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[1]Passed away on October 20, 2007
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EDITORIAL

Role of cannabinoids in chronic liver diseases

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Abstract

Cannabinoids are a group of compounds acting primarily *via* CB1 and CB2 receptors. The expression of cannabinoid receptors in normal liver is low or absent. However, many reports have proven up-regulation of the expression of CB1 and CB2 receptors in hepatic myofibroblasts and vascular endothelial cells, as well as increased concentration of endocannabinoids in liver in the course of chronic progressive liver diseases. It has been shown that CB1 receptor signalling exerts profibrogenic and proinflammatory effects in liver tissue, primarily due to the stimulation of hepatic stellate cells, whereas the activation of CB2 receptors inhibits or even reverses liver fibrogenesis. Similarly, CB1 receptor stimulation contributes to progression of liver steatosis. In end-stage liver disease, the endocannabinoid system has been shown to contribute to hepatic encephalopathy and vascular effects, such as portal hypertension, splanchnic vasodilatation, relative peripheral hypotension and probably cirrhotic cardiomyopathy. So far, available evidence is based on cellular cultures or animal models. Clinical data on the effects of cannabinoids in chronic liver diseases are limited. However, recent studies have shown the contribution of cannabis smoking to the progression of liver fibrosis and steatosis. Moreover, controlling CB1 or CB2 signalling appears to be an attractive target in managing liver diseases.

Key words: Hepatic fibrosis; Endocannabinoids; Endocannabinoid receptors; CB1; CB2

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INTRODUCTION

Hepatic fibrosis is a dynamic process resulting from liver tissue injury. Previously, it was believed that hepatic fibrosis is irreversible. However, current knowledge allows us to consider fibrosis as an active, potentially reversible process originating from wound-healing responses to chronic liver injury of various etiology. The continuous influence of injuring stimuli leads to an imbalance between the accumulation and degradation of extracellular matrix (ECM) components, which include mainly deposits of fibrillar collagens, proteoglycans and glycoproteins. The major sources of ECM elements are hepatic stellate cells (HSCs), which physiologically constitute about 5%-8% of liver cells. In the course of chronic liver injury, HSCs are activated and transformed from fat-storing cells (Ito cells) to myofibroblast-like cells. Along with this transformation, they undergo morphological and functional changes into contractile, smooth muscle α-actin-positive cells expressing profibrogenic and proinflammatory properties[1,2]. Among mediators activating HSCs, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are of special concern. PDGF stimulates proliferation and migration of HSCs, whereas TGF-β, which is the most potent profibrogenic cytokine, acting through its receptor, induces downstream signalling involving Smad family mediators. Thus, it regulates transcription of TGF-β target genes. The results of the above-mentioned signalling are complex; these include increased synthesis of fibrillar collagens, especially collagen type 1, and other ECM components, reduced expression of matrix metalloproteinase, along with augmented production of tissue inhibitor of metalloproteinase-1 (TIMP-1). So far, the perfect non-invasive biomarkers of hepatic fibrosis are under investigation include TGF-β, ECM components and TIMPs[3-5]. The overproduction of ECM components and imbalanced processes of synthesis-degradation eventually lead to progressive liver fibrosis[2]. Thus, reduction of fibrosis

can be obtained by either reduced liver myofibroblast activity, resulting from inhibition of ECM components synthesis, or enhanced degradation of ECM. The clinical data demonstrated that even advanced liver fibrosis can be inhibited and reversed[6]. However, the compounds with antifibrotic activities, potentially useful in clinical practice, are still under investigation.

CANNABINOIDS AND ENDOCANNABINOID SYSTEM IN PHYSIOLOGY AND PATHOLOGY

Cannabinoids are a group of compounds acting primarily *via* CB1 and CB2 receptors. The first cannabinoid discovered, in 1964, was a plant-derived Δ^9-tetrahydrocannabinol (THC), the psychoactive component of *Cannabis sativa*[7]. Following this finding was the discovery and determination of receptors for cannabinoids in nervous tissue. The first results were obtained by Matsuda *et al*[8] and presented the effects of cloning cDNA of G protein-coupled receptor found in neural cells, recognized then as CB1 receptor. However, this receptor was shown to be responsible primarily for psychoactive and neuronal effects of cannabinoids, which did not explain the other effects exerted by THC. Hence, the research for other receptors led to the discovery of CB2 receptor, expressed in macrophages of the spleen[9]. The presence of cannabinoid receptors in mammalian tissues prompted the research for its endogenous ligands and resulted in isolation of anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG)[10,11]. So far, among several endogenous discovered ligands for cannabinoid receptors, AEA and 2-AG are the best characterized. The interesting feature of endocannabinoid mediators is that they are not stored in cells, instead, they are synthesized from lipid precursors in cellular membranes and released in response to specific stimuli[12].

The endocannabinoid system is comprised of at least two types of receptors for THC (CB1 and CB2), endogenous lipid compounds acting as ligands, and molecules regulating the synthesis and degradation of endocannabinoids. The expression of CB1 receptors was initially associated with the nervous system, as they were shown to control perception, cognitive, motor and behavioral functions. Nevertheless, the CB1 receptors are present peripherally in endothelial cells, adipocytes, gut and liver cells[13-16]. It has been shown that cannabinoid CB1 signalling regulates intake of high-energy-containing food and alcohol, energy homeostasis and hepatic lipogenesis. As to CB2 receptors, they are largely expressed in several lines of peripheral blood immune cells, tonsils, spleen and testis[17]. Moreover, their presence at low levels has been confirmed in various other tissues and cells, like hepatic myofibroblasts[18].

The imbalance in endocannabinoid system signaling is observed in various pathological conditions, including nervous system disorders, metabolic disturbances, impaired immunological responses (both allergy and hypersensivity), cardiovascular and gastrointestinal diseases, and carcinogenesis[19].

CANNABINOIDS IN HEPATIC FIBROSIS

The expression of cannabinoid receptors in normal liver is very low, partially because they are not expressed in hepatocytes. However, many studies have demonstrated the up-regulation of the expression of CB1 and CB2 receptors in hepatic myofibroblasts and vascular endothelial cells, as well as increased concentration of endocannabinoids, especially AEA, in liver in the course of chronic progressive liver diseases[18,20,21]. Teixeira-Clerc *et al*[20] have provided evidence for the involvement of CB1 receptor in regulation of hepatic fibrosis and the profibrogenic effect of CB1 signaling. Increased expression of CB1 receptors has been observed in human cirrhotic liver samples, primarily in HSCs during their transformation into myofibroblasts during the course of chronic liver injury. Moreover, the effect of CB1 inactivation was demonstrated in three experimental models of liver injury induced by CCl_4, thioacetamide or biliary cholestasis. The favorable antifibrogenic results were obtained either by pharmacological inactivation with rimonabant (SR141716), a selective antagonist of CB1 receptor, and *via* genetic inactivation in homozygous CB1-deficient mice. Decreased progression of fibrosis was accompanied by reduced hepatic TGF-β expression, and growth inhibition and increased apoptosis of myofibroblasts. These effects seemed to result from reduced phosphorylation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK), thus affecting the pathways responsible for cell proliferation and survival.

Although CB1 receptor is believed to have profibrogenic effects, studies on the CB2 receptor have proven its antifibrogenic activity in liver, as CB2 knockout mice developed augmented cirrhosis when exposed to CCl_4, compared to wild type[18]. It has been demonstrated in human cirrhotic liver samples that the expression of CB2 receptor is limited primarily to the cells positive for smooth muscle α-actin located within fibrotic septa; however, it is also detected in non-parenchymal cells, inflammatory cells and bile duct epithelial cells adjacent to fibrotic septa. The supporting results were obtained in separate research on cultured human hepatic myofibroblasts and activated rat HSCs, which were shown to express CB2 receptor. The final effects of the stimulation of CB2 receptor with THC or selective agonist JWH-015 are dose-dependent and expressed as growth inhibition or apoptosis. Interestingly, these two endpoints result from two distinct pathways, the induction of cyclooxygenase-2 (COX-2) in growth inhibition and intracellular oxidative stress for apoptosis, as they are diminished by the selective COX-2 inhibitor and two potent antioxidants, respectively.

Additionally, apart from receptor-dependent mechanisms of endocannabinoid actions on HSCs, the direct mechanism exerted by AEA leading to cell death have been observed in the research of Siegmund *et al*[22]. Stimulation of cultured human HSCs with AEA induces cell death in the necrotic pathway. This event is preceded by reactive oxygen species (ROS) formation and an increase in intracellular Ca^{2+} in HSCs. The pharmacologi-

cal inactivation of CB1, CB2 and vanilloid receptor-1 (VR1) does not prevent AEA-triggered cell death, which appears to be mediated by membrane cholesterol. Furthermore, the distinction between cholesterol content in the cellular membrane of HSCs and hepatocytes results in selective elimination of HSCs that are richer in cholesterol[22]. The conclusion of their analysis is that AEA exerts a potential antifibrogenic effect by inhibition of HSC proliferation and induction of necrotic death. The elevated levels of circulating AEA in cirrhotic patients might reflect the regulatory antifibrotic response to progression of fibrosis[22,23]. However, due to disadvantageous properties, such as triggering a local inflammatory response to necrosis and tissue damage, as well as systemic vasodilatation, the usefulness of AEA in the treatment of liver fibrosis is limited.

CANNABINOIDS IN LIVER STEATOSIS

Metabolic syndrome, leading to liver steatosis, has emerged as an important and frequent cause of chronic liver injury, ranging from simple steatosis to steatohepatitis, which is accompanied by inflammatory reaction and progressive fibrosis of liver tissue. The involvement of the endocannabinoid system in the pathogenesis of fatty liver disease has been shown recently. Since endocannabinoids are essential in regulation of energy balance, food intake and lipogenesis, impairment of this homeostasis results in various metabolic disturbances. Apart from central control of energy homeostasis *via* CB1 receptors localized in the brain, endocannabinoids seem to exert, as well CB1-receptor-dependent, peripheral effects on lipid metabolism in adipocytes, liver tissue and skeletal muscle[14]. This could be partially explained by increased expression of lipogenic transcription factor and activation of downstream enzymes, which result in increased fatty acid synthesis. Moreover, fat-rich diet has been shown to contribute to enhanced hepatic expression of CB1 in liver tissue and increased levels of endocannabinoids, thus increasing the metabolic imbalance[15].

ROLE OF ENDOCANNABINOID SYSTEM IN CONDITIONS ACCOMPANYING END-STAGE LIVER DISEASE

The role of the endocannabinoid system in liver diseases is complex. It has been particularly examined in end-stage liver disease and shown to contribute to hepatic encephalopathy and vascular effects such as portal hypertension, splanchnic vasodilatation, relative peripheral hypotension and probably cirrhotic cardiomyopathy.

There is limited, but reliable data on the neuroprotective role of the endocannabinoid system in hepatic encephalopathy. It has been demonstrated in a murine model that during fulminant hepatic failure, the levels of 2-AG in the brain are elevated, probably as a response to liver damage. The administration of CB2 endogenous ligand 2-AG, an antagonist of CB1 receptor, SR141716A, or an agonist of CB2 receptor, HU308, accomplished a marked improvement in neurological score. Hence, influencing the endocannabinoid system with exogenous cannabinoid derivates specific for the CB2 or CB1 receptor might have a beneficial therapeutic effect on neurological dysfunction in liver diseases[24]. Further research has indicated the impact of CB2 signaling on the activity of cerebral AMP-activated protein kinase (AMPK) in conditions of liver failure. It has been shown in wild type mice that administration of THC leads to increased activity of AMPK in the brain and neurological improvement, possibly *via* stimulation of CB2 receptors, as this effect is absent in CB2 knock-out mice[25].

Numerous hemodynamic vascular effects contributing to the poor prognosis of disease outcome accompany end-stage liver disease. The cirrhotic rebuilding of hepatic tissue results in increased resistance in portal circulation and eventually in elevated portal pressure. Additionally, the arterial vasodilatation in splanchnic and systemic circulations contributes to hyperdynamic state, arterial hypotension and increased blood inflow from mesenteric arteries, which augments the unfavorable effect of portal hypertension. There are many reports linking these vascular effects with the improper activity of the endocannabinoid system, particularly with stimulation of CB1 receptor in vascular endothelial cells with endogenous cannabinoids. The cirrhotic state is often accompanied by the endotoxemia caused by release of bacterial lipopolysaccharide (LPS) synthesized by the intestinal flora into the systemic circulation, while its hepatic elimination is insufficient[26]. The effects of blood LPS on systemic circulation correspond to the hemodynamic changes observed in cirrhosis[27]. Batkai *et al*[21] have provided evidence that explains the association between the endocannabinoid system and its influence on circulatory changes in cirrhosis. It was demonstrated in an animal model of cirrhosis complicated by hemodynamic alterations, that treatment with CB1 receptor antagonist (SR141716A) sufficiently improved hemodynamic state, which manifested in elevation of arterial pressure and reduction of mesenteric blood flow and portal pressure. It was shown that the intravenous injection of the monocyte fraction isolated from the blood of both cirrhotic rats and a patient with cirrhosis, was able to induce hypotension. This was reversible by treatment with SR141716A, whereas the injection of monocytes from controls did not exert such an effect. Moreover, the examination of monocytes from cirrhotic and control individuals and animals demonstrated increased levels of AEA in the monocytes in cirrhotic state, which may have reflected the stimulation of endocannabinoid synthesis by bacterial LPS shown in previous studies[21]. Additionally, upregulation of CB1 receptors in hepatic arterial endothelial cells isolated from cirrhotic livers was observed, thus, indicating its increased sensitivity to vasodilatory stimuli, such as endocannabinoids secreted by the monocytes and platelets adhering to endothelium[21].

Vascular effects exerted by endocannabinoids are divergent and complex. It is postulated that endocannabinoids might contribute to potency disorders in cirrhosis, as was observed in animal models. AEA was shown to

augment the relaxation of samples of corpus cavernosum from biliary cirrhotic rats, probably through CB1 and VR1 signalling[28].

Recently, the role of endocannabinoid signaling in the development of cardiomyopathy during liver cirrhosis has been investigated. It is characterized by decreased β-adrenergic responsiveness, impaired cardiac conduction and insufficient heart muscle contraction to excitation stimuli, whereas cardiac output remains increased compared to baseline. Gaskari *et al*[29] have confirmed in an animal model the role of CB1 signaling in the pathogenesis of cirrhotic cardiomyopathy. It has been shown that, when the cardiac muscle probes from cirrhotic rats are pre-incubated with the CB1 antagonist, AM251, their contractility is similar to the controls[29]. The significance of CB1 signaling has been demonstrated *in vivo* in cirrhotic rats presenting late symptoms of decreased cardiac contractility, hypotension and tachycardia. These symptoms were ameliorated in cirrhotic rats, by the bolus injection of AM251, whereas its administration in controls had no effect. Hence, the authors concluded that the above-mentioned cardiac effects might have resulted from increased concentration of AEA in cardiac tissue in liver cirrhosis, as the cardiac expression of CB1 receptors was similar in cirrhotic and non-cirrhotic control rats[30]. These observations are consistent with the study of Bonz *et al*[31] assessed the influence of AEA on the contractility of human heart atrial muscle upon electrical stimulation. The inotropic negative effect exerted by AEA and other examined CB1 agonists was predictably abolished by pre-incubation with CB1 antagonist ation[31]. Thus, blocking the CB1 signaling might have advantageous therapeutic effects on various clinical aspects of cirrhotic cardiomyopathy and other related conditions.

CLINICAL ASPECTS

There are limited clinical data on the effects of cannabinoids in chronic liver diseases. According to clinical research of Hezode *et al*[32], daily cannabis smoking appears to be an independent factor of fibrosis progression in chronic hepatitis C (CHC) patients. The research was performed on a group of 270 CHC patients, divided into non-cannabis users (52.2%), occasional cannabis users (14.8%) and daily cannabis users (33.0%). The collected data on epidemiological, demographic, metabolic and virological aspects, and history of cannabis, alcohol and tobacco abuse, allowed them to specify the factors for fibrosis progression. This study confirmed the well-recognized independent fibrosis predictors such as necroinflammatory activity ⩾ A2 (METAVIR score), age > 40 years at the time of exposure, steatosis and serious alcohol abuse, but also rated the daily cannabis use as a distinct factor that influenced alone the progression of liver fibrosis. This could result from profibrogenic activity of CB1 signaling, thus implying the beneficial therapeutic potential of CB1 antagonists.

Moreover, it has been proven that regular daily cannabis use has a significant impact on the severity of steatosis, which may eventually contribute to fibrosis progression in the course of CHC[33]. It has been shown that high fat dietary supply increases the hepatic levels of AEA, expression of CB1 receptor and augments fatty acid synthesis, thus contributing to obesity and other metabolic disorders proceeding to liver steatosis[15,34]. The mechanisms, in which endocannabinoids lead to obesity-associated fatty liver, or even steatohepatitis, are CB1-receptor-dependent and include increase in fatty acids intake, induction of lipolysis in adipocytes, stimulation of hepatic lipogenesis and downregulation of adiponectin in adipose tissue. Interestingly, CB1-knockout mice are resistant to obesity induced by high-energy-containing food intake[35]. Similarly, the pharmacological inactivation of CB1 with rimonabant (SR141716) results in reduction of obesity and hepatic steatosis in rodents[36,37].

ENDOCANNABINOID SYSTEM AS A THERAPEUTIC TARGET

The beneficial effect of the regulation of endocannabinoid signaling is postulated in management of various pathological conditions, including obesity and metabolic syndrome; addiction to alcohol, tobacco and opiates; Alzheimer's disease, Parkinson's disease, schizophrenia, memory loss, chronic pain, liver fibrosis, and numerous inflammatory conditions and allergies[38].

Due to their regulatory functions in chronic hepatic disorders, especially fibrosis, influencing the endocannabinoid receptors seems to be an advantageous therapeutic target. It seems that treatment with CB1 antagonist, CB2 agonist or both, may offer clinical benefits, resulting in at least deceleration of disease progression. It has also been shown that blocking CB1 signaling is favorable in maintaining the proper blood pressure in hypotensive cirrhotic rats[21,39]. Moreover, in clinical studies, rimonabant has exerted additional beneficial actions influencing the profile of blood lipids and glycemia control in obesity, metabolic syndrome and type 2 diabetes mellitus. It also had an impact on lifestyle modification, for instance, cigarette smoking cessation rates were significantly higher during treatment with rimonabant[40]. Interestingly, Wang *et al*[41] have suggested a possible link between CB1 signaling and ethanol preference in immature mice, and this effect was diminished after administration of rimonabant. This observation might be particularly useful in patients with alcoholic liver disease who persist in drinking.

CONCLUSION

The role of the endocannabinoid system in hepatic physiology and pathologic conditions has been studied recently. Unquestionably, influencing endocannabinoid signaling may have a beneficial effect on delaying or even reversing hepatic fibrosis. It is particularly important due to the lack of antifibrotic drugs with established advantageous profiles of activity, despite years of investigations into this subject. Thus, further research may provide the valuable means of management in hepatic fibrosis in the future.

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REVIEW

Ste20-related proline/alanine-rich kinase: A novel regulator of intestinal inflammation

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Abstract

Recently, inflammatory bowel disease (IBD) has been the subject of considerable research, with increasing attention being paid to the loss of intestinal epithelial cell barrier function as a mechanism of pathogenesis. Ste20-related proline/alanine-rich kinase (SPAK) is involved in regulating barrier function. SPAK is known to interact with inflammation-related kinases (such as p38, JNK, NKCC1, PKCθ, WNK and MLCK), and with transcription factor AP-1, resulting in diverse biological phenomena, including cell differentiation, cell transformation and proliferation, cytoskeleton rearrangement, and regulation of chloride transport. This review examines the involvement of Ste20-like kinases and downstream mitogen-activated protein kinases (MAPKs) pathways in the pathogenesis and control of intestinal inflammation. The primary focus will be on the molecular features of intestinal inflammation, with an emphasis on the interaction between SPAK and other molecules, and the effect of these interactions on homeostatic maintenance, cell volume regulation and increased cell permeability in intestinal inflammation.

Key words: Inflammatory bowel diseases; WNK; NKCC1; Barrier function; Ste20-related proline/alanine-rich kinase

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INTRODUCTION

Inflammatory bowel diseases (IBD), primarily ulcerative colitis (UC) and Crohn's disease (CD), are chronic idiopathic inflammatory disorders of the gastrointestinal tract that are thought to arise as a result of an interplay of genetic and environmental factors. The mechanisms implicated in the pathogenesis of IBD (Figure 1) include: (1) inappropriate regulation of the innate immune response at the level of the intestinal mucosa; (2) deregulation of the adaptive immune system stemming from an imbalance between regulatory and effector-cell immune responses to luminal antigens; and (3) increased permeability across the mucosal epithelial barrier due to loss of structural integrity and/or abnormal transepithelial transport[1,2]. The loss of barrier function, in particular, has gained increasing support as an IBD pathogenic mechanism because the epithelium represents a potential intersection of both genetic and environmental influences. The intestinal mucosa is composed of a single layer of polarized intestinal epithelial cells (IECs) that protects against direct contact with enteric antigens, bacteria and other pathogens (Figure 1). The integrity of the epithelium is maintained primarily through a combination of intercellular adhesion structures and specialized junctions. In addition, other factors such as the presence of mucins, rapid turnover of epithelial cells, and peristaltic movement of the gastrointestinal tract, all help to protect against colonization and invasion of the intestinal mucosa by pathogens[3]. Moreover, epidemiological and genetic linkage studies have confirmed a strong link between modulation of the barrier function and IBD; these include, for example, the loci IBD1-9, corresponding to regions on chromosomes 16, 12, 6, 14, 5, 19, 1, 16 and 3, respectively[4-13], and a new IBD locus on chromosome 2[14].

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) ARE INVOLVED IN INTESTINAL INFLAMMATION

Intracellular signaling cascades are the main route of communication between the plasma membrane and

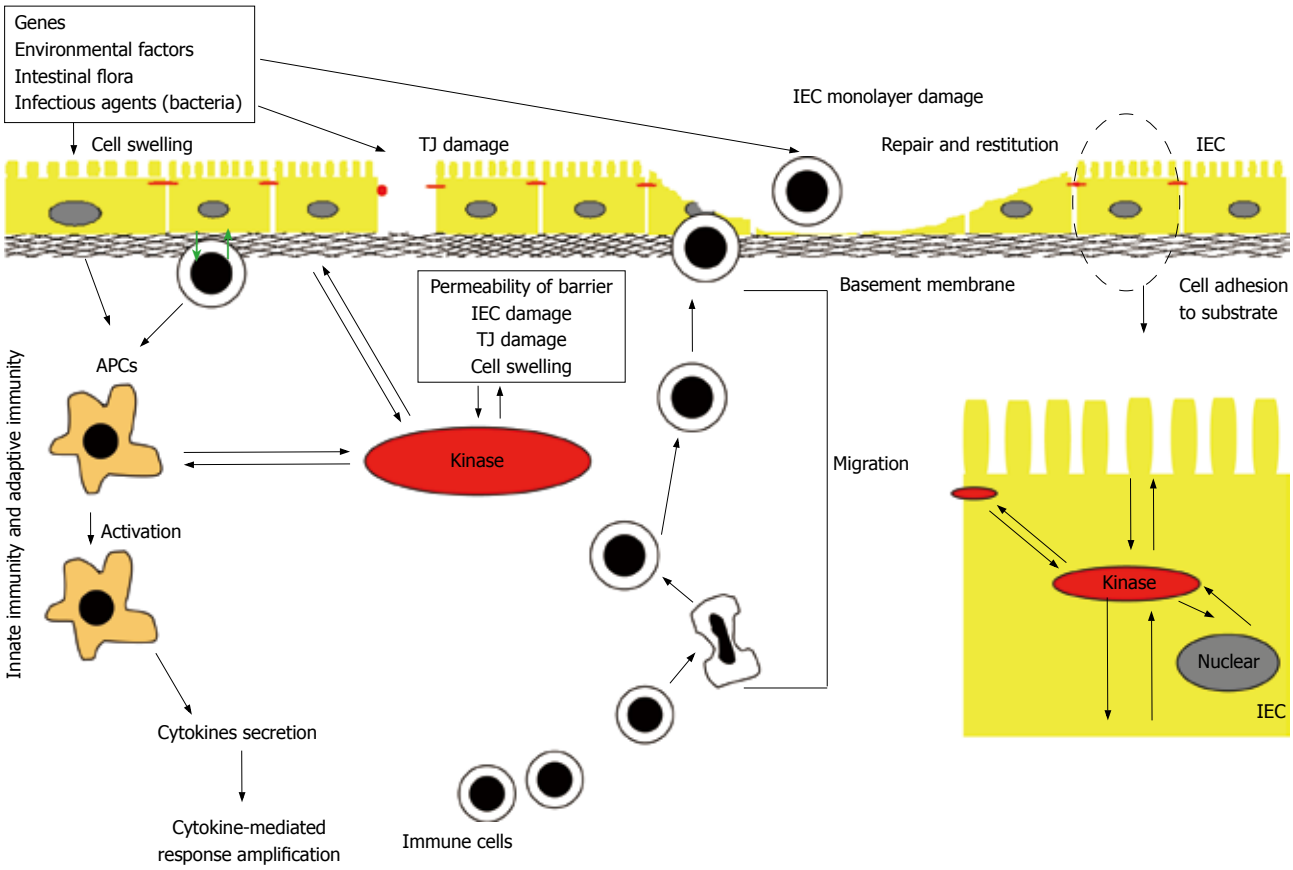

Figure 1 Pathogenesis of IBD. Many different factors, such as genetic factors, environmental factors, and intestinal non-pathogenic or pathogenic bacteria can damage the mucus, epithelium, or the tight junction, to initiate the inappropriate regulation or deregulation of the immune response, leading to the secretion of pro-inflammatory cytokines, decrease in epithelial barrier function and initiation of the inflammation-related signaling pathways. IEC: Intestinal epithelial cell; APC: Antigen presenting cell; TJ: Tight junction.

regulatory targets in various intracellular compartments. The evolutionarily conserved MAPK signaling pathway plays an important role in transducing signals from diverse extra-cellular stimuli (including growth factors, cytokines and environmental stresses) to the nucleus in order to affect a wide range of cellular processes, such as proliferation, differentiation, development, stress responses and apoptosis. MAPK signaling cascades, which comprise up to five levels of protein kinases that are sequentially activated by phosphorylation, are also involved in intestinal inflammation[15-17] (Figure 2).

