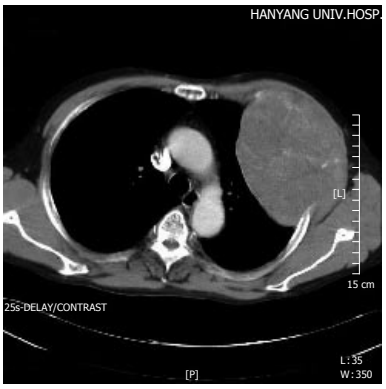

Figure 1 Chest helical CT. A mass on the anterolateral aspect of the left chest wall. The inner part of the mass is heterogeneously attenuated and consistent with mottled calcification. There is no evidence of metastasis in the mediastinum or the lung parenchyma.

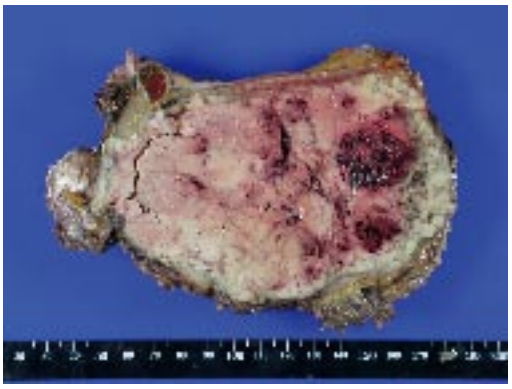

Figure 2 Gross findings of the chest wall tumor. The cut surface shows an encapsulated, yellowish-white solid mass with areas of hemorrhage and necrosis.

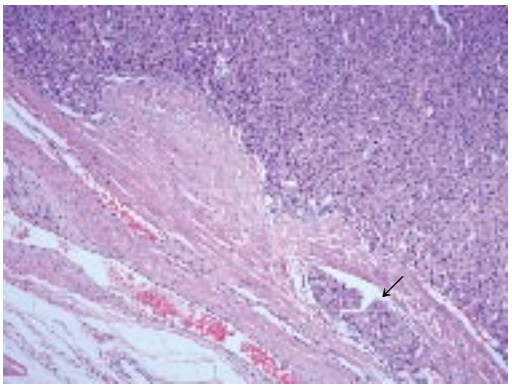

Figure 3 Microscopic findings of the chest wall tumor. The tumor displays typical features of well-differentiated, trabecular liver cell carcinoma with intravascular tumor emboli (arrow) (H&E stain, ×200).

as follows: WBC 10 000/mm^3, hemoglobin 134 g/L, platelets 216 000/mm^3, serum protein 56 g/L, albumin 34 g/L, total cholesterol 214 mg/dL, serum glucose 113 mg/dL, total bilirubin 0.9 mg/dL, AST 38 U/L, ALT 52 U/L, alkaline phosphatase 104 U/L, and prothrombin time 135%. Patient's viral markers were as follows: HBsAg(+), HBcAb(+), HBeAg(-), HBeAb(+) and HBV DNA < 2.5pg. Serum alpha-fetoprotein was 308.3 ng/mL.

The density of the soft tissue protruding inwards towards the lung was noted, with accompanying evidence of rib destruction along the anterolateral aspect of the third left rib on the chest film. A 9.5 cm× 12.7 cm sized heterogeneous tumor with a mottled calcification was noted on the anterolateral aspect of the left chest wall on the CT, but there was no evidence of metastasis within the lung parenchyma or the mediastinum (Figure 1). An additional study of abdominal ultrasonography and whole body bone scan revealed no evidence of an intrahepatic mass, abdominal lymphadenopathy, or bone metastasis. A primary bone tumor on the left upper chest wall was suggested, and radical resection of the tumor around the rib and soft tissue was performed.