MAPK signaling pathways are involved in regulating crucial inflammatory mediators and could thus serve as molecular targets for anti-inflammatory therapy. At least six distinct MAPK pathways have been identified in multicellular organisms, of which three, the extra-cellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 cascades, are significantly activated and directly involved in inflammatory diseases such as IBD (Figure 2). In this context, cross-talk between these pathways and other inflammatory signaling pathways, including the NF-κB and Janus kinase/signal transducers, and activation of transcription (STAT) cascades[18-20], is also relevant to the action of MAPK pathways.

The involvement of some MAPK members in IBD is suggested by linkage studies. For example, the ERK1 gene is located in a major IBD susceptibility region on chromosome 16[4], and the p38α gene is located in a major IBD susceptibility region on chromosome 6[9]. Activation of p38 MAPK is also known to induce the production and secretion of pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α)[21], and increased activity of p38 MAPK has been observed in patients with IBD[18,22]. Inhibition of p38 has been well documented to suppress IBD[17], and the guanylhydrazone compound, CNI-1493, which inhibits both JNK and p38, strongly reduces clinical disease activity in CD patients. In addition, inhibition of either ERK or p38 kinase pathway decreases lipopolysaccharide (LPS)-induced production of the cytokines, IL-6 and TNF-α[23]. The involvement of JNK pathways in intestinal inflammation has been intensively studied both in patients with IBD and in an experimental colitis model[18,24,25]. JNK inhibitors, which affect either JNK signaling pathway indirectly (e.g. CEP1347) or block the catalytic domain of JNK (e.g. SP 600125), have been tested for their potential value in treating IBD. Collectively, these observations demonstrate a very important role for MAPK pathways in the control and therapy of IBD.

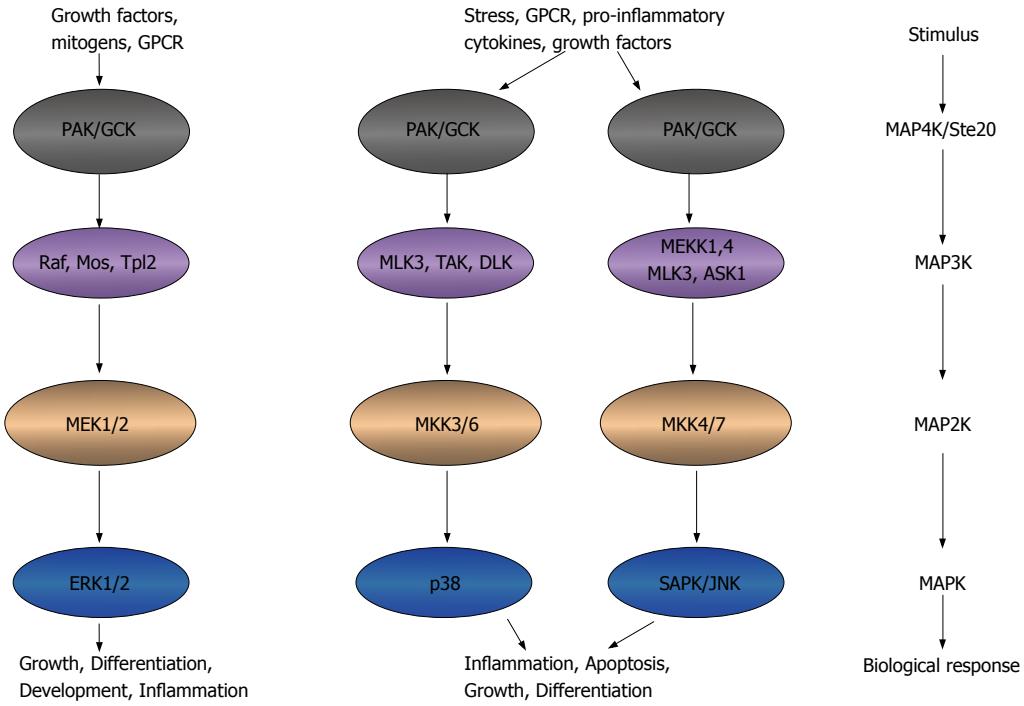

Figure 2 Ste20 kinases participate in inflammation. Ste20 kinases that function as an MAP4K can activate MAP3K, MAP2K and MAPK, leading to the inflammatory functions. This model adapted from the model presented in http://www.cellsignal.com/pathways/map-kinase.jsp. MAPK: Mitogen-activated protein kinase. GPCR: G-protein coupled receptor; PAK: p21 activated kinase; GCK: Germinal central kinase; MLK: Multiple lineage kinase; TAK: Tat-associated kinase; DLK: Dual leucine zipper-bearing kinase; MEK: MAPK/Erk kinase; MEKK: MEK kinase; ASK: Aspartate kinase; MKK: MAPK kinase; Erk: Extracellular signal-regulated kinase; SAPK: Stress-activated protein kinase; JNK: Jun-amino-terminal kinase.

STE20-LIKE KINASES ACT UPSTREAM OF MAPK PATHWAYS

The various MAPK pathways share a common family of upstream mediators: the Ste20 kinases. Ste20 was originally identified as a component of the pheromone-response pathway in budding yeast, and has also been shown to participate in the signaling pathways that regulate osmotic responses, including those to high osmolarity glycerol (HOG)[26]. Several mammalian Ste20 homologs have been identified. The Ste20 family includes two subfamilies that share basic structural and functional properties. The first subfamily includes the p21-activated kinases (PAKs), which are characterized by a C-terminal catalytic domain and an N-terminal binding site for the small G proteins, Rac1 and Cdc42. The second family comprises of the germinal center kinases (GCKs), which contain an N-terminal kinase domain and a C-terminal regulatory domain.

Ste20-like kinases function as MAP4Ks, triggering activation of MAPK cascade[27-29] and transmitting signals from extra-cellular stimuli that activate transcription factors (Figure 2). The resulting changes in gene expression, in turn, regulate cellular functions[27-31] that are important in the maintenance of epithelial barrier function, apoptosis, growth, morphogenesis, cell permeability, and rearrangements of the cytoskeleton that lead to changes in cell shape and motility. For example, members of the PAK subfamily of Ste20 kinases have been shown to increase endothelial permeability[32,33]. The pro-inflammatory cytokine, TNF-α, stimulates expression of the yeast Ste20 homolog, Map4k4, through TNF-α-receptor-1-mediated signaling to c-Jun[34], the chemokine CXCL12 and the complement factor C5a. The resulting increase in Map4k4 activity triggers cell migration *via* a PAK1/2-p38α MAPK-MAPKAP-K2-HSP27 pathway[35]. Other relevant examples include: (1) Ste20-like kinase (SLK)[36], Ste20-like oxidant stress-activated kinase (SOK)[37] and prostate-derived Ste20-like kinase 1-α (PSK1-α)[38], which induce apoptosis by activating the JNK pathway; (2) lymphocyte-oriented kinase (LOK)[39] and SLK[40], which regulate Rac1-mediated actin reorganization during cell adhesion and spreading; (3) mixed lineage kinase-3 (MLK-3)[41], which activates the SAPK/JNK and p38/RK pathways *via* SEK1 and MKK3/6; and (4) hematopoietic progenitor kinase 1 (HPK1), which is activated by prostaglandin E2 (PGE2) through a G-protein coupled receptor (GPCR) pathway, and negatively regulates transcription of the *fos* gene[42].

Ste20-like kinases has been reported to be activated by at least three pathogen-associated molecular patterns (PAMPs)-lipopolysaccharide, peptidoglycan, and flagellin-produced by invading microbial pathogens, and has been shown to initiate innate immune responses by binding to pattern recognition receptors (PRRs)[43]. PAMPs activate GCKs (Ste-20 family of kinases), which signal through MLK-2 and -3 to recruit JNK, p38 and their effectors[43]. These findings indicate an important role for GCKs and MLKs in PAMP-stimulated MAPK pathway activation, and therefore in stimulating the expression of pro-inflammatory genes involved in intestinal inflammation.

STE20-RELATED PROLINE/ALANINE-RICH KINASE (SPAK) IS A STE20-LIKE KINASES INVOLVED IN INTESTINAL INFLAMMATION

The GCKs may be divided into eight subfamilies based on homologies in their C-terminal domains (GCKI-VII). The Ste20-like kinase SPAK[44], PASK (the rat SPAK homolog)[45,46] and OSR1[47] share GCK Ⅵ homologies. Among these, SPAK and OSR1 are ubiquitously expressed. PASK is also expressed in most rat tissues, but its expression is particularly notable in cells with high ion-transport activity[45,48]. Both SPAK and PASK are highly expressed in epithelia and neurons[49]. On the other hand, PASK is found only in negligible levels in the liver and skeletal muscle[50]. SPAK, OSR1 and PASK contain a series of proline and alanine repeats (PAPA box) at the extreme N-terminus, followed by a serine/threonine kinase domain, a nuclear localization signal, a consensus caspase cleavage recognition motif, and a C-terminal regulatory region. However, the colonic SPAK isoform is unique in that it lacks the PAPA box and N-terminal F-alpha helix loop, due to the presence of a 5' splice junction-like sequence within exon-1[51]. Given its ubiquitous expression and diverse functional domains, the SPAK protein may be associated with diverse biological roles. It has been shown that under hyper-osmotic (but not hypo-osmotic) stress conditions, SPAK translocates from the cytosolic pool to a Triton X-100-insoluble fraction; although present in both fractions, SPAK associated with the Triton X-100-insoluble pool is dephosphorylated[52]. Our laboratory has observed that upon SPAK over-expression[51] or under TNF-α stress conditions (unpublished data), SPAK is cleaved and the N-terminal fragment is translocated to the nucleus.

The Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1), a member of the Slc12 family of solute carriers and target of SPAK, plays a crucial role in cell volume regulation, cell proliferation and survival, and epithelial transport[53]. The activity and expression of NKCC1 can be regulated by cell volume[53] and intracellular chloride concentration[54], which act through NKCC1's N-terminal (R/K) FX (V/I) binding motif. The pro-inflammatory cytokines IL-1β, TNF-α[55] and IL-6[56] also regulate NKCC1 activity. In addition, NKCC1 can be activated by α- and β-adrenergic stimulation *via* the cAMP/PKA-dependent pathway[57-59] and can be stimulated by PKC in a cell-specific manner[60,61]. Notably, NKCC1 can be phosphorylated by hyperosmolarity and, *in vitro*, by JNK, which can also be activated by hyperosmolarity[62,63]. As an upstream kinase to NKCC1, SPAK can associate through its conserved C-terminal domain with the (R/K) FX (V/I) motif of NKCC1 and phosphorylate Thr203, Thr207, and Thr212 residues on NKCC1, thereby playing an important role in inflammation[45,64,65]. However, SPAK alone is unable to activate NKCC1. SPAK is a substrate of WNK1/4, which are serine threonine kinases lacking a lysine in subdomain I of the catalytic domain[66]. SPAK physically associates through its conserved C-terminal domain with the C-terminus of WNK, resulting in phosphorylation and activation of SPAK by WNK. WNK is also unable to activate NKCC1 in the absence of SPAK, indicating that this association of SPAK with WNK is required for SPAK-dependent phosphorylation and activation of NKCC1. A mutation of WNK1 is involved in the pathogenesis of pseudohypoaldosteronism type Ⅱ (PHA Ⅱ), characterized by hypertension and hyperkalemia[67].

SPAK can also activate p38 pathways in different cell types[51,68,69] to play a role in cell differentiation; an observation that may be relevant in the context of the known relationship between the p38 pathway and inflammation[17,70-74]. Interestingly, p38 activation has been noted in damaged corneal epithelial tissue and in an *in vitro* intestinal epithelial restitution model[75-78], suggesting that under some circumstances p38 may be involved in regulating cell motility and wound healing. Protein kinase C θ (PKCθ) is known to be an intestinal inflammation-related kinase[79]. By associating with Rho GTPases, PKCθ migrates from the cytosol to the membrane and the actin cytoskeleton[80], where SPAK may act as both a substrate and target of PKCθ in a TCR/CD28-induced signaling pathway that leads selectively to AP-1 activation, T-cell transformation and proliferation, and IL-2 production[81]. SPAK is also known to associate with F-actin under conditions of stress, which, along with the activation and phosphorylation of myosin light chain kinase (MLCK), leads to cytoskeleton rearrangement[47,52]. Fray, the Drosophila orthologue of mammalian SPAK, has been shown to participate in the activation of the JNK pathway by sorbitol[47]. Fray probably functions by activating MAP3K, leading to activation of MAP2K/MEK4 and MEK7, and ultimately, JNK activation.

Accumulating evidence points to the important role that SPAK plays in the physiology and pathogenesis of intestinal inflammation (Figure 3). First, by activating and phosphorylating p38, Ap-1, NKCC1, as well as p21-activated protein kinase 1 (PAK1, another Ste20 line kinase), SPAK induces the transcription of inflammation-related genes or modulates the function of inflammation-related proteins. Second, SPAK is activated and phosphorylated by WNK1/4, PKCθ and MLCK. In addition, SPAK has been reported to associate with the heat shock protein HSP105, the cytoskeleton protein gelsolin, and the apoptosis-associated tyrosine kinase AATYK. We have observed that SPAK can increase the permeability of Caco2-BBE cells (unpublished observations). Additional unpublished data indicate that colonic epithelial SPAK expression is increased in IBD patients and in mice with experimentally induced colitis. Importantly, we have also found that the pro-inflammatory cytokine, TNF-α, increases colonic SPAK expression, an observation that underscores the importance of SPAK in the pathogenesis of intestinal inflammation.

PERSPECTIVE

Increased permeability across the mucosal epithelial

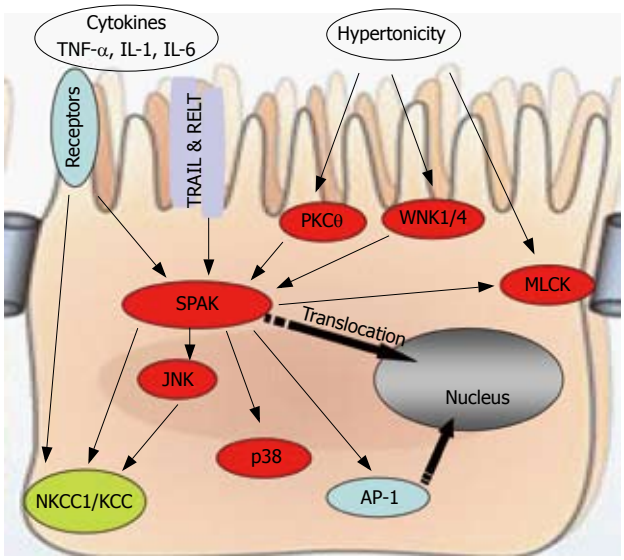

Figure 3 SPAK interacts with other molecules to maintain cellular homeostasis. SPAK can be a substrate, indirectly or directly, for pro-inflammatory cytokines, environmental stress including hypertonicity, some other kinases such as PKCθ, WNK1/4, or other receptors, for example TRAIL & RELT. Also SPAK can function as upstream kinase to JNK, p38, or ion transport NKCC1/KCC, transcription factor AP-1, as well as MLCK. WNK: With no lysine kinase 1/4; TRIL: TNF-related apoptosis-inducing ligand; RELT: Receptor expressed in lymphoid tissues; MLCK: Myosin Ⅱ regulatory light chain kinase; NKCC1: Sodium potassium chloride chloride transporter 1; KCC: Potassium chloride chloride transporter; AP-1: Activating protein 1.

barrier resulting from loss of structural integrity and/or abnormal transepithelial transport is thought to be one of the main functional changes that lead to IBD. Numerous studies have focused on epithelial barrier function, measuring transepithelial electrical resistance (TER), which is known to be decreased in intestinal epithelium by over-expression of SPAK[51]. Other studies have assessed cell adhesion and migration, providing a measure of wound healing. The pro-inflammatory cytokine TNF-α is both necessary and sufficient to trigger the onset of IBD. In fact, nearly half of the drugs used for the treatment of IBD target TNF-α. In *in vitro* studies, we have found that TNF-α increases SPAK expression in intestinal epithelial cells in a dose- and time- dependent manner (unpublished data). It is therefore reasonable to speculate that the regulation of SPAK by TNF-α could account for TNF-α-mediated alterations of barrier function and inflammation in intestinal epithelial cells. Additional studies on the role of SPAK in intestinal barrier function would likely substantially advance the field of IBD.

Intestinal inflammation is usually associated with hyper-osmotic status in the lumen. The WNK1/4-SPAK-NKCC1 pathway has been highlighted in this context as a molecular mechanism that may contribute to ion transport and cell volume changes. This pathway, together with its interactions with other related molecules, such as MLCK, claudin and zo-1, may play an important role in maintaining cell shape, since the epithelial cell tight junctions that play a dominant role in TER would collapse in IBD. In short, more attention should be paid to tight junction and cell volume regulation as important contributing factors in IBD.

It should be evident from this review that SPAK occupies an important intracellular position, integrating extra-cellular pro-inflammatory signals and converting them into pro-inflammatory cellular responses. Given its unique position at the crossroads of multiple pathways, SPAK appears to represent an attractive target for developing effective and efficient strategies to treat IBD. Continuing work along the lines suggested above could make important contributions to the effort to realize the potential of this therapeutic approach.

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REVIEW

Natural heme oxygenase-1 inducers in hepatobiliary function

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Abstract

Many physiological effects of natural antioxidants, their extracts or their major active components, have been reported in recent decades. Most of these compounds are characterized by a phenolic structure, similar to that of α-tocopherol, and present antioxidant properties that have been demonstrated both *in vitro* and *in vivo*. Polyphenols may increase the capacity of endogenous antioxidant defences and modulate the cellular redox state. Changes in the cellular redox state may have wide-ranging consequences for cellular growth and differentiation. The majority of *in vitro* and *in vivo* studies conducted so far have attributed the protective effect of bioactive polyphenols to their chemical reactivity toward free radicals and their capacity to prevent the oxidation of important intracellular components. However, in recent years a possible novel aspect in the mode of action of these compounds has been suggested; that is, the ultimate stimulation of the heme oxygenase-1 (HO-1) pathway is likely to account for the established and powerful antioxidant/anti-inflammatory properties of these polyphenols. The products of the HO-catalyzed reaction, particularly carbon monoxide (CO) and biliverdin/bilirubin have been shown to exert protective effects in several organs against oxidative and other noxious stimuli. In this context, it is interesting to note that induction of HO-1 expression by means of natural compounds contributes to protection against liver damage in various experimental models. The focus of this review is on the significance of targeted induction of HO-1 as a potential therapeutic strategy to protect the liver against various stressors in several pathological conditions.

Key words: Heme oxygenase; Hepatobiliary function; Natural inducers; Carbon monoxide; Oxidative stress

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INTRODUCTION

Heme oxygenase (HO) is the first, and the rate limiting enzyme in the catabolism of heme[1], to yield equimolar amounts of biliverdin, carbon monoxide (CO) and free iron (Figure 1). To date, two isoforms of HO designated as HO-1 and HO-2 have been identified in mammals[2]. HO-1 is also known as heat shock protein 32. Its human form is composed of 288 amino acids with a molecular mass of 32800 Da and shares about 80% amino acid sequence identity with rat HO-1[3]. On the other hand, human HO-2 is a 36-kDa protein that consists of 316

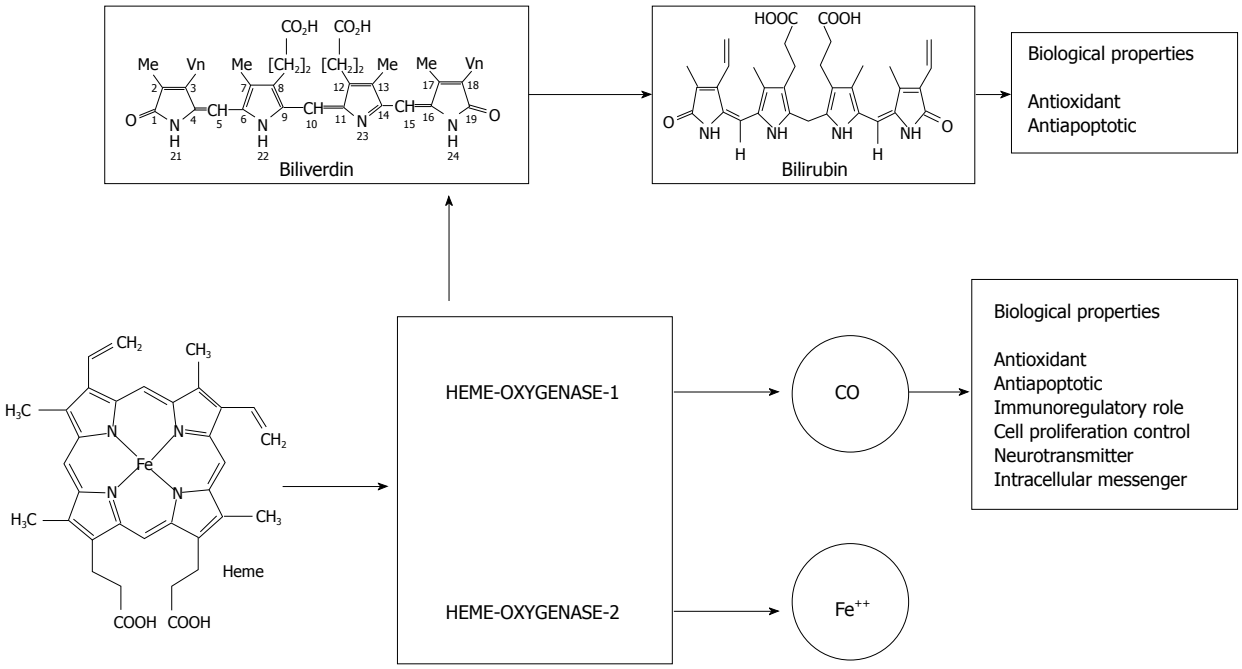

Figure 1 Schematic representation of heme degradation with biological properties of its byproducts.

amino acids with three cysteine residues[4]. HO-1 is highly inducible by hemin and other chemical and physical agents such as ultraviolet, hydrogen peroxide, heavy metals, hypoxia, and nitric oxide[5,6]. Immunohistochemical studies with specific monoclonal antibodies have revealed the distribution of HO-1 and HO-2 in rat liver with distinct topographical patterns[7]: HO-1 has been shown to be expressed principally in Kupffer cells, while HO-2 is expressed in parenchymal cells[8]. Trakshel *et al*[2] have demonstrated that under unstimulated conditions, the activity of HO-2 was two- to three-fold higher than that of HO-1, while the activity of HO-1 increased more than 100-fold in the presence of cadmium or cobalt. Under conditions of oxidative stress, hypoxia or hyperthermia, the induction of HO-1 accounts for the majority of heme breakdown, leading to the formation of bilirubin and CO. Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this stress-response enzyme may be considered as an important therapeutic strategy for the protection against inflammatory processes and oxidative tissue damage (Figure 1). In this article, recent findings on the implications of HO-1 induction on the cellular adaptive cytoprotective response to various insults and inflammatory conditions are reviewed, with particular emphasis placed on targeted HO-1 induction by natural compounds for hepatoprotection.

NATURAL INDUCERS OF HO-1

A number of natural antioxidant compounds contained in foods and plants have been demonstrated to be effective non-stressful and non-cytotoxic inducers of the response protein HO-1 in hepatic cellular models. Most of these compounds are contained in plants that, besides being widely used as food, spices or flavoring, since ancient times, also represent locally traditional medicinal plants.

Curcumin

Curcumin (diferuloylmethane, Figure 2) is the most investigated natural HO-1 inducer. Curcumin is a yellow pigment obtained by populations living in Asian tropical regions by drying and powdering the rhizome of turmeric (*Curcuma longa Linn*). Widely used as food flavouring, it also plays an important role in traditional medicine because of its anti-inflammatory, anticarcinogenic and antioxidant properties. Curcumin has been demonstrated to be a potent HO-1 inducer in several cellular models (for a Review see Lin[9]). However, the ability of curcumin to induce HO-1 in human hepatocytes has been demonstrated only recently by McNally *et al*[10]. Interestingly, curcumin is able to confer, at non-toxic doses, a significant protective effect in two transplant-related models of cellular injury, such as cold preservation and warm reperfusion. In a successive study[11], these authors confirmed the HO-1 induction ability of curcumin and elucidated the possible biochemical mechanism. Indeed, at both non-toxic and toxic doses, curcumin treatment resulted in ROS generation, activation of Nrf2 and mitogen-activated protein kinases (MAPKs) and in the inhibition of phosphatase activity. They concluded that at non-toxic doses these multiple pathways converged to induce HO-1.

Flavonoids

Flavonoids are naturally occurring antioxidants belonging to the large family of polyphenols. They are widely distributed in plants used as food, as well as traditional medicines, because of their peculiar variety of clinically relevant properties, such as anti-tumor, antiplatelet, anti-ischemic, and anti-inflammatory activities. Antioxidants with strong free-radical scavenging properties contribute

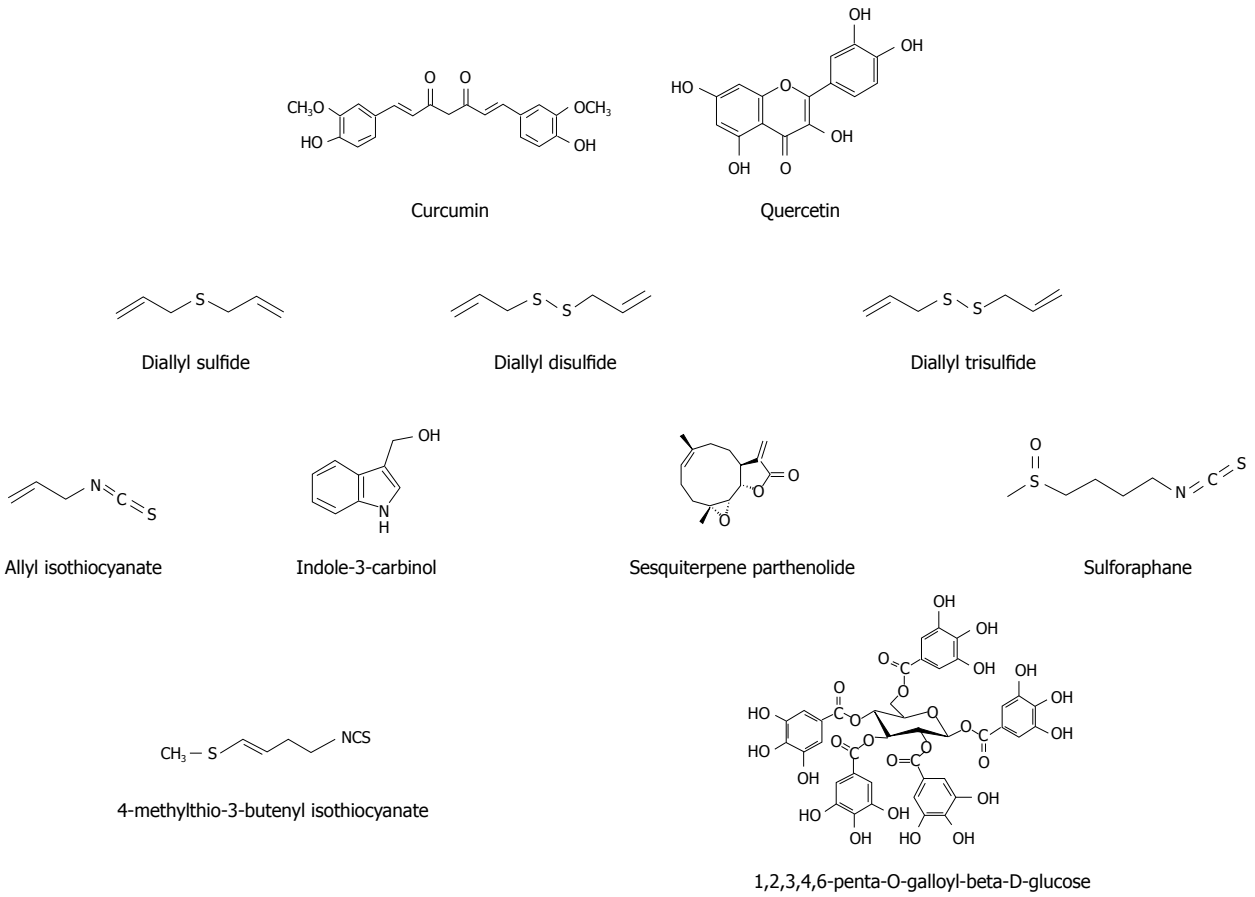

Figure 2 Chemical structures of various natural HO-1 inducers.

to their biological effects mainly by the Michael reaction acceptor function. However, recent studies[12] have demonstrated the ability of flavonoids to exert their protective properties also by influencing signaling pathways, thus indirectly interacting with the endogenous antioxidative defense system.

Quercetin (Figure 2) is one of the most common flavonoids and, probably, overall the most investigated. In human hepatocytes, quercetin is able to attenuate ethanol-induced oxidative damage by HO-1 induction *via* p38 and, especially, *via* ERK/Nrf2 transduction pathway[13,14]. Recently, in the same cellular model, Kluth *et al*[15] have confirmed that quercetin is able to activate the gene expression regulated by the EpRE of HO-1, although its ability was about 10 times less than that of thyme.

Garlic-derived organosulfur compounds

Diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) (Figure 2), the three major garlic (*Allium sativum*) organosulfur compounds, have been demonstrated to be HO-1 inducers in hepatic cellular models. In human hepatoma HepG2 cells, Chen *et al*[16] observed that garlic organosulfur compounds induced HO-1 as the result of Nrf2 activation. Gong *et al*[17] have confirmed the involvement of Nrf2 activation in the induction of HO-1 by garlic DAS in HepG2 cells. Additionally, in this latter study a different pathway of HO-1 induction has been revealed for DATS, leading the authors to argue that structural differences in terms of the number of sulfur moieties and the length of alkyl side chains can explain the differential effects of garlic-derived organosulfur compounds on the MAPK-mediated activation of Nrf2 and HO-1 induction.

Isothiocyanates

Cruciferous vegetables, particularly Brassica vegetables (i.e. broccoli, Brussels sprouts and cabbage), contain high concentrations of glucosinolates (β-thioglucoside *N*-hydroxysulfates) that are the precursor of isothiocyanates, potent inducers of cytoprotective enzymes and inhibitors of carcinogenesis. Noteworthy, the isothiocyanate sulforaphane, due to its peculiar ability to inhibit phase I enzymes and induce phase Ⅱ enzymes (i.e. HO-1), exerts valuable pleiotropic pharmacologic effects.

Jeong *et al*[18] have investigated the regulatory role of allyl isothiocyanate, indole-3-carbinol, the sesquiterpene parthenolide and sulforaphane (Figure 2) in the expression and degradation of Nrf2 and the induction of the antioxidant enzyme HO-1. Allyl isothiocyanate is an effective inducer of Nrf2 protein expression, ARE-reporter gene and HO-1, but had little effect on delaying the degradation of Nrf2 protein. Parthenolide and indole-3-carbinol also induced ARE-reporter gene expression and Nrf2, although to a lesser extent if compared to sulforaphane and allyl isothiocyanate. Nonetheless, parthenolide considerably induces the HO-1 expression at a level comparable to sulforaphane, while indole-

3-carbinol shows no effect. Of note, sulforaphane strongly induces Nrf2 protein expression and ARE-mediated transcription activation, retards degradation of Nrf2 through inhibiting Keap1, thereby activating the transcriptional expression of HO-1. In the same cellular model, Keum *et al*[19] have confirmed that transcriptional activation of Nrf2/ARE is critical in sulforaphane-mediated induction of HO-1. Further evidence of the ability of isothiocyanate to activate ARE-mediated HO-1 gene transcription through Nrf2/ARE signaling pathway has been provided by a study from Prawan *et al*[20] on HepG2-C8 cells.

Hanlon *et al*[21] have demonstrated that 4-methylthio-3-butenyl isothiocyanate (Figure 2), the isothiocyanate metabolite of glucoraphasatin, a glucosinolate uniquely contained at high concentrations in Spanish black radishes, significantly induces HO-1.

Other compounds and plant extracts

1,2,3,4,6-Penta-*O*-galloyl-beta-d-glucose (PGG), a bioactive tannin contained in many medicinal plants, is able to induce HO-1 in hepatic cells (Hep-G2)[22]. PGG confers hepatoprotection against oxidative injury by inducing HO-1 expression *via* stimulating NF-E2-related factor 2 nuclear translocation in an ERK-dependent manner.

In an *in vivo* study, Yao *et al*[14] have demonstrated that a standardized *Ginko biloba* extract, containing not identified terpenes and flavonol heterosides, is able to induce hepatic microsomal HO-1 on mRNA, protein expression and enzymatic activity, providing a hepato-protective effect in ethanol challenged animals.

Hep-G2 cells have been used as a cellular model to demonstrate that the α-methylene-γ-butyrolactone moiety in dehydrocostus lactone, one of the bioactive constituents of the medicinal plant *Saussurea lappa*, increases cellular resistance to oxidant injury in HepG2 cells, presumably through Nrf2/ARE-dependent HO-1 expression[18]. The same biochemical mechanism is supposed to explain the upregulation of HO-1 synthesis induced by inchinkoto, a Chinese/Japanese herbal medicine[23].

HEME OXYGENASE AS A REGULATOR OF HEPATOBILIARY FUNCTION

In rat liver, HO-1 is prominent in Kupffer cells, whereas HO-2 is most abundant in hepatocytes[7]. Upon stimulation with lipopolysaccharide (LPS), HO-1 not only occurs in tissue macrophages, but is also markedly induced in hepatocytes. On the other hand, HO-2 does not change. Sinusoidal endothelial cells and/or hepatic stellate cells have little if any HO-1, but in culture, appear to express HO-2[8]. HO mRNA levels are high in fetal rat liver during prenatal maturation (9 d before birth) and reach a maximum 24 h after birth, when levels decline but remain above adult levels for at least 1 mo. This correlates with a greater capacity of the liver for bilirubin production in fetuses compared with adults and such circumstances could render the fetus more susceptible to drug injuries because of a depressed heme-cytochrome P450 system[24]. However, Dennery *et al*[25] have found that serum bilirubin protects against oxidative damage in the first few days of life in neonatal Gunn rats exposed to hypoxia.

Another condition in which there is induction of HO-1 is liver regeneration following 2/3 hepatectomy[26]. In HO-1-deficient mice, there is an increased susceptibility of the liver to endotoxin and interruption of a major pathway of iron recycling[27]. Most importantly, the phenotype of the first human case of HO-1 deficiency includes endothelial cell damage, iron accumulation in the liver and kidney, and an increasing cell susceptibility to heme overloading *in vitro*[28].

The major source of CO in animals is the degradation of heme by HO; CO produced by HO may serve as an important cellular signal in the microenvironment. In 1994, Suematsu *et al*[29] have shown that CO is present at submicromolar levels in the liver effluent and that inhibition of HO with ZnPP increases perfusion pressure in the isolated perfused rat liver, an effect that can be reversed by adding CO or a cGMP analogue to the perfusate. CO serves as an endogenous factor that reduces sinusoidal tone involving, at least in part, hepatic stellate cells[30]. The importance of CO as an endogenous modulator of vascular portal perfusion has been confirmed by Pannen *et al*[31] who demonstrated how NO serves as a potent vasodilator in the hepatic arterial circulation, but exerts only a minor vasodilatory effect in the portal venous vascular bed, while CO does not regulate hepatic artery tone, but is able to maintain portal vascular tone in a relaxed state. Wakabayashi *et al*[32] have confirmed these results, by showing that the induction of HO-1 with hemin causes a decrease in baseline resistance and in the response to endothelin-1 through an increase in CO production in the extrasinusoidal compartment. We have also shown that overproduction of CO by induction of HO with $CoCl_2$, reduces the response to the vasoconstrictor endothelin-1, but not to phenylephrine, in the isolated perfused rat liver[33].

Elimination of constitutive CO generation through administration of ZnPP not only increases sinusoidal tone, but also stimulates bile-acid-dependent bile flow[34]. The choleretic action coincides with an increase in microvascular tone and oxygen consumption and may thus reflect a prolonged duration of bile acid uptake by hepatocytes. Exogenous CO at micromolar concentrations completely reverses these changes. The effect of ZnPP is mimicked by administration of methylene blue, a soluble guanylate cyclase inhibitor, but not fully reversed by membrane-permeable 8Br-cGMP, suggesting some involvement of cGMP-independent mechanisms. CO generated in hepatocytes may also affect bile excretion by altering the contractility of the bile canaliculus (BC)[34]. Inhibition of CO with ZnPP shortens intervals of contraction of BCs and increases intracellular Ca^{2+}, an effect that is reversed by CO at a micromolar level without increasing cGMP[35].

As to mechanisms of the CO effect, several lines of experimental evidence suggest that CO modulates BC functions through its action on cytochrome P450-mediated calcium mobilization[36].

Jaundice

Bilirubin production is two- to three-fold greater in newborns than in adults. This increase in plasma bilirubin levels is due in large part to the combination of the rapid degradation of fetal hemoglobin in the first few days of life and the immaturity of the hepatic bilirubin conjugating system, thus leading to an increase in unconjugated bilirubin. If the levels of unconjugated bilirubin become too high, the bilirubin may cross the blood-brain barrier, resulting in bilirubin encephalopathy or kernicterus. Phototherapy is the method of choice to lower serum bilirubin levels, but its safety and efficiency have been called into question and is still a matter of debate. The clinical use of HO inhibitors is an alternative therapy. Sn-PP produces a significant decrease in the levels of serum (mean decrease, 38%) and biliary bilirubin (mean decrease, 47%) in normal subjects. The decrease in these parameters lasts for a minimum of 4 d after administration of the metalloporphyrin[37]. The tin porphyrins have been used on newborns with ABO incompatibility[38], on patients with hereditary porphyria[39], liver disease[37], or Crigler-Najjar type I syndrome[40]. Results indicate that the use of SnMP within 24 h of birth in premature newborns substantially moderates the development of hyperbilirubinemia and reduces the requirement for phototherapy markedly (> 75%) in inhibitor-treated infants compared with control subjects[41]. When administered at the appropriate time to near-term and term newborns with hyperbilirubinemia, it can entirely eliminate the need for phototherapy. In patients with biliary cirrhosis and hemochromatosis, Sn-PP is able to reduce bilirubin levels for about 4 d. Biliary bilirubin concentrations decreased (mean decrease, 49%) in hemochromatosis patients after Sn-PP administration. No decrease in biliary bilirubin concentrations can be detected in primary biliary cirrhosis patients under the same conditions[37].

HEME OXYGENASE CONFERS PROTECTION VERSUS DIFFERENT TYPES OF INSULT

Hypoxia, ischemia/reperfusion (IR) and transplantation

Hemorrhagic shock (HS) causes severe hepatic dysfunction or acute failure related to decreased hepatic microcirculatory flow, and results in enhanced hepatic expression of HO-1[8]. Furthermore, the increase in portal resistance, upon blockade of the HO-CO pathway, is much more pronounced after HS compared with sham controls. After HS is endogenously generated, CO preserves sinusoidal perfusion, mitochondrial redox state, and secretory function in the isolated perfused rat liver[42]. This protective role of CO is mediated *via* a relaxing mechanism, in part, involving Ito cells. Similar results have been obtained by Kyokane *et al*[43] in endotoxemic rats overexpressing both inducible nitric oxide synthase (i-NOS) and HO-1. In this condition, inhibition of CO, but not of NO, causes marked vasoconstriction and cholestasis. Thus, CO may exert a protective function against hepatobiliary dysfunction after HS and endotoxemia.

Reperfusion injury has been defined as the conversion of reversibly injured cells (myocardial, endothelial, *etc.*) to irreversibly injured cells, and is mediated by a burst of free-radical generation as the previously hypoxic cells are flooded with oxygen. HO mRNA increases within 4 h of reperfusion of non-necrogenic ischemic rat liver[44]. Redaelli *et al*[45] have shown that the significant effects of heat preconditioning on liver transplantation after cold storage are prevented by inhibition of HO with tin protoporphyrin, and can be reproduced by administration of cobalt protoporphyrin, an inducer of HO. Thus, overexpression of HO-1 improves post-transplantation survival from 3 d to 3 wk and graft function after prolonged cold ischemia preservation. The mechanism underlying these beneficial effects does not appear to be the prevention of apoptosis. The same beneficial effects of induction of HO-1 with cobalt protoporphyrin (CoPP) or with adenoviral HO-1 (Ad-HO-1) transfection have been shown in steatotic livers[46]. Following cold ischemia/isotransplantation, HO-1 over-expression extended animal survival from 40% in untreated controls to about 80% after CoPP or Ad-HO-1 therapy. This effect is correlated with the preserved hepatic architecture, improves liver function, depresses infiltration by T cells and macrophages, causes suppression of local expression of i-NOS, and modulates the pro- and anti-apoptotic pathways[47]. More recent data have shown that HO-1 modulates pro-inflammatory responses that are triggered *via* TLR4 signaling, a putative HO-1 repressor[48].

The role of CO in protecting liver grafts from cold I/R injury associated with liver transplantation has been studied by Kaizu *et al*[49]. Inhalation of CO reduces hepatic injury and is associated with marked downregulation of early mRNA expression for tumor necrosis factor TNF-α, interleukin IL-6, and NOS. CO significantly inhibits phosphorylation of ERK1/2 MAPK and its upstream MEK1/2 and downstream transcriptional factor c-Myc. CO also significantly inhibits I/R injury-induced STAT1 and STAT3 activation. In contrast, CO does not inhibit p38 or JNK MAPK pathways during hepatic I/R injury. These results demonstrate that exogenous CO suppresses early pro-inflammatory and stress-response gene expression and efficiently improves hepatic I/R injury by downregulation of the MEK/ERK1/2 signaling pathway with CO. CO production, evaluated by CO-Hb, is associated with improved function in liver-transplanted patients[50]. Furthermore, an increase in HO-1 during transplantation is more protective than high HO-1 expression before transplantation[51].

Buis *et al*[52] have shown that donor HO-1 genetic polymorphism may influence the outcome of liver transplantation. Allele genotype is associated with increased graft survival. Graft survival at 1 year is significantly better for A-allele genotype compared to TT-genotype (84% *vs* 63%). Graft loss, due to primary dysfunction (PDF), occurs more frequently in TT-genotype compared to A-receivers ($P = 0.03$). Recipients of a liver with TT-genotype have significantly higher serum transaminases after transplantation and hepatic HO-1 mRNA levels are significantly lower compared to the A-allele livers. No differences are found for any outcome variable between class S and LL-variant of the (GT) (n) polymorphism. Haplotype analysis has confirmed dominance of the A (-413) T single nucleotide polymorphism over the (GT) (n) polymorphism. In conclusion, HO-1 genotype is associated with outcome after liver transplantation, suggesting that HO-1 mediates graft survival after liver transplantation. Excessive shear stress secondary to portal hypertension is probably involved in the augmented HO-1 expression in small-for-size graft liver[53]. In this model, recombinant Ad-HO-1 administered to donors 48 h before transplantation enhances HO-1 expression in both whole and small-for-size allografts, with a predominant augmentation in the small-for-size allografts, suggesting favorable conditions for the induction of HO-1 expression in small-for-size allografts. In close relation to the expression level of HO-1, Ad-HO-1 significantly prolongs both whole and small-for size allograft survivals, with a remarkable effect in the small-for-size allograft group. The prolongation of allograft survival is blocked by the HO-1 inhibitor (Zinc protoporphyrin Ⅸ). The non-treated small-for-size allografts demonstrate impaired liver function during the early period after reperfusion, which can be improved by over-expression of HO-1, but reversed by the HO-1 inhibitor. The markedly increased expression of HO-1 in small-for-size allografts is associated with lower levels of adhesion molecules and pro-inflammatory cytokines in the early phase after reperfusion[54]. Also, in aged liver, HO-1 overexpression can provide potent protection against cold I/R injury. This effect depends, at least in part, on HO-1-mediated inhibition of the anti-apoptotic mechanism, as an active form of pro-apoptotic caspase-3 (p20) protein, and was found to be 2.9-fold lower at 24 h in the hemin-pretreated group, as compared to saline liver transplant controls[55].

The other product of HO activity, biliverdin, also exerts protective effects against liver I/R injury[56]. Adjunctive biliverdin improves portal venous blood flow from the beginning of reperfusion and increases bile production as compared with the control group. I/R-induced hepatocellular damage, as measured by GOT/GPT release, is diminished by biliverdin. Improved liver function by biliverdin is accompanied by preservation of the histologic structure. Additionally[56], biliverdin adjuvant after orthotopic liver transplantation (OLT) decreases endothelial expression of cellular adhesion molecules (P-selectin and intracellular adhesion molecule 1), and decreases the extent of infiltration by neutrophils and inflammatory macrophages. Biliverdin also inhibits expression of i-NOS and pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in OLTs. Finally, biliverdin therapy promotes an increased expression of anti-apoptotic molecules independently of HO-1 expression, consistent with biliverdin, being an important mediator through which HO-1 prevents cellular death.

Alcohol and non-alcoholic steatosis

Steato-hepatitis is a liver disease characterized by fat accumulation, inflammation, necrosis, and fibrosis. It can be caused by alcohol, or be independent from alcohol and defined as non-alcoholic steato-hepatitis (NASH). In hepatic NASH, Malaguarnera *et al*[57] have shown that HO-1 expression is significantly increased, and the increase reflects the severity of the disease. They observed a significant correlation between the increased levels of HO-1 and ferritin, and between the increased levels of HO-1 and lipid peroxidation. Moreover, NASH patients with lower levels of GSH exhibit higher expression of HO-1. Thus, the induction of HO-1 seems an adaptive response against oxidative damage elicited by lipid peroxidation, and it may be critical in the progression of the disease.

The only data on alcoholic steato-hepatitis are those of Yao *et al*[14] who have shown that the induction of HO-1 by *Ginkgo biloba* is associated with a decrease in liver damage caused by ethanol feeding for 90 d in rats. This is probably due to the enhanced anti-oxidative capacity against the ethanol-induced oxidative stress and the maintenance of cellular redox balance.

Liu *et al*[13] have shown that ethanol dose-dependently induces HO-1 and increases HO activity in human hepatocytes in culture, and that HO-1 mRNA increases after 30 min of exposure. Induction of HO-1 with CoPP prevents damage from ethanol. These results have been confirmed by Yao *et al*[43], who showed that quercetin prevents ethanol toxicity in human hepatocytes, an effect which is mediated by HO-1 induction. HO activity is also increased by chronic ethanol consumption in rats[58]. While 2.5-mo-old rats respond to acute ethanol intoxication by displaying increased expression of liver HO-1 mRNA, and 6-mo-old rats exhibit a mild response, 18-mo-old rats do not show any response, probably because of a decreased transcriptional ability to respond to stress in older animals[59].

Cirrhotic and pre-hepatic portal hypertension

In cirrhosis induced by bile duct ligation, Wei *et al*[60] have shown that HO-1 mRNA and protein expression is increased in hepatocytes and some Kupffer cells in the early phase of the disease, while HO-2 expression is unchanged. HO-1 induction is also related to iNOS induction.

In cirrhotic livers, mainly biliary cirrhosis, both HO-1 and HO-2 were found to be increased by Goh *et al*[61]. HO-1 was localized mainly in Kupffer cells, while HO-2 was localized in the cytoplasm of the

hepatocytes. Similar results were obtained in patients with post-hepatic cirrhosis by Makino *et al*[62]. They have shown that HO-1 is increased in the liver, being mainly distributed in Kupffer cells and hepatocytes. By contrast, in livers in which portal hypertension is idiopathic and due to increased perisinusoidal resistance, there is a decreased expression of HO-1 in Kupffer cells and an absence in hepatocytes. A study in cirrhotic patients undergoing liver transplantation has shown that HO-1 is up-regulated through heme-independent stimuli according to the development of portal hypertension and that induced HO-1 plays a pathophysiological role in portal hypertension through CO production[63].

In cirrhotic patients CO-Hb is increased, as demonstrated by Tran *et al*[64], but does not correlate with disease severity (MELD score, Child Turcotte Pugh score, or other biochemical or clinical measurements). In cirrhotic patients with spontaneous bacterial peritonitis, CO production, evaluated as CO concentration in the exhaled air and blood CO-Hb level, is further increased and may participate in circulatory alterations[65].

A clear role of the HO-CO system in the pathophysiology of hemodynamic alterations related to experimental cirrhotic portal hypertension in the rat is now established.

Decreased HO-2 expression in the liver is associated with increased portal resistance[33], while mesenteric artery dilatation and hypo-reactivity to vasoconstrictors, phenylephrine, KCl, endothelin-1, is associated with induction of HO-1 and increased HO-2. As a confirmation of these findings, transfection of normal rats with human HO-1 mimics mesenteric arterial alterations of portal hypertension[66]. Hyper-expression of HO-1 is particularly relevant in cirrhotic rats with ascites and its function is mediated by large-conductance calcium-activated potassium channels[67]. The alpha subunits of these channels, in particular, are increased in cirrhotic animals and their increase may be mediated by the increased HO-1[68].

In experimental pre-hepatic portal hypertension, obtained by partial portal vein ligation in rats, Fernandez *et al*[69] have shown that HO activity is increased in the liver. HO-1 expression is present in hepatocytes and Kupffer cells of portal hypertensive rats but not of normal animals, while HO-2 is similarly expressed in all liver cell types of normal and portal-vein ligated rats. They have also evaluated the role of CO in hyporeactivity of the mesenteric vascular beds of prehepatic portal hypertension in rat[69]. In this model, inhibition of HO with ZnMP does not modify the hypo-reactivity to KCl that is partially attenuated by NOS inhibition and completely corrected by simultaneous inhibition of HO and NOS. Also the hypo-reactivity to methoxamine is not affected by ZnMP, but it is completely overcome by L-NAME, without any increase in response after combined inhibition of NOS and HO. In cirrhotic patients with hepatopulmonary syndrome (HPS), characterized by decreased arterial pO_2 levels and increased alveolar-arterial oxygen gradient, CO-Hb levels are increased, compared to those without the syndrome, and are correlated with pO_2 ($P < 0.001$) and Aa pO_2 ($P < 0.001$) levels. Thus, CO may contribute to human HPS[70]. These data confirm what was experimentally found: in cirrhosis experimentally induced in the rat by bile duct ligation, NO-mediated up-regulation of HO-1 expression has been shown to participate in HPS[71]. In the same model, HO-1 mRNA transcription and protein expression are significantly increased in cirrhotic hearts compared with sham-operated controls, whereas there is no difference in HO-2 mRNA or protein levels. Total HO activity and cGMP levels are significantly increased in cirrhotic ventricles *vs* controls, and treatment with ZnPP significantly decreases cGMP production and improves the blunted papillary muscle contractility, whereas it has no effect on control muscles. CO perfusion inhibits papillary muscle contractility, an effect completely blocked by methylene blue and partially blocked by ZnPP. Thus, activation of the HO-CO-cGMP pathway is involved in the pathogenesis of cirrhotic cardiomyopathy[72].

Renal HO-1 expression is decreased in cirrhotic rats (bile ligation) in renal tubules and interlobular arterioles, while it is increased in the liver. The decreased HO-1 is related to renal dysfunction[73].