The resected tumor was well-encapsulated with dimensions 14 cm× 10 cm× 7.5 cm and weighed 650 mg including the excised rib tissue. The cut surface of the tumor was whitish-brown with accompanying hemorrhage and necrosis, and was solid and hard but tended to crumble easily (Figure 2). Microscopic examination revealed large polygonal cancer cells separated by thin vessels resulting in a pole-like arrangement, and multiple cells undergoing mitosis were seen. Cancer cell emboli were seen inside the vessels surrounding cancer cells, and there was no evidence of normal liver tissue within the microscopic field (Figure 3). Immunohistochemical staining showed positive results for alpha-fetoprotein, hepatocyte specific antigen and cytokeratin, and showed negative results for vimentin and smooth muscle actin (Figure 4).

Abdominal computerized tomography (CT) and abdominal magnetic resonance imaging (MRI) were performed in search of the primary focus of the hepatocellular carcinoma. The surface of the liver was slightly irregular which was suspicious of liver cirrhosis, but there was no evidence of hepatocellular carcinoma, and no lipiodol uptake nor tumor vessels were seen in the hepatic arteriography. Mild lobular necrosis with mild portal inflammation and macronodular cirrhosis on the portal area were noted upon liver biopsy, but there was no evidence of a tumor. Upon esophagogastroduodenoscopy, antral gastritis was present but there were no gastric or esophageal varices, and there was no significant sigmoidoscopic finding. Postoperative alpha-fetoprotein level decreased from 308.3 ng/mL to 42.4 ng/mL. Follow-up abdominal CT was performed three weeks after the first hepatic arteriography, and like before, no lipiodol uptake was seen. There were no operation-related complications, and the patient was discharged to be regularly monitored as an outpatient.

Four months after discharge, the patient visited the emergency room due to back pain radiating to both lower extremities. On physical examination, there was evidence of motor weakness in both lower extremities, and the patient complained of paresthesia and hyperesthesia in the dermatome below the forth thoracic vertebra. Immediate thoracic MRI revealed spinal cord compression due to a metastatic lesion to the third and forth thoracic vertebrae, with spinal deviation to the right of the posterolateral position (Figure 5). Emergent decompressive laminectomy was performed. Further evaluation with a whole body bone scan revealed a newly developed increased uptake at the left side of the third to fifth thoracic vertebrae, while abdominal ultrasonography showed no significant change from the initial diagnosis of metastatic hepatocellular carcinoma in the chest wall. Histological examination of the metastatic vertebral lesions revealed metastatic hepatocellular carcinoma, which was the same finding observed dur-