During HPS caused by liver cirrhosis, pulmonary endothelial NOS expression and NO production are increased. Increased NO contributes to the blunted hypoxic pressure response (HPR) during cirrhosis and may induce HO-1 expression and CO production, exacerbating the blunted HPR. We hypothesized that NO regulates the expression of HO-1 during cirrhosis, contributing to HPS. Cirrhosis was induced in rats by common bile duct ligation (CBDL). Rats were studied 2 wk and 5 wk after CBDL or sham surgery. Lung HO-1 expression was elevated 5 wk after CBDL. Liver HO-1 was increased at 2 wk and remained elevated at 5 wk. In catheterized rats, the blunted HPR was partially restored by HO inhibition. Rats treated with the NOS inhibitor *N*(G)-nitro-l-arginine methyl ester for the entire 2 wk or 5-wk duration had normalized HO-1 expression and HPR. These data provide *in vivo* evidence for the NO-mediated up-regulation of HO-1 expression and support the concept that HPS is multifactorial, involving not only NO, but also HO-1 and CO.

In kidneys from CBDL rats, Miyazono *et al*[73] have shown that HO-1 protein expression is increased slightly at 2 wk but is abolished at 5 wk. In addition, histologically, HO-1 expression was suppressed in renal tubules and interlobular arterioles in 5-wk-old CBDL rats. Conversely, HO-1 expression in liver was strongly increased. Consistent with the development of cirrhosis and renal dysfunction, mean arterial pressure (MAP), glomerular filtration rate (GFR), and renal blood flow (RBF) are decreased in CBDL rats, compared with sham-operated controls. In sham rats, treatment with the selective HO inhibitor ZnPP markedly decreases GFR and RBF to values similar to those measured in CBDL rats without decreasing MAP. In conclusion, decreased renal HO-1 expression contributes to deteriorated renal function and hemodynamics during cirrhosis. This finding provides a novel mechanism for the pathophysiology of renal dysfunction during cirrhosis.

Hepatitis

Interactions between hepatitis viruses B and C and HO have been described, both directly and through the effects on the immune response.

Hepatitis B

Protzer *et al*[74] have investigated the effects of HO-1 induction in models of human hepatitis B virus (HBV) infection. Adenoviral transfer of an HBV 1.3 genome into wild-type mice was used as a model for acute hepatitis B. HBV transgenic animals were used as a model for chronic HBV infection. To investigate HO-1 effects on HBV replication at a molecular level, stably HBV-transfected hepatoma cells were used. In the acute hepatitis B model, liver injury was reduced significantly after HO-1 induction. In addition, HO-1 showed a pronounced antiviral effect, which was confirmed in stably HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. HO-1 induction repressed HBV replication directly in hepatocytes at a post-transcriptional step by reducing stability of HBV core protein and thus blocking refill of nuclear HBV covalently closed circular DNA. Small interfering RNA directed against HO-1 proved that this effect was dependent on the expression level of HO-1. The authors[74] concluded that, besides its hepato-protective effect, HO-1 showed a pronounced antiviral activity in HBV infection.

Hepatitis C

Conflicting data are available on HO-1 in hepatitis C. Ghaziani *et al*[75] have shown that human hepatoma cells expressing HCV have increased HO-1 and decreased Bach1 expression. Abdalla *et al*[76], on the contrary, have found a clear decrease in HO-1 and HO-1 mRNA in liver biopsies from HCV-infected patients. The expression of HO-1 was also reduced in cell lines that stably express HCV core protein, which suggests that core gene products are capable of regulating the expression of HO-1. These results are confirmed by Wen *et al*[77] have who shown that HCV core protein attenuates the induction of HO-1 by heme, heavy metals, and peroxides and contributes to hepatocellular damage by increasing both steady-state levels of pro-oxidants and the susceptibility of hepatocytes to damage by impairing their response to other sources of oxidative stress. Concerning the effects of HO-1 induction on hepatitis C, Shan *et al*[78] have shown a decrease in HCV replication, an effect similar to that described by Protzer *et al*[74] in HBV hepatitis.

CRITICAL CONSIDERATIONS AND FUTURE STUDIES

The amount of experimental data that demonstrate important properties of many ingredients and/or bioactive substances from plants and food plants is vast and continues to increase rapidly. The use of terms such as nutraceuticals, functional foods, herbal extracts, bioactive dietary constituents, phytochemicals and similar is becoming copious. In many cases marketing strategies abuse these terms and health properties are claimed although far from being scientifically demonstrated. Thus, researchers are requested to have scientific objectivity in evaluating health properties of food ingredients. It is possible to sustain those diverse bioactive substances from plants and food plants are promising candidates as natural HO-1 hepatic inducers. However, some critical evaluations on literature data are necessary. It is important to note that the majority of studies were conducted in cellular models, whereas only two studies were conducted on rats. Thus, the reproduction of natural HO-1 hepatic inducers in more relevant *in vivo* models is certainly necessary. With regards to the inductive mechanism of natural HO-1 hepatic inducers, although other pathways cannot be excluded, it seems quite clear that the prevalent mechanism is an ARE-mediated HO-1 gene transcription through the Nrf2/ARE signaling pathway.

Other uncertainties derive from the fact that the referred studies have reported data on natural HO-1 inducers considered both as single chemicals and food extracts. In some cases, scarce or no information has been provided about (1) the quantitative measurements of the proposed active compound; (2) methods of analysis and, (3) extraction procedures. Obviously, the above information is essential to enable other researchers to reproduce the experiments and to obtain comparable data.

When considering a possible therapeutic use of future natural HO-1-inducer-based drugs, the amount of work to perform is even more significant. Indeed, exhaustive information on absorption, distribution, metabolism and excretion by the main possible routes (oral, intraperitoneal, intravenous, intrathecal) are largely insufficient. A potential point of strength of natural HO-1 hepatic inducers is that, generally, they have no toxic effects, and it is presumed that they should not have side effects or teratogenic properties.

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REVIEW

Nutritional management of newborn infants: Practical guidelines

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Abstract

The requirements of growth and organ development create a challenge in nutritional management of newborn infants, especially premature newborn and intestinal-failure infants. Since their feeding may increase the risk of necrotizing enterocolitis, some high-risk infants receive a small volume of feeding or parenteral nutrition (PN) without enteral feeding. This review summarizes the current research progress in the nutritional management of newborn infants. Searches of MEDLINE (1998-2007), Cochrane Central Register of Controlled Trials (The Cochrane Library, Issue 3, 2007), abstracts and conference proceedings, references from relevant publications in the English language were performed, showing that breast milk is the preferred source of nutrients for enteral feeding of newborn infants. The number of nutrients found in human milk was recommended as a guideline in establishing the minimum and maximum levels in infant formulas. The fear of necrotizing enterocolitis and feeding intolerance are the major factors limiting the use of the enteral route as the primary means of nourishing premature infants. PN may help to meet many of the nutritional needs of these infants, but has significant detrimental side effects. Trophic feedings (small volume of feeding given at the same rate for at least 5 d) during PN are a strategy to enhance the feeding tolerance and decrease the side effects of PN and the time to achieve full feeding. Human milk is a key component of any strategy for enteral nutrition of all infants. However, the amounts of calcium, phosphorus, zinc and other nutrients are inadequate to meet the needs of the very low birth weight (VLBW) infants during growth. Therefore, safe and effective means to fortify human milk are essential to the care of VLBW infants.

Key words: Breast milk; Infant formula; Trophic feeding; Parenteral nutrition

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INTRODUCTION

The requirements of growth and organ development create a challenge in nutritional management of newborn infants. The stress of critical illness further complicates the delivery of adequate nutrients. Enteral feeding has several advantages over parenteral nutrition (PN), such as preservation of the gastrointestinal mucosa and decreasing the occurrence of sepsis related to bacterial translocation. Although feeding through the gastrointestinal tract is the preferred route for nutritional management, there are specific instances when PN as an adjunctive or sole therapy is necessary to meet nutritional needs. When meticulous attention is paid to the requirements of fluid, calory, protein, and fat along with monitoring the metabolic status of patients, it is possible to provide full nutritional support for critically ill newborn infants.

MACRO-NUTRIENTS OF BREAST MILK AND INFANT FORMULA

Breast milk is the preferred source of nutrients for newborn infants, and the number of nutrients found in human milk is recommended as a guideline in

establishing the minimum and maximum levels in infant formulas[1]. Following macronutrients (e.g. proteins, fatty acids and carbohydrates) for infant formulas based on scientific investigations of breast milk during the last decades were recommended by the American Academy of Pediatrics, Committee on Nutrition (AAP-CON) in 2003, and approved by Food Safety and Applied Nutrition, Food and Drug Administration (FDA)[2].

Proteins and amino acids

A minimum protein content of 1.7 g/100 kcal (i.e. total nitrogen × 6.25) and a maximum total protein content of 3.4 g/100 kcal in infant formulas have been recommended[2,3]. The current maximum protein content of 4.5 g/100 kcal is too high because there is no physiological reason to provide protein at this level. Milk contains two primary sources of protein: caseins and whey[2,3]. It has been reported that human breast milk contains whey/caseins at a ratio of 9/1 to 6/4 in different lactating periods. However, most of the marketing formulas for infants contain whey/caseins at a ratio of 6/4 to 4/6[2,3].

Whey, a protein complex derived from milk, is touted as a functional food with a number of health benefits. The biological components of whey, including lactoferrin, β-lactoglobulin, α-lactalbumin, glycomacropeptide, and immunoglobulins, demonstrate a variety of immune-enhancing properties[2,3]. In addition, whey, an antioxidant, can act as an antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent. The primary mechanism by which whey exerts its effects is by intracellular conversion of amino acid cysteine to glutathione, a potent intracellular antioxidant. A number of clinical trials have successfully been performed using whey in the treatment of cancer, human immunodeficiency virus (HIV) infection, hepatitis B, cardiovascular disease, osteoporosis, and as an antimicrobial agent[3].

Lactoferrin is an important protein in human milk (range 0.02-0.2 g/dL) at different lactating stages[2,3]. Although it is technically feasible to add bovine lactoferrin or transgenic human transferrin to infant formulas, bovine lactoferrin does not bind consistently to human lactoferrin receptors and whether it increases iron absorption remains unknown. The efficacy and safety of adding human lactoferrin to infant formulas have not been adequately evaluated. Given the emerging knowledge about the biological importance of human lactoferrin in infant nutrition, lactoferrin supplementation is worthy of consideration. However, clinical studies will be essential to demonstrate the efficacy and safety of such addition.

A minimum carnitine content of 1.2 mg/100 kcal (a level similar to that in human milk) and a maximum content of 2.0 mg/100 kcal in infant formulas (a value similar to the upper limit in human milk), have been recommended[2,3]. Although evidence that dietary carnitine is essential for infants, biochemical changes are noted when infants are fed with a carnitine-free diet. A few anecdotal reports are available on the abnormal clinical manifestations associated with diets low in carnitine[2]. Infants nourished with soy-protein-based formulas with a low carnitine content have lower plasma and urine carnitine levels and altered lipid metabolism, but no significant difference in rates of growth compared with those not nourished with soy-protein-based formulas with a low carnitine content.

Glutamine and taurine are free amino acids commonly detected in human breast milk[2-4]. The addition of glutamine to infant formulas is not recommended because no convincing evidence is available on glutamine requirement in diet. Also, no compelling evidence mandates the addition of taurine to formulas for infants. However, taurine has been used in some commercially available formulas. Currently, a minimum taurine content of zero and a maximum taurine content of 12 mg/100 kcal in infant formulas (a value similar to the limit in human milk) are recommended.

There are few compelling reasons for the addition of nucleotides to infant formulas[2,3,5]. The beneficial effects of nucleotide supplementation to infant formulas are intriguing, and further research in this area is strongly urged. When data from long-term, large-scale clinical trials are available, the question of adding nucleotides to infant formulas should be reconsidered. A maximum content with 16 mg/100 kcal of nucleotides and their precursors in infant formulas, a value similar to the upper limit in human milk, is recommended.

Fat and fatty acids

A minimum fat content of 4.4 g/100 kcal (40% of total energy) and a maximum fat content of 6.4 g/100 kcal (57.2% of total energy) in infant formulas have been recommended[2,3,5,6]. With the proposed minimum protein of 1.7 g/100 kcal (6.8 kcal/100 kcal) and minimum carbohydrate of 9 g/100 kcal (36 kcal/100 kcal), a maximum value for fat may not, therefore, exceed 57.2 kcal/100 kcal, which is equivalent to 6.4 g/100 kcal.

Medium-chain triglyceride (MCT) is not recommended to be supplemented in infant formulas, with the exception of certain exempt formulas for infants with impaired fat digestion or absorption.

Linoleic acid (LA) is recommended with a minimum content of 8% of total fatty acids in infant formulas. With a minimum fat content of 4.4 g/100 kcal, the minimum LA content is, therefore, 350 mg/100 kcal. Concentrations of LA in human milk vary widely as a reflection of maternal dietary intake, but values less than 8% of fatty acids are rarely reported. Currently, marketed infant formulas provide more than 8% of fatty acids as LA. A maximum LA content of 35% of total fatty acids in infant formulas is recommended. With a maximum fat content of 6.4 g/100 kcal, the maximum LA content is, therefore, 2 240 mg/100 kcal. The polyunsaturated vegetable oils (corn, safflower, and soybean oils) used in manufacture of infant formulas contain an abundant amount of LA (usually 45%-70% of total fatty acids). Historically, infant formulas, particularly corn-oil-based

formulas, contained LA exceeding 35% of fatty acids, with no adverse effects. Moreover, this value (35% of fatty acids) is within the limit reported for individual human milk samples[2,3].

α-Linolenic acid (ALA) is recommended with a minimum content of 1.75% of fatty acids in infant formulas, with the further stipulation that the ratio of LA:ALA should not exceed 16 to 1. With the minimum total fat content of 4.4 g/100 kcal, the minimum content of ALA is 77 mg/100 kcal, approximately 0.7% of energy. This recommendation is based on the essentiality of ALA as a precursor of the n-3 series of long-chain polyunsaturated fatty acids (LCPUFAs). Studies showed that formulas providing ALA at levels below this may be associated with delayed development of visual function and lower levels of docosahexaenoic acid (DHA) in the brain[6]. The recommended upper limit for the ratio of LA:ALA (16:1) is intended to prevent an inappropriate combination of high LA content with low ALA content, which might interfere with the formation of longer-chain fatty acids of the n-3 series. A maximum ALA content of 4% total fatty acids in formulas can be additionally stipulated that the ratio of LA:ALA is not less than 6 to 1. With a maximum fat content of 6.4 g/100 kcal, 4% of fatty acids from ALA amounts to 256 mg/100 kcal. The maximum is based on the long history of use of formulas containing soy oil (soybean oils typically contain 6%-9% ALA) as the source of unsaturated fatty acids. The recommended minimum ratio of 6 to 1 is intended to ensure that combination of the minimum LA content with the maximum ALA level does not interfere with the production of longer-chain fatty acids of the n-6 series[2,3].

LCPUFAs, including arachidonic acid (AA) and DHA, are not recommended in infant formulas. Breast milk contains adequate AA and DHA (range 5-20 mg/dL). However, whether AA + DHA should be added to infant-formula milk is uncertain. The results of studies on the growth and neurodevelopment in infants fed with milk formula supplemented with AA + DHA are inconsistent[6,7], suggesting that LCPUFAs are not essential in the diet of infants. Because of the uncertain efficacy and safety, LCPUFAs should not be added to infant formulas. The FDA expert panel plans to revisit this field in 5 years when more data from larger studies are available[2,3,6,7].

Carbohydrate and oligosaccharides

An energy density of 63-71 kcal/dL in infant formulas is recommended. Carbohydrate is the most important nutrient for energy. A minimum total carbohydrate content of 9 g/100 kcal in infant formula is recommended. This minimum is based on a theoretical calculation taking into account the amount of glucose needed for obligatory central nervous system oxidation. A maximum of 13 g/100 kcal is recommended for total carbohydrate in infant formulas. This value is obtained by subtracting from 100% of the total energy (63 to 71 kcal/dL), the minimum energy provided by protein (1.7 g protein/100 kcal = 6.8 kcal) and the minimum energy from fat (4.4 g fat/100 kcal = 39.6 kcal), resulting in a maximum of 53.6 kcal from carbohydrate, which is equivalent to 13.4 g/100 kcal[2,3].

The addition of glucose to infant formulas is not recommended, because inclusion of glucose in infant formulas offers no biological advantage over other carbohydrate sources and would unnecessarily increase the osmolality of formulas. Lactose is safe and appropriate for use in formulas by most healthy infants, and may be used as a sole carbohydrate source. However, it should not be used at a level higher than the recommended maximum value for total carbohydrate (i.e. 13 g/100 kcal). Also, addition of sucrose to infant formulas is safe and may be used for the palatability of some formulas (e.g. protein-hydrolysate-based formulas).

The concentration and composition of oligosaccharides in breast milk are increased in a dynamic process. The highest amount of oligosaccharides, 2 g/dL milk, is reached on the fourth day of life. On days 30 and 120 of lactation, it decreases to 20% and 40%, respectively, in comparison to that on day 4. Most studies have reported that oligosaccharide in human milk consists of approximately 70%-90% galacto-oligosaccharides (GOSs) and 10%-30% fructo-oligosaccharides (FOSs) in the first few months. The available data are insufficient at present to establish a minimum or a maximum level of these substances in infant formulas. However, some infant formulas supplemented with GOS 0.2-0.4 g/dL and FOS 0.05-0.1 g/dL are available on the market. Although glucose polymers are safe and appropriate for use in formulas by most healthy infants, either a minimum or a maximum level of such substances is not recommended. The amount of carbohydrate from glucose polymers in a formula should be within the lower and upper limits of total carbohydrate. Inclusion of modified food starches in infant formulas involves toxicologic concerns rather than nutritional concerns. Therefore, such food starches in infant formulas are not recommended[2,3,8].

TROPHIC AND ENTERAL FEEDING

The provision of adequate enteral nutrition for preterm infants is one of the major clinical challenges facing neonatologists throughout the world. Many preterm infants are too ill to receive substantial enteral feeds and require prolonged PN. It was reported that normal gastrointestinal structure and function are lost, villi become shorter, mucosal DNA is lost, protein content and enzymatic activity are reduced both in animal models and in children, although an anabolic state is maintained by PN[9]. In a rat model, atrophy occurred after only 3 d of no enteric intake, while gastrointestinal atrophy and dysfunction were reversed following enteral feeding[9].

Trophic feeding (synonyms include minimal enteral feeding or nutrition, gastrointestinal priming, gut priming, and early hypocaloric feeding) is a relatively recent concept that has been introduced into clinical practice in an attempt to counter the effects of enteral starvation[9,10]. It may be defined as the practice of feeding nutritionally insignificant volumes of enteral

Table 1 Evidence-based enteral nutrition in preterm newborns

	Evidence-based enteral nutrition
Human milk	Human milk from the preterm infant's own mother is the first choice. Human milk can be stored at room temperature for up to 24 h after expression in colostrum and up to 6 h for mature milk. Beyond that, it should be stored at 3-4℃ before use. If not used for more than 5 d, it should be frozen
Human milk fortifier	Human milk fortifier is indicated in preterm infants < 31 wk and/or < 1500 g. Human milk (100 mL/kg) is given per day and discontinued when the infant has established full breast-feeding
Formula milk	If human milk from the preterm infant's own mother is not available, the only acceptable alternative is a preterm formula. A concentration of about 60 kcal/100 mL or 20 kcal/oz is recommended, but should be increased to 80 kcal/100 mL or 24 kcal/oz when the infant has achieved full enteral feeds
Feeding methods	Gavage feeding is given via an indwelling nasogastric tube during mechanical ventilation. An indwelling orogastric tube is used after endotracheal extubation. Intermittent intragastric feeding is the first choice method, but continuous transpyloric feeding can be tried in selected preterm infants with extremely poor gastric emptying and symptomatic gastro-esophageal reflux
Commencement of feeds	Hourly feeds of 1 mL are generally used in infants weighing less than 1000 g, 2-h 2 mL for infants weighing 1000-1500 g, 3-h 3 mL for infants weighing 1500-2000 g, and 4-h 4 mL for infants weighing more than 2000 g, unless there is significant respiratory distress, when the infant remains on 1-2-h feeds. If this might not be tolerated, milk may be commenced at 1 mL every 2 h, even less than 1 mL every 4-6 h. Such trophic feeding should begin as soon as possible after birth, and definitely within the first 3-4 d
Progression of feeds	Daily increment in the range of 10-30 mL/kg of milk feeds is safe. Demand feeding is started after infants have established full milk feeds on a 4 h regimen. Non-nutritive sucking is beneficial without side effects
Supplements	Multivitamin supplement is started when the infant has established full enteral feeds, and iron is started when the infant has doubled their birth weight (usually at 2 mo). Medium-chain triglycerides can be used as an energy supplement for preterm infants who fail to thrive

substrate to sick neonates, to supply nutrients to, and directly stimulate, the developing gastrointestinal system without increasing disease severity. Typically, a milk volume of 10-20 mL/kg per day is given at the same rate for at least 5 d. Several studies have examined the clinical outcome after trophic feeding[9-11], showing that milk tolerance, liver function, metabolic bone disease, days to hospital discharge, and weight gain are improved after trophic feeding. Nosocomial infections due to PN (because of its interference with the immune system and translocation of enteric pathogenic microorganisms into the circulation) may be reduced either because of improved gastrointestinal mucosal barrier function or because of beneficial alteration of the enteric flora.

Since premature infants are unable to coordinate sucking, swallowing, and breathing, orogastric tube-feeding is necessary. The most common methods used are continuous milk infusion and intermittent (bolus) milk delivery (usually every 3 h). Recent studies have suggested that bolus feeding promotes more "normal" feed-fasting hormonal concentrations that potentially benefit intestinal development and nutrient partitioning, and marked differences are observed in feeding tolerance and growth between continuous *vs* bolus tube-feeding methods[9-13]. Since continuous feeding is associated with more significant feeding intolerance, more infants are switched to bolus feeding. Importantly, throughout hospitalization, the continuous feeding method is associated with slower growth compared with the bolus group. Thus, bolus feeding is more advantageous than continuous infusion for premature infants with relatively healthy gastrointestinal tracts.

Current data support the practice of starting GI priming early, which does not add complications of neonatal intensive care[9,10,12]. Further studies are needed to determine if early feeding can be advanced in volume so that the use of PN can be reduced. Bolus feeding results in better feeding tolerance and growth than continuous tube-feeding and also obviates the need for costly infusion pumps and support care. The use of human milk, however, may have the most profound effects because of its association with a decrease in morbidity. The evidence-based guidelines for enteral nutrition in preterm infants are listed in Table 1[14].

PN

PN can meet neonates' requirement for growth and development when their size or condition precludes enteral feeding. Although feeding through the gastrointestinal tract is the preferred route for nutritional management, there are specific conditions for which PN as an adjunctive or a sole therapy is necessary. In very low birth weight (VLBW) premature infants, enteral feeding cannot be established in the first few days of life, due to the immaturity of the gastrointestinal system. PN can successfully meet the nutritional demands in critically ill neonates, neonates with protracted diarrhea and neonates undergone a major gastrointestinal surgery. The evidence-based guidelines for PN in preterm infants are summarized in Table 2[14].

Fluids and energy requirements

PN is a fundamental part of neonatal intensive care[15,16]. Fluid intake volume varies from 60 to 150 mL/kg per day, depending on maturity of the infant and environmental conditions influencing insensible water loss from the skin. An energy intake of 50 kcal/kg per day is adequate to match ongoing expenditure but an additional energy intake of 70 kcal/kg per day is required to achieve optimal growth. The ideal distribution of calories should be 60% carbohydrate, 10%-15%

Table 2 Evidence-based PN in preterm newborns

	Evidence-based PN
Fluids	D 1: 60-80 mL/kg per day. Infants < 28 wk gestation are nursed in a maximally humidified environment (90% humidity) for at least 7 d. Postnatal weight loss of 5% per day to a maximum of 15% is acceptable, which is achieved by progressively increasing the fluid intake to 120-150 mL/kg per day at 1 wk of age
Energy	An intake of 50 kcal/kg per day is sufficient to match ongoing expenditure, but it does not meet additional requirements of growth. The goal energy intake is 120 kcal/kg per day, which is higher in infants with chronic lung diseases
Protein	Optimal parenteral amino acid intake is 3.5 g/kg per day. Parenteral amino acids can begin from day 1 at a dose of 1.75 g/kg per day
Carbohydrate	From day 1, 6-10 g/kg per day can be infused and adjusted to maintain blood glucose level of 2.6-10 mmol/L. Insulin is only used in infants whose blood glucose level is higher than 15 mmol/L and associated with glycosuria and osmotic diuresis, even after glucose intake has been decreased to 6 g/kg per day. Carbohydrate is given as a continuous infusion commencing at a rate of 0.05 U/kg per hour, and increased as required for persistent hyperglycemia
Fat	Intravenous fat, 1 g/kg per day, can be started from day 1, or when intravenous amino acids are started. The dose of intravenous fat is increased to 2 g/kg and 3 g/kg per day over the next 2 d. Twenty percent intravenous fat is delivered as a continuous infusion via a syringe pump, separated from the infusate containing amino acids and glucose. The syringe and infusion line should be shielded from ambient light
Minerals	Minerals should include sodium (3-5 mmol/kg per day), chloride (3-5 mmol/kg per day), potassium (1-2 mmol/kg per day), calcium (1.5-2.2 mmol/kg per day), phosphorus (1.5-2.2 mmol/kg per day), and magnesium (0.3-0.4 mmol/kg per day)
Trace elements	Trace elements should include zinc (6-8 μmol/kg per day), copper (0.3-0.6 μmol/kg per day), selenium (13-25 nmol/kg per day), manganese (18-180 nmol/kg per day), iodine (8 nmol/kg per day), chromium (4-8 nmol/kg per day), and molybdenum (2-10 nmol/kg per day)
Vitamins	Vitamins must be added to the fat emulsion to minimize loss of vitamins due to adherence to tubes and photodegradation

protein, and 30% fat. A 10% dextrose solution provides 0.34 kcal/mL, a 10% lipid solution provides 0.9 kcal/mL. Although protein is a potential energy substrate, it should be utilized only for tissue growth. Glucose and lipids can provide sufficient calories to avoid protein catabolism. A preterm neonate needs 100-150 kcal/kg per day, whereas a term neonate needs 100-120 kcal/kg per day.

Carbohydrate requirements

Glucose is the most widely used intravenous carbohydrate for neonates because it is readily available to the brain. A preterm infant has a higher glucose demand and hence early administration of glucose is vital. It is important to balance non-protein calories between carbohydrates and fats, and a 2:1 ratio is recommended. Excess use of glucose would result in lipogenesis, excess production of CO_2 and hyperglycemia leading to osmotic diuresis. Hyperglycaemia during PN can be minimized by starting glucose infusion at a rate of 4-6 mg/kg permin (6-8 g/kg per day) with progressive increase to 12-15 mg/kg per min (16-20 g/kg per day) for 2-3 wk after birth[15,16].

Treatment of hyperglycemia is initiated with insulin if blood glucose is > 200 mg/dL, although the dextrose infusion is below 5 mg/kg per min. Insulin can be started at a dose of 0.05-0.1 U/kg per hour. Insulin infusion rate should be adjusted to 0.05 U/kg per hour to keep the glucose level at 150-200 mg/dL. When the glucose level decreases to < 100 mg/dL, the glucose is monitored every 4 h once the target level is achieved[15,16]. Other causes of hyperglycemia like sepsis, intraventricular hemorrhage, and steroids should be ruled out before insulin is used.

Protein requirements

The goal of giving proteins is to limit catabolism, maintain endogenous protein stores, and provide sufficient energy and protein to support growth. It has been reported that early administration of PN is safe and efficacious with no metabolic derangements[17]. The concept put forth by the American Academy of Pediatrics that nutrition should support postnatal growth that approximates the in utero growth of a normal fetus should be accepted. Parenteral nitrogen requirement is 30-35 mmol/kg per day, which is equivalent to 3.0-3.5 mg/kg amino acids per day. These solutions contain nine essential amino acids and cysteine, tyrosine, taurine and arginine as the semi-essential amino acids. In the absence of an exogenous protein source, a preterm infant catabolizes 1 g/kg of its own body protein per day to meet its metabolic needs. Excess protein administration causes a rise in blood urea, ammonia and high levels of potentially toxic amino acids such as phenylalanine[18]. In our unit, we usually start amino acids (1 g/kg per day) on the second day of life for extremely low birth weight (ELBW) infants and increase to 3 g/kg per day with 1 g/kg daily increments per day. Protein with a maximum of 15% calories should be given.

Glutamine, one of the most abundant amino acids in both plasma and breast milk, is not included in amino acid preparations for PN. Glutamine, which is unstable in solution, is usually regarded as a non-essential amino acid. However, glutamine provides an important metabolic fuel for rapidly dividing cells of the gastrointestinal tract and immune system, and is an intermediate in a large number of metabolic pathways, and a precursor that donates nitrogen for the synthesis of purines, pyramidines, nucleotides and amino sugars. In addition, glutamine plays a key part in acid-base balance by acting as the most important substrate for renal ammonia production. It was reported that glutamine supplementation may decrease sepsis and mortality in critically ill adult patients[19]. In view of the

important metabolic roles of glutamine, further clinical evaluation is required in neonates.

Lipid requirements

Lipid is a major source of non-protein energy and has a nitrogen sparing effect. Serving as a source of essential fatty acids and LCPUFA, lipid is a major source of non-protein energy and has a nitrogen-sparing effect. The commercial intravenous lipid emulsions are aqueous suspensions containing neutral triglycerides derived from soybean, safflower oil and egg yolk to emulsify and adjust glycerin tonicity. Hydrolysis of triglycerides by hepatic and lipoprotein lipase results in formation of free fatty acids. Circulating free fatty acids can be used as an energy source or they enter adipose tissue where they are re-esterified to form triglycerides[15,16].

Parenteral fat is introduced at 1 g/kg per day, and gradually increased to 3 g/kg per day, given as a continuous infusion. In our unit, we usually start lipids on the third day of life in ELBW infants when the most acute phase of respiratory distress or other life-threatening events are controlled. We start 1 g/kg of lipids per day and increase it to 3 g/kg per day. At present, a 20% lipid emulsion is preferred over 10% emulsion, because the higher phospholipid content in 10% solution impedes plasma triglyceride clearance, resulting in higher concentrations of triglyceride and plasma cholesterol. Also, combined MCT/long-chain triglyceride (LCT) and lipid emulsion is preferred over LCT emulsion in preterm and critical neonates, because MCT/LCT is more easily metabolized. Advantages of lipid emulsions over concentrated glucose solutions include their isotonicity and greater energy density, the latter means that a low volume is required per calorie[15,16].

Minerals, trace elements and vitamins

Minerals and trace elements delivered with PN are calculated to meet in-utero accretion rates. Sodium, potassium, chloride, calcium, magnesium and phosphorus levels need to be closely monitored and the infusion needs to be prescribed accordingly. Neonates on long-term TPN may develop trace element deficiencies which should be checked regularly. TPN can provide the daily requirements for water and fat-soluble vitamins. The dose of water-soluble vitamins is 1 mL/kg per day, which should be added to the dextrose-electrolyte solution. The dose of fat-soluble vitamins is 1 mL/kg per day, which should be added to the lipid emulsions[14-16].

PN-associated cholestasis

In neonatal intensive care units where appropriate medical, nursing, pharmacy and laboratory experts are available, the potential benefits of PN outweigh its hazards. However, PN-associated cholestasis, onset of hyperbilirubinemia with direct bilirubin > 2 mg/dL within 2 wk after starting PN, are the common complications of PN, along with hepatomegaly, and mild elevation of conjugated bilirubin, alkaline phosphatase and transaminases[20,21]. Liver function generally becomes normal within 1-4 mo after stopping PN, but prolonged liver dysfunction and even fibrosis have been reported in certain cases[20,21]. The following factors may contribute to PN-associated liver diseases, including prolonged duration of PN therapy, sepsis, low serum albumin, excessive caloric load, enteral fasting, deficiency in nutritional taurine, carnitine, manganese, oxidative stress and hormonal factors such as elevated insulin/glucagon ratio, gut hormones and biliary stasis. Simple interventions such as minimizing the duration of therapy, early detection and treatment of sepsis, and choosing enteral nutrition rather than PN whenever possible, can minimize liver injury[20,21]. Ursodeoxycholic acid (UDCA) is used in the treatment of cholestasis[20], because it increases the hydrophilic non-hepatotoxic bile acid pool, decreases hepatocyte display of histocompatibility antigens and gives direct cytoprotection. The dose of UDCA is 20-30 mg/kg per day.

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TOPIC HIGHLIGHT

Toru Ishikawa, MD, *Series Editor*

Secondary prevention of recurrence by interferon therapy after ablation therapy for hepatocellular carcinoma in chronic hepatitis C patients

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Abstract

Chronic hepatitis C is a leading cause of hepatocellular carcinoma (HCC) worldwide. Interferon (IFN) therapy decreases the incidence of HCC in patients with chronic hepatitis C. Prevention of chronic-hepatitis-C-related HCC is one of the most important issues in current hepatology. We have previously reported that male gender and high titer of hepatitis C virus (HCV) RNA are predictive factors for the development of HCC in HCV-related cirrhosis. Clinical efforts at eradicating or reducing the viral load may reduce the risk for HCC. Furthermore, because HCC often recurs after ablation therapy, we performed a trial of IFN in patients with chronic liver disease caused by HCV to see whether IFN therapy decreases recurrence after ablation therapy of HCV-related HCC. By using IFN therapy as a secondary prevention, patients with HCC who had received complete tumor ablation showed better survival, primarily as a result of the preservation of liver function and also probably prevention of recurrence. Postoperative IFN therapy appears to decrease recurrence after ablation therapy such as radiofrequency ablation (RFA) therapy of HCV-related HCC. We believe that there is a survival benefit in secondary prevention using IFN therapy. However, a controlled study is essential to obtain conclusive evidence of the efficacy of this strategy.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Interferon; Secondary prevention

INTRODUCTION

In Japan, the overwhelming majority of hepatocellular carcinoma (HCC) is caused by chronic hepatitis and liver cirrhosis due to persistent hepatitis C or B, mostly hepatitis C virus (HCV) infection. The onset of HCC can be roughly divided into intrahepatic metastasis and multicentric carcinogenesis. The latter can be further divided into synchronous multicentric carcinogenesis, in which HCC occurs in multiple locations simultaneously, and asynchronous multicentric carcinogenesis, in which HCC occurs some time after localized therapy such as partial hepatectomy, percutaneous ethanol injection therapy (PEIT), or percutaneous radiofrequency ablation (RFA). With asynchronous multicentric carcinogenesis, the prognosis of patients can be improved by preventing carcinogenesis in the remaining liver. HCC is treated by analyzing multiple factors, including: (1) decreased hepatic function due to chronic hepatitis and cirrhosis; (2) multicentric carcinogenesis (synchronous/asynchronous) due to persistent infection; and (3) early intrahepatic metastasis due to portal invasion, which is one of the characteristics of HCC. In other words, unlike other cancers, it is necessary to assess not only cancer progression, but also the hepatic reserve. The main objective of interferon (IFN) therapy for chronic hepatitis C infection is to end persistent infection and prevent the progression of liver disease. The present article discusses the significance of IFN therapy as secondary prevention after localized therapy for HCC, particularly IFN therapy combining pegylated IFN (PEG-IFN) and ribavirin.

Table 1 Univariate and multivariate analyses on the carcinogenic factors for HCC in patients with HCV cirrhosis

Variables	Number of patients	Univariate analysis			Multivariate analysis		
		P-value[1]	RR[2]	95% CI	*P*-value[3]	RR[2]	95% CI
Sex							
Male	85	$P = 0.001$	1.971	1.298-2.991	$P = 0.005$	2.107	1.198-2.987
Female	80		1			1	
Alcohol							
Yes	49	$P = 0.012$	1.681	1.108-2.550	NS ($P = 0.496$)	1.234	0.673-2.262
No	116		1			1	
ALT							
⩾ 100	77	$P = 0.013$	1.657	1.103-2.489	NS ($P = 0.876$)	1.050	0.572-1.927
< 100	88		1			1	
LDH							
⩾ 480	74	NS ($P = 0.064$)	-	-	NS ($P = 0.112$)	0.673	0.413-1.096
< 480	91					1	
HCV-RNA							
⩾ 1.0 Meq/mL	110	$P = 0.018$	1.709	1.086-2.695	$P = 0.028$	1.658	1.125-2.315
< 1.0 Meq/mL	55		1			1	
Ant i-HBc							
Positive	78	NS ($P = 0.834$)	-	-	NS ($P = 0.577$)	1.136	0.724-1.782
Negative	87					1	

[1]*P*-values were obtained by using the log-rank test; [2]RR were calculated by comparing classes with Cox regression analysis; [3]*P*-values were obtained by using Cox regression analysis. RR: Relative risks; CI: Confidence interval; NS: Not significant.

ABLATION THERAPY FOR HCC

Unlike other cancers, the treatment of HCC involves not only the stage of the carcinoma itself, but also the stage of underlying chronic hepatitis. In patients with advanced HCC or extrahepatic metastasis, chemotherapy is mostly performed and its usefulness has been shown[1-5]. However, in patients with stage I or Ⅱ HCC, including early-stage HCC, percutaneous therapy is useful because its impact on normal hepatocytes is relatively small. Percutaneous therapy began with PEIT[6] and advanced to percutaneous microwave coagulation therapy (PMCT)[7], and today, RFA that combines the advantages associated with the previous two techniques is often performed[8-10]. In Japan, RFA was first performed in 1999, and it is still premature to discuss its long-term results, but regarding overseas results, Rossi *et al*[8-10] have reported that the survival rate for RFA was 94% at 1 year, 68% at 3 years, and 40% at 5 years, and that the rate of local recurrence was 5% with an average follow-up of 22.6 mo. At present, three RFA needles are available: Radionics Cooltip (single needle), RTC LeVeen probe (expandable needle), and RITA Model 90/70 (expandable needle). In our department, different RFA needles are used depending on tumor site and size, and according to our data, the extent of thermocoagulation per single ablation for RITA Model 90 is 43.2 mL, which is significantly greater when compared to the others. The rate of local recurrence within a range of 20 mm or 30 mm is significantly lower for RITA Model 90 (data not shown). Ablation therapy appears useful for the local control of HCC, but even if local control is sufficient, it is necessary to take into account background liver factors when suppressing recurrence. In other words, as in chronic hepatitis B [11], it is important to treat chronic hepatitis C using IFN.

CARCINOGENIC FACTORS IN HCV-RELATED CHRONIC HEPATITIS

While the onset mechanism of HCV HCC has not been elucidated, it has been suggested that persistent HCV-induced inflammation causes abnormally high levels of transaminase and results in excessive cellular turnover consisting of hepatocyte necrosis and regeneration, thus increasing the risk for genetic abnormalities leading to carcinogenesis. We examined carcinogenic factors in patients with HCV cirrhosis and advanced liver fibrosis; long-term follow-up examinations revealed that high viral titer, sex (male), and age (elderly) were significant onset factors (Tables 1 and 2)[12]. Hence, it is necessary to prevent HCC in patients with these risk factors.

PRIMARY PREVENTION OF HCV-RELATED CHRONIC HEPATITIS BY IFN THERAPY

Many studies have documented that IFN significantly suppresses the onset of HCC from chronic hepatitis and liver cirrhosis. Studies have found that IFN therapy for HCV infection is useful in suppressing carcinogenesis and improving liver function[13,14] and that IFN therapy eliminates HCV RNA and clearly suppresses the onset of HCC in patients with normalized transaminase levels[15]. Additionally, even if a complete response is not achieved, IFN therapy suppresses HCC when compared to untreated cases[16].

Furthermore, even in the presence of advanced chronic hepatitis, cirrhosis improves in about half of patients with a sustained response to IFN therapy[17], and IFN therapy lowers transaminase, maintains platelet counts, and reduces carcinogenesis[13]. This suggests

Table 2 Univariate and multivariate analyses of the carcinogenic factors for HCC in male patients with HCV cirrhosis

Variables	Number of patients	Univariate analysis			Multivariate analysis		
		P-value[1]	RR[2]	95% CI	*P*-value[3]	RR[2]	95% CI
Age (yr)							
⩾ 60	43	*P* = 0.032	1.726	1.032-2.881	*P* = 0.035	4.469	1.271-5.723
< 60	42		1			1	
Alcohol							
Yes	45	*P* = 0.826	1.058	0.632-1.771	NS (*P* = 0.676)	0.877	0.473-1.025
No	40		1			1	
Smoking							
Yes	36	*P* = 0.566	0.863	0.517-1.440	NS (*P* = 0.696)	0.893	0.504-1.580
No	49		1			1	
AST							
⩾ 100	47	*P* = 0.213	1.376	0.824-2.298	NS (*P* = 0.151)	1.863	0.797-4.350
< 100	38		1			1	
ALT							
⩾ 100	46	*P* = 0.805	1.064	0.643-1.763	NS (*P* = 0.485)	0.752	0.337-1.667
< 100	39		1			1	
γ-GTP							
⩾ 80	41	*P* = 0.509	1.182	0.714-1.954	NS (*P* = 0.561)	1.178	0.679-2.041
< 80	44		1			1	
Anti-HBc							
Positive	43	*P* = 0.111	1.522	0.898-2.577	NS (*P* = 0.099)	1.609	0.914-2.835
Negative	42		1			1	

[1]*P*-values were obtained by using the log-rank test; [2]RR were calculated by comparing classes with Cox regression analysis; [3]*P*-values were obtained by using Cox regression analysis. RR: Relative risks; CI: Confidence interval; NS: Not significant.

Table 3 Studies in which IFN was administered after treatments for HCV-related HCC in Japan

Authors	Treated *vs* untreated	Treatment	Follow-up (mo)	Recurrence (%)	Survival (%)
Ikeda[21]	20 *vs* 10	IFN-β	25	10 *vs* 70 ($P = 0.0004$)	
Kubo[22]	15 *vs* 15	IFN-α	36	33 *vs* 80 ($P = 0.037$)	
Suou[23]	18 *vs* 28	IFN-α	60	28 *vs* 82 ($P < 0.01$)	0 *vs* 27 ($P < 0.05$)
Shiratori[24]	49 *vs* 25	IFN-α	84	80 *vs* 92[1]	53 *vs* 23

[1]IFN therapy did not markedly lower the rate of recurrence the first time, it significantly lowered the rate of recurrence the second and third times.

that IFN suppresses persistent hepatitis in liver cirrhosis and carcinogenesis. Regarding the onset of HCC, it is not clear if it is important to maintain low transaminase levels or suppress liver fibrosis, but it is highly likely that blocking fibrosis is important in suppressing carcinogenesis. Therefore, IFN therapy appears to prevent liver fibrosis in liver cirrhosis.

Ever since the national health insurance system began covering IFN therapy in 1992, antiviral therapy for hepatitis C has steadily advanced and at present, therapy combining PEG-IFN and ribavirin is considered the most potent. The combination therapy was markedly effective in about 90% of patients with genotype-2 HCV when administered for 24 wk[18], and it was markedly effective in about 50% of patients with intractable hepatitis (genotype-1 HCV or high viral load) when administered for 48 wk[19]. In Japan, PEG-IFN and ribavirin combination therapy has improved the therapeutic results for intractable chronic hepatitis C.

IFN THERAPY AS SECONDARY PREVENTION FOR RECURRENT HCC

IFN therapy has been performed to prevent recurrent HCC (Table 3). One study retrospectively investigated recurrence after curative resection of HCV HCC, and found that alanine aminotransferase levels remained high[20]. In other words, hepatocyte necrosis and inflammation appear to be closely involved with recurrence. If IFN is successful in lowering HCV to an undetectable level, necrotic inflammation is naturally improved. At the same time, carcinogenesis is believed to be suppressed even in biochemical responders. Ikeda *et al*[21] have investigated the suppression of recurrent HCC by IFN-β following surgical resection or PEIT for HCC in patients with HCV cirrhosis. They have reported that intermittent IFN-β administration following surgical resection or PEIT for HCV HCC suppresses recurrence.

Kubo *et al*[22] have conducted a randomized controlled trial of postoperative IFN therapy in patients with HCV HCC and have reported that the rate of recurrence is significantly lower for patients with IFN therapy.

Suou *et al*[23] administered 6 MU of IFN-α for 24 wk and reported that the 3-year survival rate for patients without IFN-α was 18% and that of patients with IFN-α was 63%. Additionally, Shiratori *et al*[24] have reported that while IFN therapy does not markedly lower the rate of recurrence the first time, it significantly lowers the rate for the second and third times. Hence, IFN may initially act on tumors to suppress intrahepatic micrometastases following therapy for HCC, and then it may act on the virus to suppress recurrence 3-5 years later. Furthermore, it is reported to the contrary that although the cumulative recurrence rate in the IFN group was found to be lower than in the control group during the first 3 years after commencement of IFN administration, the recurrence rate in the IFN group increased with the lapse of time over 3 years. However, long-term, low-dose, intermittent IFN therapy successfully delayed clinical recurrence of HCC after radical RFA therapy[25]. In these studies, IFN therapy consisted of non-PEG-IFN monotherapy and the rate of sustained viral response was low, at 13%-33%. Therefore, if PEG-IFN and ribavirin combination therapy further improves antiviral effects[26], then recurrence may be suppressed even more. However, many patients with HCV HCC are elderly or have cirrhosis, and the dose and duration of PEG-IFN and ribavirin combination therapy have not been established in these patients. Further investigations are warranted.

IFN therapy following therapy for HCC is safe in selected patients. However, IFN therapy for the prevention of recurrent HCC is different from that for the treatment of primary HCC. Because prevention involves not only inflammation, fibrosis, and HCV, but also HCC-related factors, further investigations, including randomized controlled trials, are needed. Furthermore, antiviral therapy itself may improve liver reserve and expand the therapeutic options at the time of recurrence, thus improving the prognosis of HCC, and this issue also needs to be addressed by further studies including randomized controlled trials.

CONCLUSION

Secondary prevention of HCC is an important clinical issue because the recurrence rates of HCC are extremely high even after effective local treatment with hepatic resection or percutaneous ablation. This involves multicentric carcinogenesis in which new lesions are formed as a result of underlying hepatitis. Therefore, IFN therapy following the treatment for HCC is safe in selected patients and IFN therapy is an effective secondary prevention. In the future, PEG-IFN and ribavirin combination therapy may prove to be effective in preventing recurrence, and further investigations involving more cases are needed.

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Diallyl sulfide protects against *N*-nitrosodiethylamine-induced liver tumorigenesis: Role of aldose reductase

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Abstract

AIM: To evaluate the protective effect of diallyl sulfide (DAS) against *N*-nitrosodiethylamine (NDEA)-induced liver carcinogenesis.

METHODS: Male Wistar rats received either NDEA or NDEA together with DAS as protection. Liver energy metabolism was assessed in terms of lactate, pyruvate, lactate/pyruvate, ATP levels, lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD) activities. In addition, membrane disintegration of the liver cells was evaluated by measuring lipid-peroxidation products, measured as malondialdehyde (MDA); nitric oxide (NO) levels; glucose-6-phosphatase (G6Pase), catalase (CAT) and superoxide dismutase (SOD) activities. Liver DNA level, glutathione-S-transferase (GST) and cytochrome c oxidase activities were used as DNA fragmentation indices. Aldose reductase (AR) activity was measured as an index for cancer cells resistant to chemotherapy and histopathological examination was performed on liver sections from different groups.

RESULTS: NDEA significantly disturbed liver functions and most of the aforementioned indices. Treatment with DAS significantly restored liver functions and hepatocellular integrity; improved parameters of energy metabolism and suppressed free-radical generation.

CONCLUSION: We provide evidence that DAS exerts a protective role on liver functions and tissue integrity in face of enhanced tumorigenesis caused by NDEA, as well as improving cancer-cell sensitivity to chemotherapy. This is mediated through combating oxidative stress of free radicals, improving the energy metabolic state of the cell, and enhancing the activity of G6Pase, GST and AR enzymes.

Key words: *N*-nitrosodiethylamine; Diallyl sulfide; Liver cancer; Energy metabolism; Aldose reductase

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INTRODUCTION

Primary liver cancer has been classified as the fifth most common cause of cancer and the fourth most common cause of cancer mortality in the world. One of the main pathological subtypes of liver cancer is hepatocellular carcinoma, which constitutes a major contributor to cancer incidence and mortality[1]. The population of Egypt has a heavy burden of liver disease, mostly due to chronic infection with hepatitis C virus. Since the liver offers a very important site for detoxification of xenobiotics, the use of synthetic chemoprotective agents offers potential risk factors[2,3]. Several reports have stressed the importance of many dietary habits in modifying the initiation, promotion and progression stages in carcinogenesis[4]. Garlic (*Allium sativum*), an important flavoring agent, exhibits medicinal properties that include immunomodulatory, hepatoprotective, antioxidant, antimutagenic, and anticarcinogenic effects[5,6]. The anticarcinogenic property of garlic has been documented from both epidemiological and experimental studies which suggests that the consumption of garlic can decrease the incidence of several cancers[2,7,8]. The ability of garlic to reduce the incidence of cancer has been attributed to its content of organosulfur compounds which reportedly suppress carcinogen-induced tumors in various organs of animals including the colorectum, breast and liver[9-11].

A major constituent of garlic, diallyl sulfide (DAS), has been shown to inhibit chemical toxicity and tumorigenesis in several animal models[12]. Nonetheless, the possibility that DAS may exert a protective role against *N*-nitrosodiethylamine (NDEA)-induced liver tumorigenesis cannot be ruled out. In this study, we investigated the cellular and molecular mechanisms of the protective effects of DAS against liver damage induced by NDEA, a potent inducer of liver cancer. We determined the histopathological effect of DAS on liver tissue, as well as on enzymatic and non-enzymatic liver functions. In addition, we investigated the possibility that DAS might act on maintaining liver tissue functions, which were assessed by investigating energy metabolism, membrane disintegration and DNA integrity indices.

MATERIALS AND METHODS

Animals

We used a total of 36 male albino rats of the Wistar strain, weighing 170-200 g, that were obtained from the central animal facility at the Faculty of Pharmacy, Cairo University, Cairo, Egypt. All rats were housed in a room with a controlled environment, at a constant temperature of 23 ± 1℃, humidity of 60% ± 10%, and a 12 h light/dark cycle. The animals were housed in groups and kept at constant nutritional conditions throughout the experimental period. The experimental protocols were approved by the Ethical Committee of Cairo University.

Drugs and chemicals

NDEA, DAS, enzymes and coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of Analar grade. NDEA was prepared as 8 mg/mL saline, whereas DAS was prepared as 80 mg/mL corn oil.

Induction of liver cancer

Each rat received an oral dose of 20 mg/kg per day NDEA for 5 d per week for 9 wk, followed by 10 mg/kg per day for 5 d per week for another 6 wk.

Protocols and experimental groups

Animals were divided into three groups: Group Ⅰ was the NDEA-induced cancer group, and Group Ⅱ was the DAS-treated group. Cancer was induced in this group by the same protocol. In addition, DAS was co-administered at a daily oral dose of 200 mg/kg per day for 5 d per week for the total period of the experiment (i.e. 15 wk). Group Ⅲ consisted of normal rats that received an oral dose of vehicles (saline, corn oil) for the total period of the experiment.

Biochemical estimations

Blood analysis: At the end of the experimental period, all animals were killed by cervical dislocation. The separated plasma was analyzed for total protein[13] and albumin[14]. The separated serum was analyzed for aspartate aminotransferase (AST), using a kit provided by Bicon, Germany[15]; alkaline phosphatase (ALP) using a kit provided by Biolabo, France[16,17], and gamma glutamyltransferase (GGT) using a kinetic photometric method[18].