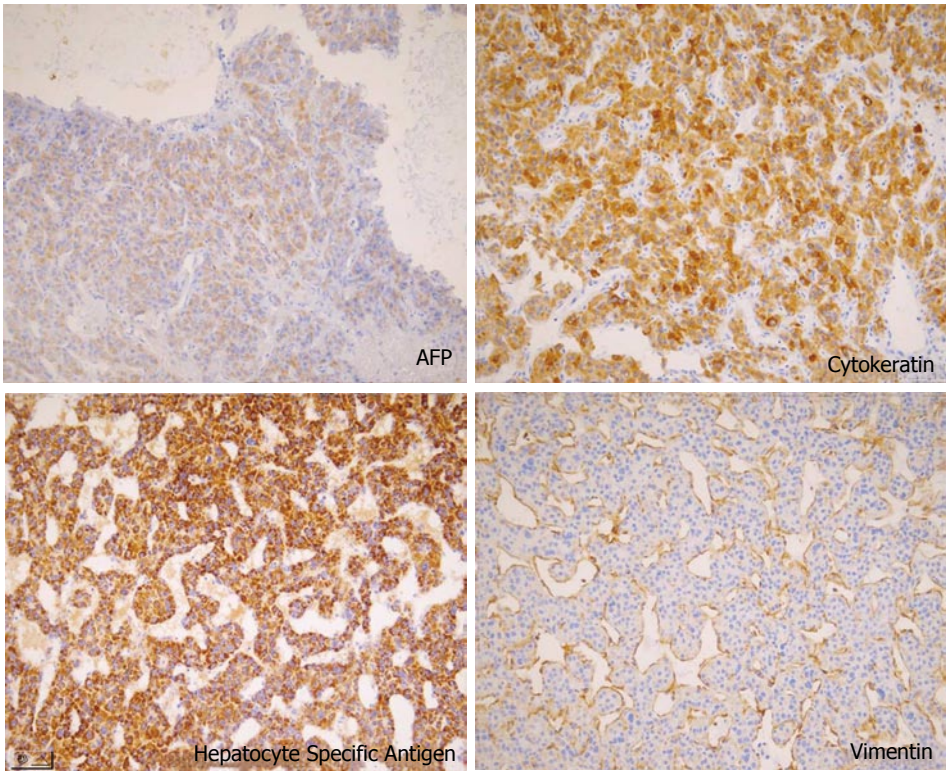

Figure 4 Microscopic findings of the chest wall tumor. The tumor cells are positive for AFP, cytokeratin, and hepatocyte specific antigens. This section shows positive staining for hepatocyte-specific antigens (IHC, ×400).

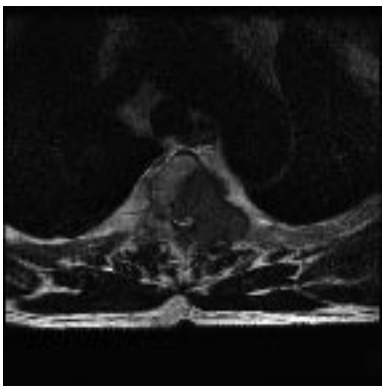

Figure 5 Thoracic spine MRI. The metastatic tumor of the third vertebra compressing the spinal cord (T2 weighted image).

ing microscopic examination of the chest wall mass. Afterwards the patient received radiotherapy and adriamycin-based chemotherapy. One year later, no lesion suggesting primary hepatocellular carcinoma has been found in the follow-up abdominal CT scan.

DISCUSSION

An extrahepatic hepatocellular carcinoma without a primary intrahepatic hepatocellular carcinoma can be explained in two different ways. The first is ectopic liver carcinogenesis and the second is a hepatoid adenocarcinoma.[6-8]

Ectopic liver is a rare developmental abnormality that can originate in the gall bladder, hepatic ligament, omentum, posterior peritoneal cavity, or the thorax.[8-11] Because ectopic liver does not have perfect functional architecture as does a normal liver, it is more likely to develop hepatocellular carcinoma, if carcinogenic factors such as viral hepatitis infection or liver cirrhosis are involved.[6]

Characteristically, the pathologic examination of hepatocellular carcinoma arising from ectopic liver reveals normal liver tissue, including portal triads. It may be connected to the liver by a fibrous stalk, which is composed of the portal vein, hepatic artery, or bile duct. If no evidence of primary cancer of the liver is present after a long term follow-up with various liver imaging studies, a malignancy originating from ectopic liver should be suspected .[7] In Japan, 25 cases of hepatocellular carcinoma of ectopic liver were described. This can be treated with complete resection of the tumor, which yields a good prognosis.

Hepatoid adenocarcinoma is a variant form of adenocarcinoma, characterized by vast hepatic differentiation. It produces alpha-fetoprotein, while having the same function and form as hepatocellular carcinoma, but originates from the endoderm and mucosa of urogenital organs and therefore can manifest itself in the gastrointestinal tract, ovaries, pancreas, lungs, kidneys, uterus or bladder. It contains a characteristic moderately to well-differentiated tubular or papillary shaped adenocarcinoma[8].

The present case differs from the two aforementioned examples because it was discovered as a solitary metastatic hepatocellular carcinoma of the chest wall, with an unknown primary focus. First, it was composed solely of hepatocellular carcinoma, and microscopic examination revealed no evidence of adenocarcinoma nor normal liver tissue including portal triads. This finding was entirely different from that expected in the carcinogenic sequence of ectopic liver or hepatoid adenocarcinoma. Second, cancer cell emboli were found within the vessels near the cancer cells, which is a characteristic microscopic finding of metastatic cancer. Thirdly, there was no evidence of an intrahepatic tumor in the liver imaging studies, and this result was again seen during the follow-up abdominal CT one year later. Fourthly, four months after resection of the chest wall tumor, metastatic hepatocellular carcinoma was found in the thoracic vertebrae. The thoracic vertebrae

are a region of intense bone marrow replication, and are among the most important metastatic sites for hepatocellular carcinoma[12].