Tissue analysis: The liver was removed, rinsed with ice-cold saline and blotted dry. Accurately weighed pieces of liver tissue were treated differently for the separation and estimation of the studied parameters.

Measurement of liver malondialdehyde (MDA) content: A 10% homogenate was prepared in 1.15% KCl, centrifuged at 1000 × *g* at 4℃ for 20 min, and the resultant supernatant was used for the assay of liver MDA content[19].

Measurement of liver cytochrome c oxidase activity and nitric oxide (NO) content: Liver tissue was homogenized in Tris-sucrose buffer, pH 7.4 (5% homogenate), using Potter-Elvejhem glass homogenizer, and centrifuged at 2000 × *g* at 4℃ for 10 min. The resultant supernatant was used for the estimation of cytochrome c oxidase activity[20] and NO content[21].

Measurement of liver glutathione-S-transferase (GST), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD) and catalase (CAT) activities: A 10% homogenate was obtained in Tris-sucrose buffer, pH 7.4, and centrifuged at 105 000 × *g* at 4℃ for 30 min, using a Dupont Sorvall Ultracentrifuge (USA), to isolate the cytosolic fraction which was used for the assay of GST[22], SOD[23], LDH[24], G6PD[25] and CAT[26] activities.

Estimation of liver pyruvate and lactate concentrations: Liver tissue was homogenized in 5% meta-phosphoric acid, and centrifuged at 3000 × *g* at 4℃ for 15 min. The resultant supernatant was used for the estimation of pyruvate and lactate concentrations according to the method of Mohun and Cook[27] and David[28], respectively.

Estimation of liver ATP content: Liver tissue was homogenized with 3 mL ice-cold 3 mol/L perchloric acid, using Potter-Elvejhem glass homogenizer. Following that, 12.5 mL of 1 mmol/L EDTA was added and the mixture was centrifuged at 1000 × *g* at 4℃ for 1 h. The supernatant was further treated for the estimation of ATP[29].

Determination of liver glucose-6-phosphatase (G6Pase) activity: Liver tissue was homogenized in ice-cold solution containing 0.15mol/L KCl; 4 mmol/L $MgSO_4$; 4 mmol/L EDTA and 4 mmol/L N-acetylcysteine, pH 7, and centrifuged at 12000 × *g* at 4℃ for 10 min. The resultant supernatant was analyzed for G6Pase activity[30].

Estimation of liver DNA content: Liver tissue was homogenized in 0.25 mol/L sucrose/in TKM buffer (0.05 mol/L Tris-HCl, 0.025 mol/L KCl, 0.005 mol/L

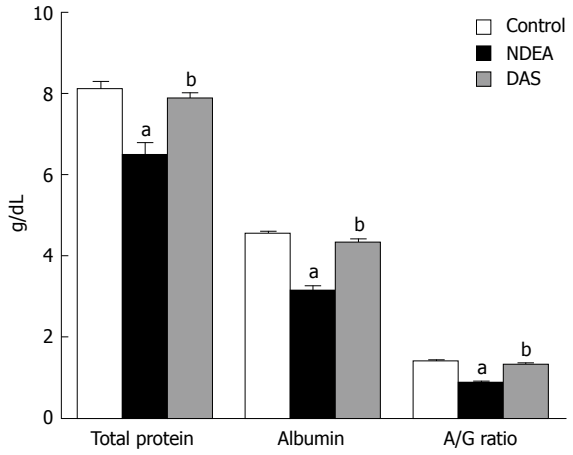

Figure 1 Non-enzymatic liver functions in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group ($^aP = 0.045$, $^bP = 0.042$, respectively).

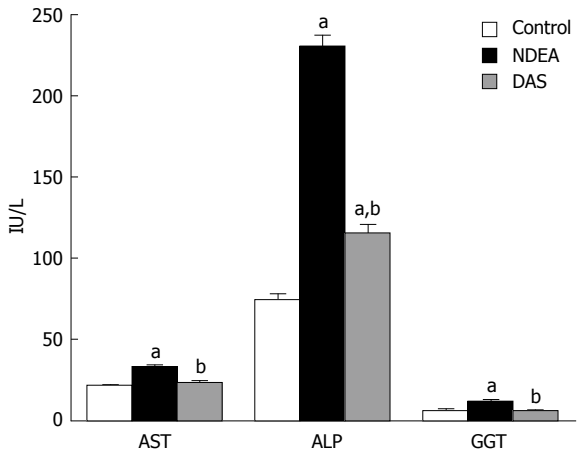

Figure 2 Enzymatic liver functions in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group ($^aP = 0.048$, $^bP = 0.044$, respectively).

$MgCl_2$), pH 7.5, to prepare a 15% homogenate. Then 0.1 mL of 0.3 mol/L perchloric acid was added, left to stand at 0℃ for 15 min, centrifuged at 2000 × *g* at 4℃ for 10 min, and the precipitate was used for the estimation of DNA content[31].

Determination of liver aldose reductase (AR) activity: Liver tissue was homogenized in potassium phosphate buffer, pH 7 (20% homogenate), centrifuged at 105000 × *g* for 45 min at 4℃, and the resultant supernatant was used for the estimation of AR activity[32].

Determination of protein concentrations: Protein concentrations of the above supernatants were estimated by the method of Lowry *et al*[33].

Histopathological examination

The portions of liver tissue embedded in paraffin were sectioned at 5 μm. Following sectioning, liver tissue was stained with hematoxylin and eosin. Light microscopy was used to evaluate the pathological changes in liver tissue.

Statistical analysis

The values were expressed as mean ± SE. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Kruskal-Wallis comparison test. $P \leqslant 0.05$ was considered significant.

RESULTS

The results showed that there were no differences between the various control groups (vehicle-treated groups). Thus, the data from all of the control animals were po-oled and are shown as one normal group.

Effect of NDEA in absence or presence of DAS on non-enzymatic liver functions

NDEA significantly decreased total protein, albumin, and A/G ratio. Co-administration of DAS restored total protein, albumin and A/G ratio (Figure 1). Moreover, NDEA significantly increased total bilirubin (2.43 ± 0.151 mg/dL), compared to their control counterparts (1.19 ± 0.090 mg/dL). DAS significantly decreased total bilirubin (1.68 ± 0.078 mg/dL, $P = 0.045$) when compared to the NDEA-treated group value.

Effect of NDEA in absence or presence of DAS on enzymatic liver functions

NDEA significantly elevated serum AST, ALP and GGT activities when compared to the normal group. DAS co-administration restored serum AST and GGT activities, together with a significant decrease in ALP activity, compared to the NDEA group value ($P = 0.048$) (Figure 2).

Changes in liver energy metabolism indices following NDEA in absence or presence of DAS

Table 1 showed that NDEA significantly decreased ATP level and increased G6PD activity compared to normal group values ($P = 0.046$). Treatment with DAS resulted in a significant increase in ATP level compared to the NDEA-treated group value. In addition, DAS significantly elevated and reduced pyruvate and lactate levels respectively, with a consequent significant reduction in lactate/pyruvate ratio compared to both the NDEA and control counterparts. Also, DAS significantly increased LDH and G6PD activities compared to either NDEA or control groups.

Changes in oxidative stress and membrane disintegration indices following NDEA in absence or presence of DAS

As shown in Table 2, NDEA treatment significantly ($P = 0.048$) elevated MDA and NO contents and reduced G6Pase, CAT and SOD activities when compared to control counterparts. Administration of DAS restored MDA level and G6Pase activity, significantly decreased NO level, and non-significantly changed SOD and CAT activities when compared to NDEA-treated rats.

Table 1 Effect of NDEA in absence or presence of DAS on liver energy metabolism indices (mean ± SE)

Parameters	Normal	NDEA	DAS
Lactate (μmol/g liver)	2.27 ± 0.16	2.08 ± 0.094	1.41 ± 0.08[a,b]
Pyruvate (μmol/g liver)	0.11 ± 0.007	0.103 ± 0.008	0.15 ± 0.007[a,b]
Lac/Pyr	20.6 ± 1.65	17.37 ± 0.9	11.31 ± 0.76[a,b]
LDH (μmol/mg protein per min)	0.9 ± 0.021	0.9 ± 0.043	1.05 ± 0.028[a,b]
ATP (μmol/g liver)	5.69 ± 0.32	4.04 ± 0.12[a]	4.91 ± 0.085[a,b]
G6PD (μmol/mg protein per min)	27.9 ± 2.29	47.4 ± 1.48[a]	54.1 ± 4.04[a,b]

Significantly different from baseline values at [a]P = 0.046; Significantly different from NDEA treatment at [b]P = 0.043.

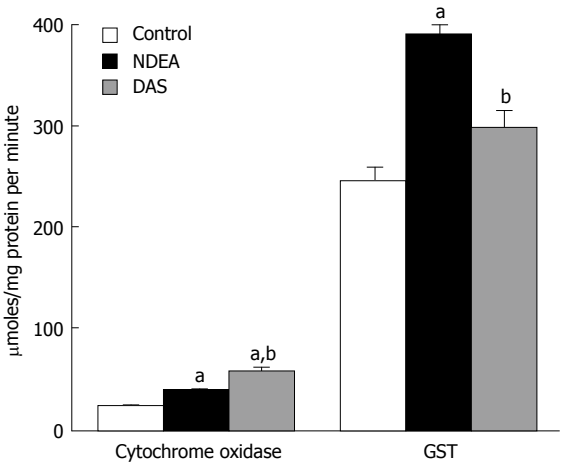

Figure 3 Changes in cytochrome oxidase and GST activities in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group values ([a]*P* = 0.043, [b]*P* = 0.045, respectively).

Effect of NDEA in absence or presence of DAS on DNA fragmentation indices

NDEA significantly elevated DNA level (3.97 ± 0.189 mg/gt) compared to control level (2.5 ± 0.199 mg/gt) (P = 0.043). DAS normalized DNA levels (2.38 ± 0.08 mg/gt). NDEA significantly elevated cytochrome c oxidase and GST activities. DAS significantly lowered GST , which approached the normal values. However, DAS significantly enhanced cytochrome c oxidase activity when compared to either control or NDEA-treated group levels (Figure 3).

Effect of NDEA in absence or presence of DAS on AR activity

NDEA significantly elevated AR activity (P = 0.045). Co-administration of DAS restored such enzymatic activity (Figure 4).

Histopathological findings

Examination of liver sections of the different groups illustrated that: Liver tissue of the normal group showed hepatic lobules with normal architecture (Figure 5A). Liver tissue of the NDEA-treated rats showed pleomorphism. Some cells exhibited multiple nucleoli, some of the cells were pyknotic, while others showed

Table 2 Effect of NDEA in absence or presence of DAS on oxidative stress and membrane disintegration indices (mean ± SE)

Parameters	Normal	NDEA	DAS
MDA (nmol/g liver)	55.6 ± 3.4	90.4 ± 8.01[a]	66.8 ± 2.4[b]
NO (nmol/g liver)	139 ± 9.7	344 ± 14.9[a]	187 ± 9.2[a,b]
G6Pase (nmol/mg protein/min)	6.39 ± 0.46	3.38 ± 0.26[a]	7.36 ± 0.3[b]
CAT (IU/mg protein)	173 ± 9.1	141 ± 6.44[a]	138 ± 6.64[a]
SOD (IU/mg protein)	88.3 ± 6.55	55.7 ± 2.7[a]	57.8 ± 5.38[a]

Significantly different from baseline values at [a]P = 0.048; Significantly different from NDEA treatment at [b]P = 0.044.

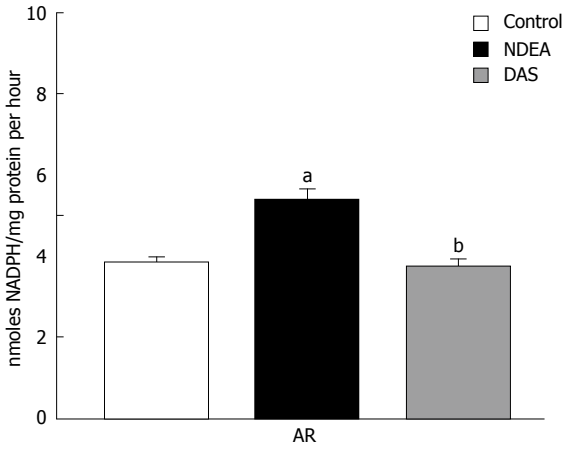

Figure 4 Changes in AR in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group values ([a]*P* = 0.045, [b]*P* = 0.046, respectively).

intranuclear vacuoles and cellular infiltration (Figure 5B). Other sections showed massive areas of vacuolated hepatocytes, cellular infiltration, and some cells possessed pyknotic nuclei (Figure 5C). Other section showed hyperchromatic nuclei and numerous Kupffer cells (Figure 5D). In other sections, hyperchromatic malignant nuclei were evident (Figure 5E). Liver tissue from the DAS-treated rats showed fewer degenerative changes, such as vacuolated cytoplasm, few pyknotic nuclei and dilated sinusoids (Figure 5F). Other section showed more or less normal hepatic lobular architecture (Figure 5G).

DISCUSSION

The liver is a multifunctional organ that plays essential roles in metabolism, biosynthesis, excretion, secretion and detoxification. These processes require energy, making the liver a highly aerobic, oxygen-dependent tissue. These processes also cause vulnerability of the liver to anoxia, increased susceptibility to noxious insults, and create a demand for cell replacement after tissue loss. Enhanced liver cell death and impaired regeneration are indeed features of most liver disorders. Proteins play a big role in fighting off infections and building or repairing muscle tissue. Low albumin is a sign of poor health and a predictor of a bad outcome. Thus, a

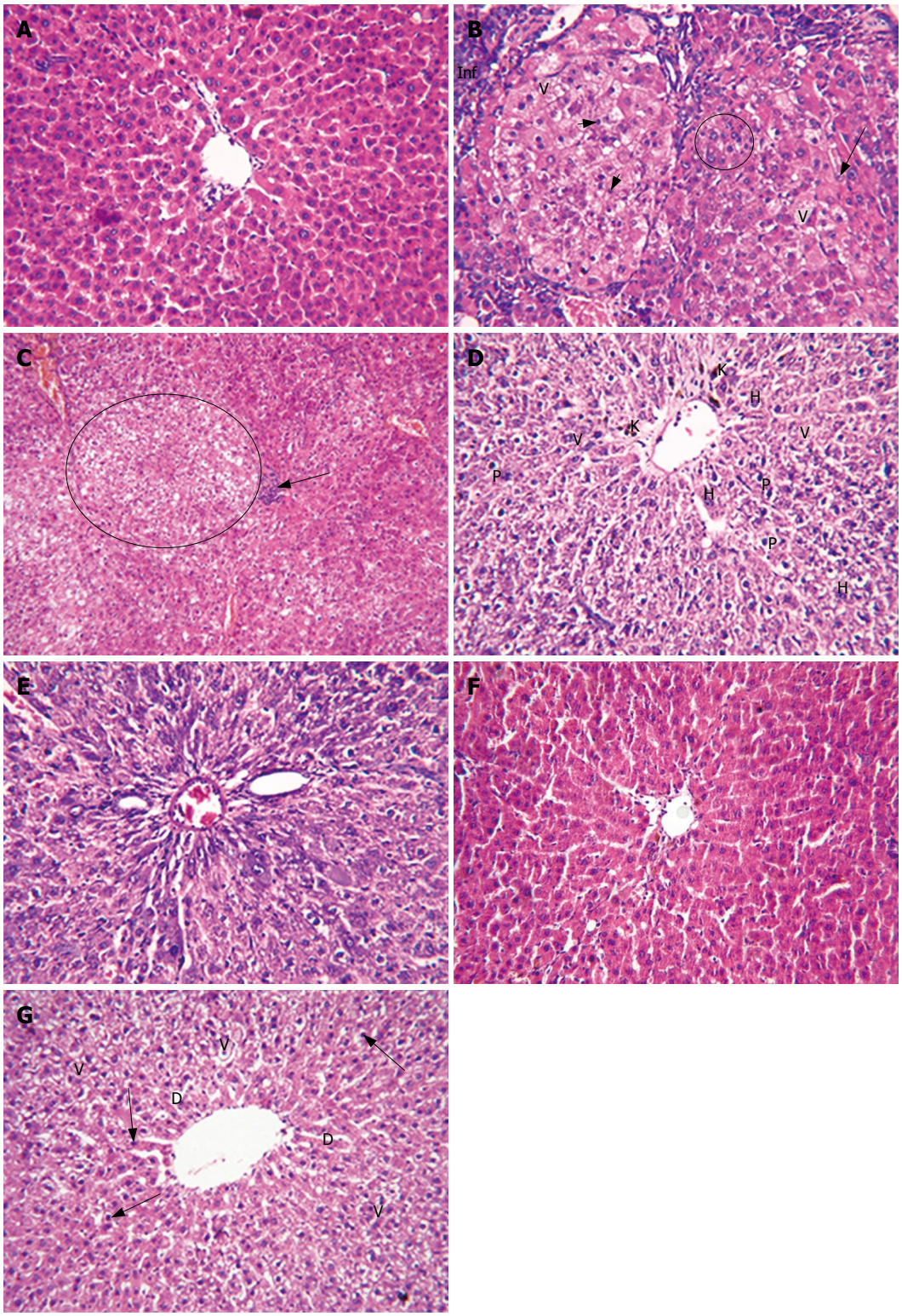

Figure 5 Liver sections of the different groups. A: Liver tissue of the normal group (control) showed hepatic lobule having normal architecture; B: Liver tissue of the NDEA-treated rats showed nuclear pleomorphism; some cells exhibited multiple nucleoli (encircled), pyknotic cells (short arrows), intranuclear vacuoles (arrow), some showed cytoplasmic vacuoles (V) and cellular infiltration (Inf); C: Other sections showed massive areas of vacuolated hepatocytes (encircled); cellular infiltration (arrow) and some cells possessed pyknotic nuclei; D: Other slides showed vacuolated cytoplasm, hyperchromatic nuclei, pyknotic nuclei and numerous Kupffer cells; E: Other section showed hyperchromatic malignant nuclei (H); F: Liver tissue of the DAS-treated rats showed some degenerative changes, vacuolated cytoplasm (V), few pyknotic nuclei (arrows) and dilated sinusoids (D); G: Other slides showed more or less normal hepatic lobular architecture.

decrease in the A/G ratio often indicates the presence of impaired liver function. As shown in our results, NDEA decreased significantly total protein, albumin and A/G levels, which was indicative of poor liver function and inability to fight infections. On the contrary, DAS administration normalized total protein, albumin and A/G levels, indicating the ability of DAS to improve liver function in face of NDEA-induced liver damage.

Serum AST, ALP and GGT are sensitive indicators of hepatic injury. Several reports have shown an increase in the activities of AST and ALT during NDEA-induced hepatocarcinogenesis[34]. Elevated activities of serum AST, ALP and GGT observed in NDEA-treated rats may be due to the NDEA-induced hepatic damage and the subsequent leakage of these enzymes into the circulation. Administration of DAS restored the activities of these enzymes to near normal values, which may be an indication of the hepatoprotective role of DAS.

As shown in our results, NDEA produced a significant decrease in hepatic ATP level. Oncotic necrosis is most often the consequence of metabolic injury, leading to ATP depletion. ATP depletion in hepatocytes is associated with ATP-depletion-dependent cytoskeletal alterations, after which a metastable state develops, characterized by mitochondrial depolarization and lysosomal breakdown. This metastable state culminates in outright rupture of plasma membrane, irreversible breakdown of the plasma membrane permeability barrier, and leakage of cytosolic enzymes and metabolic intermediates[35]. Treatment with DAS resulted in a significant decrease in lactate and lactate/pyruvate ratio, along with significant elevation of pyruvate, ATP levels and liver LDH activity, compared to NDEA-treated rats. The increased activity of LDH could favor pyruvate (aerobic carbohydrate metabolism) against lactate (anaerobic), thus enhancing energy metabolism in the cell and reflecting restoration of normal cellular/metabolic function. The histopathological findings observed in this study support the biochemical ones that liver tissue of the NDEA-treated rats showed drastic changes in the morphology of the liver cells, whereas the DAS-treated rats showed more or less normal hepatic lobular architecture. Accordingly, we presented evidence that DAS substantially improved the liver cell metabolic indices, as well as its synthetic capacity, and further protected against its malignant transformation.

The present data revealed that NDEA produced significant increases in the activity of liver G6PD, which agrees with findings from other studies[36,37]. G6PD is a housekeeping enzyme that produces riboses, which are incorporated into nucleotides and nucleic acids, and NADPH, the major cytoplasmic reducing compound[38]. NADPH is necessary for reduction of oxidized glutathione by glutathione reductase[39], and is a substrate for phase I and Ⅱ biotransformation and detoxification enzymes[36]. G6PD is elevated in response to external stimuli, toxic and oxidative stress[40,41]. G6PD activity is strongly upregulated in proliferating cells such as malignant cells[42]. There is increasing evidence that G6PD activity is of major importance for NADPH production for defense against oxidative stress, rather than for ribose production during proliferation[43]. Interestingly, treatment with DAS resulted in further elevation in the activity of G6PD. This result provides new evidence that DAS might exhibit a compensatory mechanism in enhancing the production of NADPH as a further defense mechanism against proliferating cancer cells, as well as for enhancing cellular antioxidant capacity.

In hepatocellular carcinoma, there is disequilibrium between oxidant and antioxidant balance, which is tilted towards the oxidant side[44]. Reactive oxygen species (ROS) are believed to cause genetic oxidation and damage to DNA and other macromolecules. Unchecked, this oxidative damage may lead to a host of conditions including cancer. Normally, this process is held in check by elaborate endogenous or exogenous antioxidant processes. Various enzymatic and non-enzymatic systems have been developed by the cell to cope with ROS and other free radicals[45]. Since many of the anomalies that are induced by NDEA can arise from oxidative stress, which is also known to accompany cancer development, it was of a prime interest to evaluate oxidative stress levels under those circumstances. As shown in the present study, NDEA produced a significant increase in hepatic MDA and NO levels, along with significant decreases in SOD and CAT activities. MDA was one of the main lipid peroxidation products; its elevated levels can reflect the degree of lipid-peroxidation-induced injury in hepatocytes[46]. On the other hand, it has been reported that SOD and CAT constitute a mutually supportive defense against ROS[47]. The decreased activity of SOD in liver of NDEA-treated rats may have been due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. This may have caused an increased accumulation of superoxide radicals, which could have further stimulated lipid peroxidation[48]. Decreased activities of SOD and CAT in NDEA-treated rats, which is in agreement with other reported studies[49], could have been due to over-utilization of these enzymatic antioxidants to scavenge the products of lipid peroxidation. Tumor cells have been reported to sequester essential antioxidants from the circulation in order to meet the demands of the growing tumor cells. On the other hand, the current data demonstrated the ability of DAS to reduce formation of ROS and reactive nitrogen species, measured as MDA and NO, which agrees with findings from other laboratories[7]. These findings conform to previous results on the established, specific antioxidant profile for DAS as an inhibitor of the hepatic ROS generating enzyme CYP2E1. The latter enzyme is known as a prominent trigger of hepatic oxidative stress[50]. Treatment with DAS showed no significant enhancement of the activity of the endogenous antioxidant enzyme SOD or CAT.

G6Pase plays a critical role in blood glucose homoeostasis and its activity can also be considered as an index of the stability of the microsomal membrane[51]. Decreased activity of liver G6Pase was shown in the NDEA-treated rats, which might be attributed to the increased lipid peroxidation caused by NDEA. Consistent with previous studies[52], DAS administration enhanced G6Pase activity significantly, compared to the NDEA-treated group value, suggesting the ability of DAS to preserve membrane integrity.

In this study, an increased activity of liver GST was observed in NDEA-treated rats, with respect to their control counterparts. In addition, we also showed an increased activity of serum GGT in the NDEA-treated

rats, which might have been responsible for the increased level of GST in this group of animals[44]. Initial reports from nitrogen-mustard-resistant cell lines have shown these cells to over-express GST, which also holds true for a number of tumors. The increased level of GST is likely to be the key mediator of drug resistance in cancer chemotherapy[44]. Restoration of GST activity was observed with DAS treatment, suggesting a preservation of the redox system, which reflects a decrease in free-radical production, as well as improving cancer cell sensitivity to chemotherapy.

Cancer is well known to induce uncontrolled cellular proliferation. In this context, our results demonstrated that NDEA increased total DNA level, suggesting enhanced cellular proliferation. Notably, treatment with DAS significantly reduced DNA levels to near normal values, suggesting interference with mitotic pathways and enhancing apoptosis of cancer cells[53]. In addition, the current results showed an enhanced activity of cytochrome c oxidase enzyme in the NDEA-treated rats, which was further enhanced by the co-administration of DAS. However, for this investigation, we measured total cytochrome c oxidase, so it was difficult to delineate whether the increase we observed in the levels of cytochrome c oxidase was attributed to the mitochondrial or cytosolic fraction. According to the observed elevation in hepatic MDA and NO contents in the NDEA-treated group, a state of oxidative stress can exist in such animals, which contributes to mitochondrial membrane leakage and in turn, allows the translocation of cytochrome c oxidase to the cytosolic fractions. Thus, we suggest that the increase in cytochrome c oxidase with NDEA treatment might be of cytosolic origin. Interestingly, we demonstrated, and to the best of our knowledge, for the first time, that DAS markedly enhanced the activity of cytochrome c oxidase. We suggest that such increased cytochrome c oxidase activity might be attributed to a mitochondrial rather than cytosolic origin, which is supported by the observed increase in ATP and decrease in oxidative-stress biomarkers shown in DAS-treated animals. DAS might induce direct perturbation of mitochondria, resulting in apoptotic damage of the cancer cells. This effect has been reported recently with some anticancer agents[54,55].