In summary, when hepatocellular carcinoma is found outside the liver without a primary focus within the liver, a tissue biopsy must be obtained. Tissue biopsy must confirm the presence of normal liver tissue, including portal triads and cancer emboli within vessels near the tumor, for an accurate differential diagnosis. Chest CT, abdominal ultrasonography, and whole body bone scans must be used in order to confirm the presence of metastasis to other organs. To discern between hepatocellular carcinoma of ectopic liver and solitary metastatic hepatocellular carcinoma without a primary focus in the liver, a follow-up study is essential after the operation in order to find the primary site of origin.

Although the patient had no evidence of an intrahepatic tumor, he was a hepatitis B carrier with liver cirrhosis which suggested a latent primary hepatocellular carcinoma metastasizing to the chest wall that spontaneously regressed. In cases like this, in which extrahepatic hepatocellular carcinoma exists without evidence of tumor within the liver, one has to consider the possibility of a tumor originating from ectopic liver tissue, hepatoid adenocarcinoma, or, although rare, metastatic hepatocellular carcinoma without an intrahepatic mass.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

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Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
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10th International Congress of Obesity
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Vienna, Austria
Kenes International

Prague hepatology 2006
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Seatlle - Washington
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Canadian Digestive Disease Week
24–27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
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MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4–8 February 2006
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European Multidisciplinary Colorectal Cancer Congress 2006
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www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
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21–25 October 2006
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World Congress on Controversies in Obesity, Diabetes and Hypertension
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Asia Pacific Obesity Conclave
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Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
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6th Annual Gastroenterology And Hepatology
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Hepatitis 2006
25 February 2006–5 March 2006
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World Congress on Gastrointestinal Cancer
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c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
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5th International Congress of The African Middle East Association of Gastroenterology
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7th World Congress of the International Hepato-Pancreato-Biliary Association
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Edinburgh Convention Bureau
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13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
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laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26–28 January 2006
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Gastrointestinal Cancers Symposium Registration Center
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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
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American Society of Gastrointestinal Endoscopy
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American Society of Colon and Rectal Surgeons
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3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

5 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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6 **Morse SS.** Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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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	

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10	>	*t*>5	*t* > 5	Space before ">" and after ">" is 0.5 character
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12	⩽	Ages⩽6 mo	Ages ⩽ 6 mo	Space before "⩽" and after "⩽" is 0.5 character
13	()	Bardeen-Cooper-(BCS)theory[4]	Bardeen-Cooper-Schrieffer (BCS) theory[4]	Space before "(" and after ")" is 0.5 character
14	[]	△△Gint = -RTIn [(θwwθmm)/(θwmθmw)]	△△Gint = -RTIn [(θwwθmm)/(θwmθmw)]	Space between "[" and " (" , and between ") " and "] " is 0.5 character
15	,	γ-3,γ-4 andγ-8	γ-3, γ-4 andγ-8	Space between "," and following word is 1 character
16	;	LDT;yellow	LDT; yellow	Space between " ;" and following word is 1 character
17	:	Discovering Dorothea: The life of the Pioneering Fossil-Hunter Dorthea Bate	Discovering Dorothea: The life of the Pioneering Fossil-Hunter Dorthea Bate	Space between ":" and following word is 1 character
18	?	If he had not wavered for two years?We shall never know.	If he had not wavered for two years? We shall never know.	Space between "?" and following word is 1 character
19	…	It is very near to our thoughts, while the ignorance regarding these lands is being very rapidly dispelled…China stands not at the parting of the ways.	It is very near to our thoughts, while the ignorance regarding these lands is being very rapidly dispelled… China stands not at the parting of the ways.	Space between "…" and following word is 1 character
20	' '	Simpler 'prokaryotic'cells	Simpler 'prokaryotic' cells	Space between " ' " and preceding word, and between " ' " and following word is 1 character
21	" "	The PTO calls situation a"workload crisis" in its strategic plan.	The PTO calls situation a "workload crisis" in its strategic plan.	Space between ' " ' and preceding word, and between ' " ' and following word is 1 character
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