Our results showed an almost 1.5-fold increase in AR activity in NDEA-treated animals. AR belongs to the aldo-keto reductase (AKR) superfamily. Most of the AKR superfamily proteins are involved in the detoxification of a wide variety of substrates. Several reports have shown that over-expression of AR, in many tumor cells, renders these cells resistant to chemotherapy, and also demonstrate that inhibition of AR enhances cancer cell sensitivity to chemotherapeutic drugs[56,57]. In addition, over-expression of AR enhances production of ROS, which cause membrane damage and cellular leakage[58]. To the best of our knowledge, the present study is the first to show enhanced production of AR in an *in vivo* model of liver tumorigenesis. To our knowledge, this is the first report that identifies the ability of DAS to reduce the expression of AR in NDEA-treated rats. This provides new evidence for its very important potential role in cancer protection. The ability of DAS to reduce the expression of AR suggests that DAS is effective against *in vivo* tumorigenesis by suppressing AR production and subsequently lowering the production of ROS, as well as enhancing cancer cell sensitivity to chemotherapeutic drugs.

Our findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of NDEA-treated rats showed damage, manifest as nuclear pleomorphism, intranuclear vacuoles, cellular infiltration, hyperchromatic nuclei, pyknotic nuclei, numerous Kupffer cells and hyperchromatic malignant nuclei. On the contrary, liver tissue of the DAS-treated rats showed more or less normal hepatic lobular architecture.

To conclude, we provide evidence that DAS exerts a protective role on liver tissue in face of enhanced tumorigenesis caused by NDEA, as demonstrated by the following points: (1) DAS could normalize almost all the non-enzymatic and enzymatic liver function tests, indicating its ability to improve liver functions; (2) DAS significantly decreased lactate and lactate/pyruvate ratio, along with elevating pyruvate, ATP levels and liver LDH activity, thus enhancing energy metabolism in the liver tissue and reflecting restoration of normal cellular/metabolic functions; (3) DAS elevated the activity of G6PD, so it might exhibit a compensatory mechanism in enhancing the production of NADPH as a further defense mechanism against proliferating cancer cells, as well as enhancing cellular antioxidant capacity; (4) DAS reduced the formation of free radicals, measured as MDA and NO, providing specific antioxidant profiles for DAS as an inhibitor of the hepatic ROS-generating enzyme; (5) DAS enhanced G6Pase activity significantly, suggesting its ability to preserve liver cell membrane integrity; (6) DAS restored GST activity, suggesting a preservation of the redox system, as well as improving cancer cell sensitivity to chemotherapy; (7) DAS significantly reduced DNA level comparable to that in the NDEA-treated group, and close to the normal value, suggesting interference with mitotic pathways and enhancing apoptosis of cancer cells; (8) DAS markedly enhanced cytochrome c oxidase activity, thus, DAS might induce direct perturbation of mitochondria, resulting in apoptotic damage of the cancer cells; (9) to the best of our knowledge, this is the first report that identifies the ability of DAS to reduce the expression of liver AR in NDEA-treated rats, which suggests a very important potential role in cancer protection and subsequently lowering the production of ROS, as well as enhancing cancer cell sensitivity to chemotherapeutic drugs; (10) our biochemical findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of the NDEA-treated rats showed damage, resulting in malignant cell formation. On the contrary, liver tissue of the DAS-treated rats showed more or less normal hepatic lobular architecture.

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COMMENTS

Background

Diallyl sulfide (DAS), a biologically active garlic constituent, has been demonstrated as a potential cytoprotective agent in many animal models. Garlic (*Allium sativum*), an important flavoring agent, exhibits medicinal properties that include immunomodulatory, hepatoprotective, antioxidant, antimutagenic and anticarcinogenic effects.

Research frontiers

Oncotic necrosis is most often the consequence of metabolic injury, leading to ATP depletion, culminating in leakage of cytosolic enzymes and metabolic intermediates. In hepatocellular carcinoma, disequilibrium exists between oxidant and antioxidant balance, which is tilted towards oxidants. Tumor cells sequester essential antioxidants from the circulation to meet the demands of the growing tumor cells. We showed that DAS reduced formation of ROS, which agrees with reported findings. Aldose reductase (AR) belongs to the aldo-keto reductase (AKR) superfamily involved in the detoxification processes of a wide variety of substrates. Over-expression of AR, in many tumor cells, renders these cells resistant to chemotherapy.

Innovations and breakthroughs

DAS protects against *N*-nitrosodiethylamine (NDEA)-induced liver cancer. DAS markedly enhanced, cytochrome c oxidase activity, thus inducing direct perturbation of mitochondria, culminating in apoptotic damage of the cancer cells. The expression of liver AR in NDEA-treated rats was reduced by DAS, subsequently lowering the production of ROS and enhancing cancer cell sensitivity to chemotherapeutic drugs. Histopathological examination illustrated that liver tissue of the NDEA-treated rats showed malignant cell formation, which was prevented by DAS.

Applications

The population of Egypt has a heavy burden of liver disease and the use of synthetic chemoprotective agents has potential risks in this population. Dietary habits may modify carcinogenesis initiation, promotion and progression. Hence, this study indicates the potential protective effect of DAS against NDEA-induced liver cancer. This could be used as a protective method to prevent exacerbation of cancer in developed countries that cannot afford the burden of expensive chemotherapy.

Terminology

DAS: Diallyl sulfide (a major constituent of garlic); NDEA: *N*-nitrosodiethylamine (inducer of liver cancer); LDH: Lactate dehydrogenase; G6PD: Glucose-6-phosphate dehydrogenase; MDA: Malondialdehyde; NO: Nitric oxide; G6Pase: Glucose-6-phosphatase; CAT: Catalase; SOD: Superoxide dismutase; GST: Glutathione-S-transferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; AR: Aldose reductase.

Peer review

The authors demonstrated in this study that DAS had anti-oxidant properties in NDEA-treated rats. This work is very interesting. The authors bring some novelty and innovation to their research. The references are appropriate and updated.

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VIRAL HEPATITIS

Liver stiffness in the hepatitis B virus carrier: A non-invasive marker of liver disease influenced by the pattern of transaminases

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Abstract

AIM: To investigate the usefulness of transient elastography by Fibroscan (FS), a rapid non-invasive technique to evaluate liver fibrosis, in the management of chronic hepatitis B virus (HBV) carriers.

METHODS: In 297 consecutive HBV carriers, we studied the correlation between liver stiffness (LS), stage of liver disease and other factors potentially influencing FS measurements. In 87 chronic hepatitis B (CHB) patients, we monitored the FS variations according to the spontaneous or treatment-induced variations of biochemical activity during follow-up.

RESULTS: FS values were 12.3 ± 3.3 kPa in acute hepatitis, 10.3 ± 8.8 kPa in chronic hepatitis, 4.3 ± 1.0 kPa in inactive carriers and 4.6 ± 1.2 kPa in blood donors. We identified the cut-offs of 7.5 and 11.8 kPa for the diagnosis of fibrosis ⩾ S3 and cirrhosis respectively, showing 93.9% and 86.5% sensitivity, 88.5% and 96.3% specificity, 76.7% and 86.7% positive predictive value (PPV), 97.3% and 96.3% negative predictive value (NPV) and 90.1% and 94.2% diagnostic accuracy. At multivariate analysis in 171 untreated carriers, fibrosis stage (t = 13.187, P < 0.001), active *vs* inactive HBV infection (t = 6.437, P < 0.001), alanine aminotransferase (ALT) (t = 4.740, P < 0.001) and HBV-DNA levels (t = -2.046, P = 0.042) were independently associated with FS. Necroinflammation score (t = 2.158, > 10/18 *vs* ⩽ 10/18, P = 0.035) and ALT levels (t = 3.566, P = 0.001) were independently associated with LS in 83 untreated patients without cirrhosis and long-term biochemical remission (t = 4.662, P < 0.001) in 80 treated patients. During FS monitoring (mean follow-up 19.9 ± 7.1 mo) FS values paralleled those of ALT in patients with hepatitis exacerbation (with 1.2 to 4.4-fold increases in CHB patients) and showed a progressive decrease during antiviral therapy.

CONCLUSION: FS is a non-invasive tool to monitor liver disease in chronic HBV carriers, provided that the pattern of biochemical activity is taken into account. In the inactive carrier, it identifies non-HBV-related causes of liver damage and transient reactivations. In CHB patients, it may warrant a more appropriate timing of control liver biopsies.

Key words: Liver elastography; Liver fibrosis; Cirrhosis; Hepatitis B virus; Chronic hepatitis B

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INTRODUCTION

Transient elastography by Fibroscan (FS)[1] has been proposed as a rapid, non-invasive technique to detect liver fibrosis[2], and many studies have confirmed its clinical usefulness, demonstrating good reproducibility

and high correlation between FS and liver fibrosis at histology[3-7]. Nevertheless, liver stiffness (LS) is influenced by factors other than fibrosis, such as major variations of alanine aminotransferase (ALT) levels[8]. We showed that during hepatitis exacerbations, LS increased, paralleling the kinetics of ALT, whereas FS values were lower than expected according to the histological stage in patients with long-lasting (≥ 12 mo) ALT normalization[8]. Similar LS profiles have been reported in patients with acute viral hepatitis[9,10].

Thus, the biochemical status (ALT levels) of the patient has to be taken into account for an accurate interpretation of LS values in clinical practice. This might be highly relevant in chronic hepatitis B virus (HBV) infection where intervening phases of disease activity and remission and asymptomatic hepatitis reactivations are observed[11-14].

In order to assess the usefulness of FS in the clinical management of chronic HBV carriers, we studied prospectively LS and evaluated its variations according to the changes of the virological, biochemical and histological profiles of liver disease.

MATERIALS AND METHODS

Patients

We studied 288 consecutive chronic HBV carriers (192 males, mean age 48.4 years, range 20-78 year) and nine patients with acute hepatitis B followed-up at the Hepatology Unit of the University Hospital of Pisa, Regional Reference Center for Chronic Liver Disease and Hepatocellular Carcinoma. The study was approved by the Ethical Committee of the hospital and patients gave their written informed consent.

HBV carriers were classified, after a monthly follow-up of at least 12 mo, as inactive or active according to their virological profile. Inactive carriers had serum HBV DNA persistently < 10^5 copies/mL (by COBAS Amplicor HBV Monitor, Roche, Basel, Switzerland) and IgM anti-HBc levels < 0.200 (by Core-M™ Axsym System, Abbott, Sligo, Ireland). Chronic hepatitis patients showed the presence of active viral replication (serum HBV-DNA levels persistently or intermittently ≥ 10^5 copies/mL during the follow-up), IgM anti-HBc ≥ 0.200 and liver histology consistent with chronic hepatitis. Exclusion criteria: hepatitis D virus (HDV) or hepatitis C virus (HCV) coinfections, Child B or C cirrhosis.

Cross-sectional study

We studied the correlation between LS and the stage of liver disease with single point FS measurements in 297 HBV carriers (288 with chronic infection: 208 untreated and 80 treated; nine with acute hepatitis B) and 50 blood donors as controls. Transient elastography was performed within 6 mo (median 3 mo, 75% of cases between 0 and 4.6 mo) from liver biopsy in 157 patients with biochemical and/or virological signs of liver disease and 21 inactive carriers. In 47 HBV carriers with inactive infection, 63 patients with cirrhosis (with previous histological diagnosis and actual ultrasonographic signs of cirrhosis) and nine patients with acute hepatitis, who did not undergo liver biopsies, FS was performed within 1 wk from US and Doppler examinations of the liver.

Prospective study

To study the correlation between LS and spontaneous or treatment-induced variations of biochemical activity, we enrolled 87 patients who underwent monthly blood controls and FS measurements at least every 6 mo. In case of ALT flares (ALT values ≥ 300 IU/L with increments of at least 2 SD above previous values), patients were monitored with blood and FS test every 2 wk for the first month and monthly thereafter until flare resolution. Transaminases and virological markers (HBV DNA and IgM anti-HBc) were tested on the same day of FS measurements. In treated patients, FS was monitored every 3 mo.

LS

Transient elastography was measured by Fibroscan (EchoSens, Paris, France). All measures were performed by trained physicians on the right liver lobe through intercostal spaces in the patient lying on his back, with right arm in maximal abduction. The US guide was used to identify a target liver area, at least 6 cm thick, without major vascular structures. The procedure was considered valid if at least 10 validated measurements were performed, with a success rate (ratio between numbers of validated and total measurements) ≥ 60% and interquartile range (IQR) < 20%. LS was recorded in kPa as the median value of all measurements.

Liver histology

Liver biopsies were obtained using 16 G disposable needles (Hepafix B; Braun, Melsungen, Germany). Liver specimens (median 27 mm, range 11-50 mm) were stained with hematoxylin and eosin. Necro-inflammatory activity and liver fibrosis were scored according to Ishak[15]. Steatosis was graded semiquantitatively, as reported previously[4]. Patients in whom liver biopsy yielded specimens shorter than 15 mm and/or with less than 11 portal tracts were excluded from the analysis.

Database

The included variables were sex, age, virological profile (HBeAg/anti-HBe status), liver disease co-factors [alcohol intake (≤ 60 or > 60 g/d), iron overload (present, in case of staining at histology and serum iron > 150 g/L and/or ferritin > 400 μg/L), hyperlipemia (cholesterol > 240 mg/dL and/or triglycerides > 250 mg/dL), diabetes (fasting plasma glucose > 140 mg/dL), overweight [body mass index (BMI) > 25 kg/m^2]. The biochemical profiles were defined as: (1) persistently elevated ALT; (2) biochemical remission (persistently normal ALT for at least 12 mo, at monthly controls); (3) ALT flares (when ALT values increased ≥ 300 IU/L, with increments of at least 2 SD above previous values). Virological profiles included HBV-DNA and IgM anti-HBc levels. Liver biopsy features were: length; number

of fragments; portal tracts number; necro-inflammation, fibrosis and steatosis scores. Cirrhosis at ultrasound (US cirrhosis) was defined when enlargement of left/caudate lobes, nodular liver boundaries, and micro-macronodular liver structure were present. We recorded in addition: the signs of portal hypertension (portal vein diameter > 12 mm; spleen volume > 45 cm^2; esophagus or gastric varices); the transient elastography performance (values, rate of successful measurements and IQRs); the characteristics of therapy (schedule, dose, duration and response).

Statistical analysis

Data are expressed as mean ± SD. The logarithmic transformation was used for quantitative data when their distributions were not normal. The Pearson's correlation coefficient was used to analyze the correlations between values of liver elastometry and fibrosis. Differences between subgroups were analysed using one-way ANOVA, Mann-Whitney rank sum test or Kruskal-Wallis test when appropriate. To identify factors independently correlated with LS, variables with statistical associations ($P < 0.05$) or trends ($P < 0.10$) at univariate analysis were included in multiple regression analyses. The diagnostic performance of transient elastography was evaluated by receiver operating characteristic (ROC) curve. By using the cut-off values with the highest sensitivity + specificity sum, we defined two different cut-off values of liver transient elastography to identify patients with significant fibrosis (Ishak score ⩾ 3/6) or cirrhosis. Statistical analysis was performed by SPSS (version 10.0, SPSS Inc., Chicago, IL, USA) software package.

RESULTS

Cross-sectional study

Overall 277 of 297 (93.3%) HBV carriers were suitable for the analysis: nine had acute hepatitis, 68 inactive infection, and the remaining 200 had chronic hepatitis. Six patients (2.1%) were excluded because their liver biopsies were < 1.5 cm and 14 (4.9%) because their elastographic measures failed (seven cases had BMI > 28). Eighty patients were under treatment [61 nucleos(t)ides, NA; 19 interferon, IFN]. Demographic and clinical characteristics of the 268 chronic carriers are reported in Table 1.

FS values were 4.6 ± 1.2 kPa in 50 blood donors, 12.3 ± 3.3 kPa in nine patients with acute hepatitis and 10.3 ± 8.8 kPa in 268 chronic HBV carriers ($P < 0.001$) (Table 2).

In 68 inactive carriers, the mean FS value was 5.0 ± 1.8 kPa. Seventeen of them had abnormal ALT and at histology showed steatohepatitis or steatosis. Their mean LS values were significantly higher as compared to HBV carriers with normal ALT and without dysmetabolic profile (6.9 ± 2.3 kPa *vs* 4.3 ± 1.0 kPa, $P < 0.001$) (Figure 1). As a result of chronic liver damage caused by factors other than HBV, these 17 inactive carriers were excluded from further analysis.

In the 171 untreated chronic HBV carriers, LS correlated significantly with fibrosis stage ($r = 0.706$, $P < 0.001$). At univariate analysis, in the 171 untreated HBV carriers, LS significantly correlated with age, sex, phase of infection (inactive *vs* active), BMI, ALT levels, biochemical remission and fibrosis stage, showing a correlation trend for HBV-DNA levels, alcohol intake and hyperlipemia (Table 3). At multivariate analysis, the phase of HBV infection ($P < 0.001$), ALT levels ($P < 0.001$), HBV-DNA levels ($P = 0.042$) and fibrosis stage ($P < 0.001$) were independently associated with LS (Table 3). In the separate analysis of the 83 untreated patients with chronic hepatitis, but without cirrhosis, the factors independently associated with LS were ALT levels ($P = 0.001$), fibrosis stage (S3-S4 *vs* S0-S2, $P = 0.001$) and necroinflammation score (⩾ 10/18 *vs* < 10/18; $P = 0.035$) (Table 4).

In 80 treated patients, LS correlated with fibrosis stage ($r = 0.453$, $P < 0.001$), but the mean values were lower than untreated patients with a comparable stage of

Table 1 Clinico-demographic characteristics of 268 chronic HBV carriers *n* (%)

	Chronic HBV carriers (*n* = 268)	Untreated HBV carriers (*n* = 188)	Treated HBV carriers (*n* = 80)
Age (yr)	48.2 ± 12.2	46.0 ± 11.8	53.3 ± 11.8
Male/Female	180/88	120/68	60/20
HBeAg/anti-HBe	31/237	22/166	9/71
Alcohol intake > 60 g/d	23 (8.6)	14 (7.4)	9 (11.3)
Diabetes	12 (4.5)	7 (3.7)	5 (6.3)
Hyperlipaemia	38 (14.2)	34 (18.1)	4 (5.0)
BMI			
25-30 kg/m^2	95 (35.4)	67 (35.6)	28 (35.0)
> 30 kg/m^2	7 (2.6)	6 (3.2)	1 (1.3)
ALT > 300 IU/L	11 (4.1)	9 (4.8)	3 (3.8)
HBV-DNA (Log_{10} IU/mL)	4.70 ± 2.17	4.97 ± 2.17	4.06 ± 2.05
Inactive carriers	68 (25.4)	68 (36.2)	-
CHB Ishak score			
S0-S2	85 (31.7)	71 (37.8)	14 (17.5)
S3-S4	19 (7.1)	12 (6.4)	7 (8.7)
S5-S6	30 (11.2)	14 (7.4)	16 (20.0)
US cirrhosis	66 (24.6)	23 (12.2)	43 (53.8)

US cirrhosis: Ultrasound signs of cirrhosis.

Table 2 Correlation between phase of infection, stage of liver disease and liver stiffness values

	n	Fibroscan values (kPa)
Blood donors	50	4.6 ± 1.2
Acute Hepatitis[1]	9	12.3 ± 3.3
Untreated HBsAg carriers overall[1]	188	8.9 ± 8.0
Inactive carriers whithout LD[2]	51	4.3 ± 1.0
Inactive carriers whit LD[2]	17	6.9 ± 2.3
CHB S0-S2	71	6.4 ± 2.4
CHB S3-S4	12	10.1 ± 3.8
CHB S5-S6	14	15.7 ± 9.0
US Cirrhosis[3]	23	23.6 ± 11.8
Treated CHB overall[1]	80	13.4 ± 9.7
CHB S0-S2	14	6.1 ± 1.7
CHB S3-S4	7	8.5 ± 2.8
CHB S5-S6	16	11.7 ± 5.2
US Cirrhosis[3]	43	17.2 ± 11.4

LD: Liver disease. [1]$P < 0.001$; [2]$P < 0.001$; [3]$P = 0.035$.

Table 3 Factors associated with FS values at uni and multivariate analysis in 171 chronic HBV carriers

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	0.006			NS
Sex	Male	0.002			NS
Phase of infection[1]	CHB	< 0.001	-9.939	-12.989-6.889	< 0.001
Alcohol introduction	> 60 g/d	0.076			NS
Diabetes	Present	NS			
Hyperlipemia	Present	0.089			NS
BMI	> 25 kg/m^2	< 0.001			NS
ALT	Log_{10} IU/mL	< 0.001	5.713	3.333-8.094	< 0.001
Biochemical remission[2]	Present	< 0.001			NS
HBV-DNA serum levels	Log_{10} IU/mL	0.051	-0.470	-0.924/-0.016	0.042
Disease stage[3]	Class	< 0.001	5.021	4.269-5.773	< 0.001

[1]CHB patients *vs* inactive carriers; [2]Biochemical remission means normal ALT ⩾ 12 mo; [3]Inactive carriers, CHB S0-S2, S3-S4, S5-S6, US cirrhosis. NS: No significance.

Table 4 Factors associated with FS values at uni and multivariate analysis in 83 untreated non-cirrhotic CHB patients

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	NS			
Sex	Male	0.062			NS
Alcohol introduction	>60 g/d	NS			
Diabetes	Present	NS			
Hyperlipemia	Present	NS			
BMI	> 25 kg/m^2	NS			
ALT	Log_{10} IU/mL	< 0.001	3.028	1.408-5.008	0.001
Biochemical remission	Present	0.017			NS
HBV-DNA serum levels	Log_{10} IU/mL	0.075			NS
Necroinflammation score	⩾ 10/18	< 0.001	1.611	0.117-3.104	0.035
Disease stage[1]	S3-S4	< 0.001	3.054	1.318-4.789	0.001

[1]CHB S0-S2 *vs* S3-S4; NS: No significance.

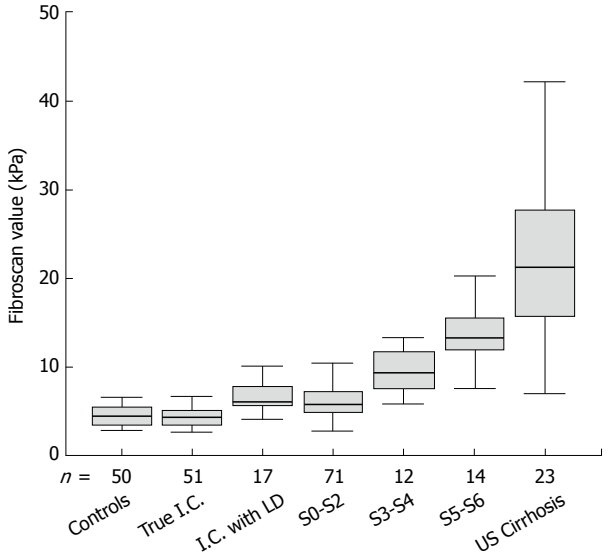

Figure 1 Distribution of Fibroscan values in Blood donors (Controls), Inactive carriers without (True I.C.) or with dysmetabolic liver disease (I.C. with LD) and in CHB patients by fibrosis stage (S0-S2, S3-S4, S5-S6, US Cirrhosis).

fibrosis (6.1 *vs* 6.4 kPa in S0-S2 patients; 8.5 *vs* 10.1 kPa in S3-S4 patients; 11.7 *vs* 15.7 kPa in S5-S6 patients; 17.2 *vs* 23.6 kPa in US cirrhosis patients) (Table 2), and the difference reached the statistical significance in patients with US cirrhosis only ($P = 0.035$). Fifty of them were under long-term NA treatment and in long-term biochemical remission, which was independently associated with FS values ($P < 0.001$, Table 5).

Diagnostic accuracy for identification of fibrosis ⩾ S3 and cirrhosis

To identify the FS cut-offs for fibrosis ⩾ S3 and cirrhosis, we analyzed untreated patients only. Area under ROC curve (AUROCs) for fibrosis ⩾ S3 and cirrhosis were 0.966 and 0.973 (95% CI 0.942-0.989 and 0.952-0.994) (Figure 2) and their cut-off values were 7.5 and 11.8 kPa, respectively.

Fibrosis ⩾ S3: The diagnostic performance of 7.5 kPa cut-off is reported in Table 6. Overall, 46 of 60 patients with elastography ⩾ 7.5 kPa had fibrosis ⩾ S3 (76.7% PPV) and 108 of 111 patients with FS < 7.5 kPa had S0-S2 fibrosis (97.3% NPV). Among the 14 patients with FS ⩾ 7.5 kPa, but a fibrosis stage < S3, five patients had ALT levels > 300 UI/L at the time of FS measurement. None of the three patients with FS < 7.5 but fibrosis ⩾ S3 had cirrhosis: one had S3 and two had S4 fibrosis at liver histology.

Table 5 Factors associated with FS values at uni and multivariate analysis in 80 treated CHB patients

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	NS			
Sex	Male	NS			
Alcohol introduction	> 60 g/d	NS			
Diabetes	Present	NS			
Hyperlipaemia	Present	NS			
BMI	> 25 kg/m^2	NS			
ALT	Log_{10} IU/mL	NS			
Biochemical remission	Present	0.001	8.705	5.277-12.133	< 0.001
HBV-DNA serum levels	Log_{10} IU/mL	NS			
Disease stage[1]	Class	< 0.001	4.374	2.982-5.766	< 0.001

[1]CHB S0-S2, S3-S4, S5-S6, US cirrhosis. NS: No significance.

Table 6 Diagnostic performance of FS for identification of fibrosis ⩾ S3 and cirrhosis by using the cut-offs of 7.5 kPa and 11.8 kPa

	Fibrosis stage ⩾ S3		Fibrosis S5-S6/US cirrhosis	
	IC + UT CHB	T CHB	IC + UT CHB	T CHB
Sensitivity (%)	93.9	78.8	86.5	54.2
Specificity (%)	88.5	71.4	96.3	90.5
Positive predictive value (%)	76.7	92.9	86.5	94.1
Negative predictive value (%)	97.3	41.7	96.3	41.3
Diagnostic accuracy (%)	90.1	77.5	94.2	63.8
Likelihood ratio for pos. test	8.18	2.76	23.18	5.69
Likelihood ratio for neg. test	0.07	0.30	0.14	0.51

IC + UT CHB: Inactive carriers + untreated CHB (171 patients); T CHB: Treated chronic hepatitis B (80 patients).

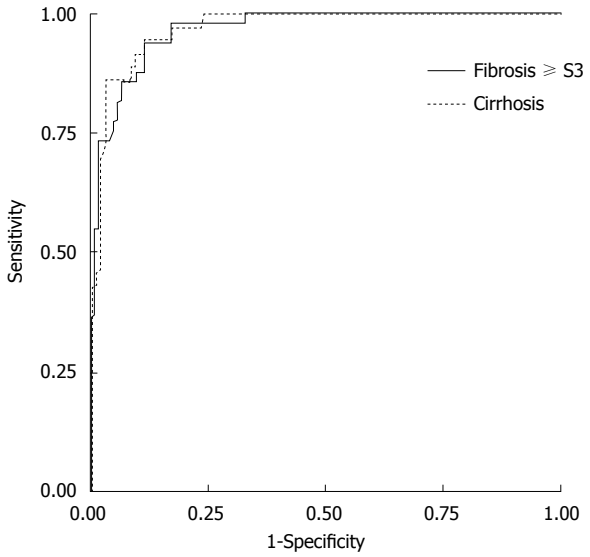

Figure 2 FS diagnostic performance: AUROCs for fibrosis S3 and cirrhosis were 0.966 and 0.973 (95% CI 0.942-0.989 and 0.952-0.994).

Cirrhosis: The diagnostic performance of 11.8 kPa cut-off is shown in Table 6. Thirty-two of 37 patients with elasticity ⩾ 11.8 kPa had histological or US cirrhosis (86.5% PPV); 129 of 134 patients with FS values < 11.8 kPa did not have cirrhosis (96.3% NPV). All but one of the five non-cirrhotic (two with S3 and three with S4 fibrosis stage) patients with FS values ⩾ 11.8 showed LS values ranging from 11.8 and 13.3 kPa; the remaining patient with 20 kPa FS value had S4 fibrosis and ALT levels > 300 UI/L at the time of FS measurement. Two of five cirrhotic patients with low FS (7.0 and 7.6 kPa respectively) were in prolonged spontaneous remission; the remaining three had elastometry values ranging between 8.9 kPa and 11.3 kPa.

Prospective study

In 87 patients, LS was monitored for a mean period of 19.9 ± 7.1 mo (range 6-36 mo): Seventy eight patients had chronic hepatitis (43 untreated and 35 treated) and nine had acute hepatitis. All patients underwent at least three FS measurements (mean 5.6, range 3-10).

Untreated patients: Thirty patients showed stable biochemical and virological profiles without disease progression: their LS did not change, showing minor fluctuations (12 mo/baseline FS mean ratio 1.00 ± 0.20; 24 mo/baseline FS mean ratio 0.99 ± 0.26). The remaining 13 patients experienced hepatitis flares. During flares, FS values increased 1.2 to 4.4-fold as compared to baseline values (mean variation 2.1 ± 1.0-fold), mean FS value during flares being 20.7 ± 12.3 kPa (range 8.6-42 kPa). LS variations paralleled the dynamic profiles of ALT: FS values reached the peak simultaneously with ALT in eight patients (61.5%), later, with 15-30 d of delay, in the remaining five (38.5%). Thereafter, FS values decreased with a latency of 30 d from the initial ALT decrease and returned to baseline values within 3 to 6 mo (Figure 3A). Patients with disease profiles characterised by ALT flares intervened by complete biochemical remission showed major variations of FS values during their hepatitis exacerbations, as compared to patients with persistent ALT elevations between flares (FS variation ranging from 1.4 to 4.4 in the former and from 1.2 to 1.6-fold in the letters, $P = 0.019$).

Acute hepatitis: In nine patients with acute hepatitis B, FS values at presentation ranged from 8.2 to 16.6 (mean

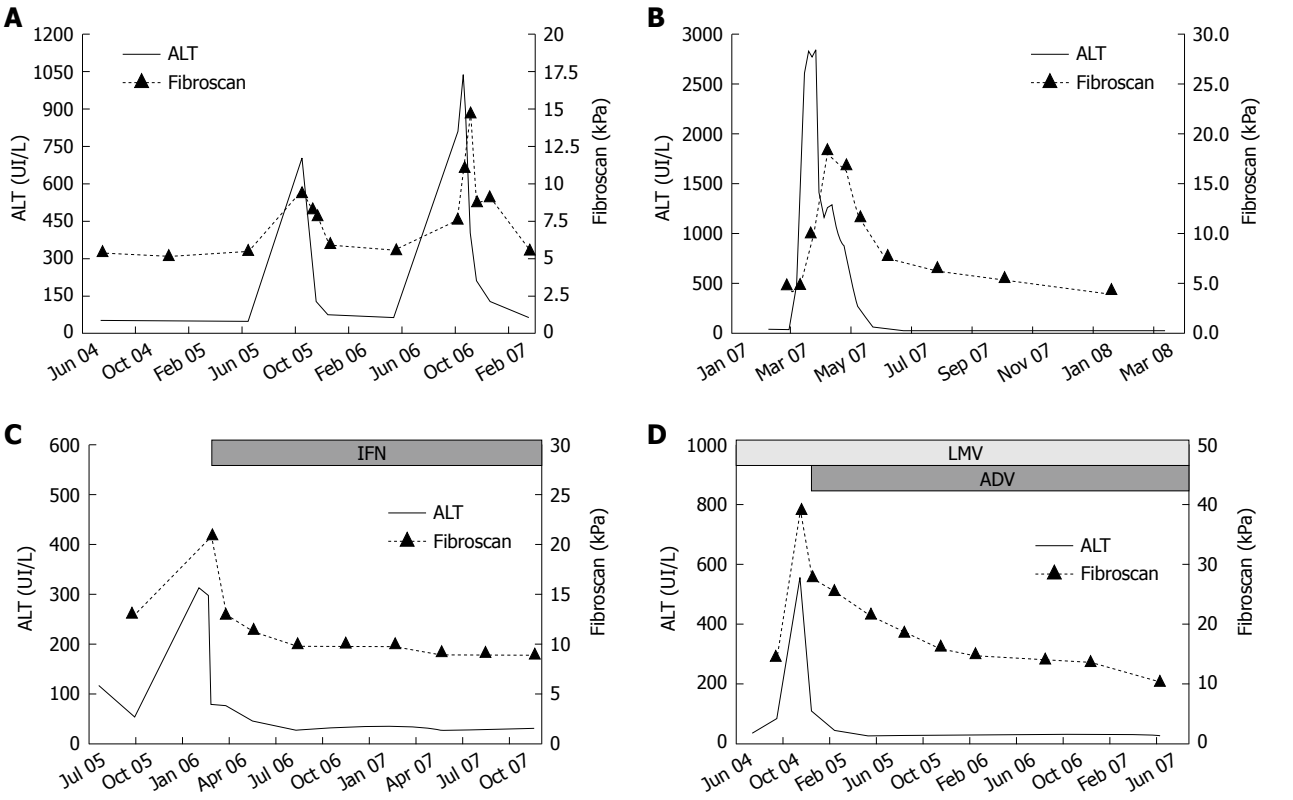

Figure 3 FS and ALT kinetics in four patients. A: CHB with S2 fibrosis stage and recurrent hepatitis flare; B: Acute hepatitis B; C: CHB with S6 fibrosis stage responding to IFN treatment; D: Cirrhosis with biochemical break-through due to lamivudine resistance and response to rescue therapy with adefovir.

12.3 ± 3.3) kPa and reached a peak of 11.8 to 45.7 kPa (20.0 ± 11.6 kPa) at the time of ALT peak. They then declined progressively to 5.6 ± 1.1 (range 4.4-6.9) kPa, in association with the reduction of ALT levels (Figure 3B).

Treated patients: The FS monitoring started with treatment in 18 patients, when treatment was already ongoing in the remaining 13. Overall, FS values showed progressive declines during therapy, with a mean on-treatment/baseline ratio of 0.9 ± 0.4 at 6 mo and 0.7 ± 0.2 at 12 mo. In patients with persistent response to long-term nucleoside/nucleotide analogues treatment, FS values decreased progressively during their follow up, with mean yearly reduction (ratio between two consecutive FS values registered at 12 mo intervals) of 0.8 ± 0.2 at 24 mo, 0.8 ± 0.1 at 36 mo and 0.7 ± 0.1 at 48 mo from the beginning of therapy.

All responders showed decreased FS values during therapy (Figure 3C and D): 0.8 ± 0.2 at 6 mo and 0.6 ± 0.2 at 12 mo, as compared to baseline values, respectively. FS value declines were similar in responders to IFN as compared to responders to NA: 0.7 ± 0.2 *vs* 0.8 ± 0.2 6 mo/baseline ratio and 0.6 ± 0.1 *vs* 0.7 ± 0.2 12 mo/baseline ratio, respectively. Two non-responder HBeAg-positive patients showed an increase of 1.1 and 2.4 times the FS values between 3 and 6 mo, during hepatitis flares, followed by a progressive decline that reached baseline values after 12 mo.

DISCUSSION

Transient elastography[1] is an easy to perform, reproducible method for the rapid and objective evaluation of LS in clinical practice[2,16] and it is proposed as a reliable, non-invasive, surrogate marker of fibrosis[3-7,17,18]. In fact, LS is a physical parameter that correlates primarily with fibrosis, but it is influenced also by other factors that modify the elasticity of the liver, such as significant variations of inflammatory infiltrate, edema and vascular congestion of the liver[8-10,19]. Accordingly, we showed that LS variations parallel ALT values during hepatitis exacerbations in the setting of both acute and chronic liver damage[8]. This evidence has important implications in clinical practice since the interpretation of the LS measure has to take into account the concurrent biochemical profile of the patient[8]. Thus, the interpretation of LS might be more difficult in the setting of CHB when major fluctuations of necrosis and inflammatory activity occur in a significant proportion of patients[11,12,18,19]. On the other hand, the availability of an easy to perform, non-invasive measure for fibrosis might improve the management of the HBV carrier. In the HBV carrier, the repeated measures of LS might help to identify the candidates for liver biopsy and to define both the phase of HBV infection and stage of liver disease that are mandatory to warrant the most appropriate treatment strategy, and to monitor liver disease progression in the single patient[20,21].

Addressing the issue of the clinical usefulness of

LS in the management of the HBV carrier we found a highly significant correlation between transient elastography and fibrosis stages ($P < 0.001$). Using 7.5 and 11.8 kPa as cut-off values for fibrosis ⩾ S3 and cirrhosis, the FS specificities were 88.5% and 96.3%, sensitivities 93.9% and 86.5%, and diagnostic accuracies 90.1% and 94.2%. These results confirm that FS is a reliable method to assess fibrosis in carriers with chronic HBV infection and disease[8].

Additional factors were independently associated with FS, such as active HBV infection ($P < 0.001$), HBV-DNA ($P = 0.042$) and ALT ($P < 0.001$) levels. In inactive HBV carriers, mean FS values were similar to normal controls and significantly lower than in CHB patients (4.3 ± 1.0 *vs* 4.6 ± 1.2 *vs* 11.2 ± 9 kPa; $P < 0.001$). These findings qualify LS as a promising tool to provide an important diagnostic assessment of the HBV carrier with inactive viral profile when the increased FS values suggest the presence of liver damage caused by factors other than HBV. In such cases, liver biopsy can be proposed for the precise characterization of liver disease. Indeed, in our study, 17 inactive carriers with metabolic liver disease had FS values higher (6.9 ± 2.3 kPa) than inactive carriers without liver disease.

In addition to the phase of HBV infection, only two other parameters, namely HBV DNA and ALT, were independently correlated with LS. Since both these parameters are linked with the extent of liver disease activity in the immune competent HBV carrier, our results further support the hypothesis that the extent of necrosis and inflammation influence LS significantly[20,22-24]. Accordingly, in the 83 untreated patients without cirrhosis, multivariate analysis showed that intra-hepatic necrosis and inflammation scores and ALT values were the only factors influencing FS ($P = 0.035$ and $P < 0.001$ respectively), in addition to the stage of liver disease.

The LS values identified as cut-offs for histological stage ⩾ S3 and cirrhosis are lower than those proposed for chronic hepatitis C. A slight variable difference between cut-offs would not be surprising when different cohorts of patients are compared, but FS values in CHB patients with cirrhosis (11.8 kPa) are consistently and persistently lower than in chronic hepatitis C (CHC) cirrhosis[4-6,8,16]. Accordingly, lower values of LS cut-offs have been proposed in preliminary reports[25,26]. These findings are consistent with the specific features of histopathology of hepatitis C, in which the combination of portal lymphoid follicles, bile duct damage, lobular activity and steatosis may contribute to the different LS, as compared to hepatitis B histopathology[27].

In this prospective study of CHB patients, we observed 1.2 to 4.4-fold increases of FS values with ALT flares, and similarly, LS values fluctuated in parallel with ALT values in nine patients with acute hepatitis. Interestingly, the extent of FS fluctuations during the hepatitis exacerbations differed according to the biochemical patterns of CHB. The range of LS variations were significantly wider in patients with ALT flares intervened by complete biochemical remissions, as compared to patients with persistent ALT elevations between flares (FS variations ranged from 1.4 to 4.4-fold in the former and 1.2 to 1.6-fold in the latter group, $P = 0.019$). Altogether these findings confirm our original observation and other more recent reports on the major influence of the biochemical profile on LS in the setting of both acute and chronic liver damage[8-10]. Finally, we found that prolonged biochemical remissions were associated with progressive reductions of FS values. LS declined yearly at about 0.2-fold in treated patients followed-up prospectively for 48 mo, and a proportion of patients who maintained evidence of cirrhosis at US achieved values of FS < 11.8 kPa. This was responsible for the worse diagnostic performance of FS in treated patients in whom the sensitivity for detecting cirrhosis fell from 86.5% to 54.2% in untreated *vs* treated patients with fibrosis ⩾ S5 (Table 6). Altogether, these data suggest a non-linear correlation between the overall kinetics of LS and histological staging during antiviral treatment. Future studies should be addressed to understand the relations among the reductions of LS, necrosis, inflammation and fibrosis in the separate settings of different fibrosis stages (i.e. ⩾ S3/< S3 and presence/absence of cirrhosis) and liver disease etiology (i.e. HBV and HCV). In fact, much of the LS changes depend on the different elastic relations among fine blocks of the liver structure. Thus, the interplay between the extent and structure of the collagen septa within the fine liver block, and the different type and extent of liver inflammatory infiltrate within them, might account for both the different FS cut-offs between CHB and CHC patients and for the different kinetics of FS and fibrosis decline during antiviral therapy.

In conclusion, our study suggests that the LS provides a useful non-invasive tool to monitor liver disease in the chronic HBV carrier. In the inactive carrier, it helps to identify non-HBV-related causes of liver damage and transient reactivation of HBV liver disease. In the CHB patient, provided that the pattern of biochemical activity is taken into account, LS values < 7.5 exclude the presence of significant fibrosis (⩾ S3) with a high NPV (97.3%) and low negative likelihood ratio (0.07). FS values ⩾ 11.8 kPa are highly specific (96.3%) for cirrhosis and show good PPV (86.5%) and positive likelihood ratio (23.18). In the HBV carrier with LS values ranging from 7.5 to 11.8 kPa, which are indicative of significant liver disease, liver biopsy remains the gold standard for an accurate grading and staging of liver disease. Finally, in CHB patients the monitoring of LS appears useful to highlight major changes in intrahepatic liver disease and warrants a more appropriate timing for control liver biopsies.

COMMENTS

Background

The old measure of liver stiffness (LS) by hand palpation has had a new appraisal after the recent introduction of the objective measure of the speed of transmission of an elastic wave across the liver (transient elastography) registered by the new instrument Fibroscan® (EchoSens, Paris, France). Originally, the new technique was proposed in clinical practice as a non-

invasive, surrogate marker of fibrosis and many studies demonstrated good reproducibility and a high correlation between LS and liver fibrosis at histology. However, liver elasticity is influenced not only by fibrosis, but also by the presence and extent of liquid, lipid and inflammatory infiltrates within the liver. The evidence that Fibroscan (FS) is significantly influenced by major variations of liver inflammation (as we previously showed), in addition to variations of staging, prompted the new frontier of testing FS values in the management of patients with chronic hepatitis.

Research frontiers

The course of liver disease in a significant proportion of chronic hepatitis B (CHB) patients is characterized by hepatitis exacerbations, intervened by prolonged remissions whose biochemical and virologic patterns can be mistaken with those of chronic inactive carriers. Thus, measuring LS might be useful to distinguish active from inactive HBV carriers. We addressed this question and present here the results of the cross-sectional and prospective studies of a large cohort of pedigreed hepatitis B virus (HBV) carriers (68 inactive carriers, 200 CHB and nine acute hepatitis B patients).

Innovations and breakthroughs

FS correlates with fibrosis in CHB patients and FS provides a reliable method to assess the overall status of liver disease in the carrier with chronic HBV infection. The mean FS values of HBV-inactive carriers were comparable to those of normal controls and significantly lower than those of CHB patients. Interestingly, in HBV inactive carriers with metabolic liver disease FS values were significantly higher than in HBV-inactive carriers without liver disease. All factors stemming for activity of liver disease, namely the phase of infection (active or inactive), HBV-DNA and ALT levels influenced LS at multivariate analysis. Accordingly, in untreated patients without cirrhosis, histological necrosis and inflammation and ALT were the only factors influencing FS in addition to fibrosis. Thus both necrosis and inflammation influence LS that qualifies as a very promising tool for the non-invasive diagnostic assessment of the liver in the HBV carrier. The best cut-off values for fibrosis and cirrhosis were significantly lower than in chronic hepatitis C (CHC) patients, studied in identical conditions (same center, instrument, operators and test timing), suggesting that FS is influenced also by the different histopathology features of CHB and CHC. This prospective study on patients with hepatitis B exacerbations confirmed 1.2 to 4.4-fold increases of FS values at the time of ALT flares. Similarly, LS paralleled ALT fluctuations in patients with acute hepatitis B. Finally, in treated patients followed up for 48 mo, LS declined yearly at about 0.2-folds, reaching values below the cirrhosis cut-off (11.8 kPa) in a proportion of patients who maintained evidence of cirrhosis. This observation may explain the worse diagnostic performance of FS in treated versus untreated patients. Altogether, these data indicate the non-linear correlation between the kinetics of LS and histological staging during antiviral treatment.

Applications

This study suggests that LS provides a useful non-invasive tool to monitor not only fibrosis, but overall liver disease in the chronic HBV carrier. In monitoring CHB patients, LS appears useful to highlight major changes of liver disease and to warrant a more appropriate timing for control liver biopsies.

Terminology

HBV-inactive carriers mean chronic HBV infection without liver damage caused by HBV, characterized by low HBV-DNA serum levels, persistently normal ALT and undetectable levels of IgM anti-HBc, a marker of HBV-induced liver disease (below the cut-off for CHB). Biochemical remission means transient ALT normalization (spontaneous or induced by antiviral treatment) in patients with CHB.

Peer review

In this study, authors perform a cross-sectional and longitudinal analysis of LS in HBV carriers, correlating this variable with stage of disease, liver inflammation and other factors that could influence FS measurements. They found a good diagnostic accuracy to detect cirrhosis and fibrosis higher than S3. The work is well performed and conclusions are correctly sustained.

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Ataxia telangiectasia-mutated-Rad3-related DNA damage checkpoint signaling pathway triggered by hepatitis B virus infection

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Abstract

AIM: To explore whether acute cellular DNA damage response is induced upon hepatitis B virus (HBV) infection and the effects of the HBV infection.

METHODS: We incubated HL7702 hepatocytes with HBV-positive serum, mimicking a natural HBV infection process. We used immunoblotting to evaluate protein expression levels in HBV-infected cells or in non-infected cells; immunofluorescence to show ATR foci ands Chk1 phosphorylation foci formation; flow cytometry to analyze the cell cycle and apoptosis; ultraviolet (UV) radiation and ionizing radiation (IR)-treated cells to mimic DNA damage; and Trypan blue staining to count the viable cells.

RESULTS: We found that HBV infection induced an increased steady state of ATR protein and increased phosphorylation of multiple downstream targets including Chk1, p53 and H2AX. In contrast to ATR and its target, the phosphorylated form of ATM at Ser-1981 and its downstream substrate Chk2 phosphorylation at Thr-68 did not visibly increase upon infection. However, the level of Mre11 and p21 were reduced beginning at 0.5 h after HBV-positive serum addition. Also, HBV infection led to transient cell cycle arrest in the S and the G2 phases without accompanying increased apoptosis. Research on cell survival changes upon radiation following HBV infection showed that survival of UV-treated host cells was greatly increased by HBV infection, owing to the reduced apoptosis. Meanwhile, survival of IR-treated host cells was reduced by HBV infection.

CONCLUSION: HBV infection activates ATR DNA damage response to replication stress and abrogates the checkpoint signaling controlled by DNA damage response.

Key words: Hepatitis B virus; DNA damage response; Cell cycle; p21; Mre11

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INTRODUCTION

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA[1-5]. There are two branches of the checkpoint response pathway, ataxia telangiectasia-mutated (ATM) pathway and ATM-Rad3-related (ATR) pathway. The major difference between ATM and ATR is the type of DNA damage to which each responds. For example, ATM responds to ionizing radiation (IR) and other agents that cause double-strand breaks (DSBs) in DNA. ATR responds to ultraviolet radiation (UV) radiation and other agents that induce the accumulation of stalled replication forks and subsequent single-stranded breaks (SSBs) in DNA. The DSBs are recognized by the Mre11-Rad50-Nbs1 complex, which recruits and activates ATM kinase[6]. The SSBs are coated

by replication protein A, and it recruits a complex of ATR kinases and ATR-interacting protein, which is then activated by the Rad9-Rad1-Hus1 complex and other factors. Accumulating evidence suggests that checkpoint signaling through ATR is intimately linked to the process of DNA replication[7,8].

A variety of checkpoint and DNA repair proteins have been identified as substrates for ATM and ATR kinases, including the checkpoint kinases Chk1 and Chk2, as well as p53, Smc1 and H2AX. Chk1 was first identified in *Schizosaccharomyces pombe* because of its role in the checkpoint arrest at G2/M, and it is mainly phosphorylated by ATR in response to UV, hydroxyurea and aphidicolin[9-11]. p21 was initially identified as a component of quaternary complex containing cdk-cyclin kinases and PCNA. Previous study has shown that p21 protein is degraded after low doses of UV; this degradation is essential for optimal DNA repair and is ATR-dependent[12]. The MRN complex consisting of Mre11, Rad50, and Nbs1 is a target of both ATM and ATR and is involved in both pathways[13-15].

Virus replication presents the host cells with large amounts of exogenous genetic material, including DNA ends and unusual structures. Therefore, infected cells recognize viral replication as a DNA damage stress and elicit DNA damage signal transduction, which ultimately induces apoptosis as part of host immune surveillance. However, recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation. For example, Epstein-Barr Virus (EBV)[16] abrogates the p53 checkpoint signaling pathway through the interaction of the BZLF1 protein and p53 to avoid apoptosis. Other viruses such as human immunodeficiency virus type 1 (HIV-1)[17-20], herpes simplex virus type 1 (HSV-1)[21,22] and human cytomegalovirus[23] can activate and exploit a cellular DNA damage response, which aids viral replication. Adenovirus blocks ATM signaling and concatemer formation through targeting the DNA repair complex of MRN for degradation and mislocalization[24,25]. Thus, under some circumstances, viruses have co-opted endogenous checkpoint regulators to ensure their own efficient replication[26,27].

Hepatitis B virus (HBV) is a partially enveloped double-stranded DNA virus with a genome of 3.2 kb. Upon infection, the viral genome is transported into the cell, where it is converted into a covalently closed circular DNA (cccDNA). The cccDNA serves as a template for transcription by host cell RNA polymerase Ⅱ. The pregenomic RNA is then reverse transcribed into DNA replicative intermediates in the cytoplasm within immature viral core particles, by the virally encoded polymerase. Integration into host chromosome may happen during its replication.

There is no evidence thus far that the ATM/ATR kinases or their downstream pathways are triggered by HBV infection. The present study was undertaken by culturing normal hepatocyte cell line HL7702 and primary hepatocytes from a healthy liver donor with HBV-positive serum, mimicking natural HBV infection. We showed here, perhaps for the first time, that HBV infection elicited acute cellular DNA damage response dependent on ATR. However, the ATR checkpoint signaling was blocked downstream of p53-dependent and p53-independent pathways to evade apoptosis.

MATERIALS AND METHODS

Chemicals

Mimosine and aphidicolin were obtained from Sigma. The stock concentration of mimosine was 100 mmol/L, the stock concentration of aphidicolin was 10 mmol/L; both were dissolved in Dimethyl Sulphoxide (DMSO).

Cell culture, synchronization and infection

The human hepatocyte cell line HL7702, which was isolated from a HBV-seronegative individual, was obtained from Shanghai Biochemistry Institute. HL7702 were cultured in RPMI-1640 with 10% heat-inactivated FBS (Gibco). Serum samples from HBV carriers were analyzed. The patient was anti-HBsAg-positive, as detected by ELISA (SIIC Ke-Hua, Shanghai), and HBV DNA in the serum sample was quantified using FQ-PCR (Da-An Gene Corp). The patient had received no antiviral therapy prior to the study and was not infected with HCV or HIV. The number of serum HBV viral particles was 7×10^9 copies/mL, as quantified by FQ-PCR. Normal serum was obtained from healthy non-infected individuals as a control. The sera were stored at -80℃ until use.

When synchronized, the HL7702 cells were cultured in RPMI-1640 containing 0.1% FBS for 2 d; the culture medium was then replaced with fresh RPMI-1640 including 10% FBS and 200 μmol/L mimosine for 24 h, in order to arrest the cells at G1-S phase. The arrested cells were then washed twice, and the culture medium was replaced with RPMI-1640 containing normal or HBV-positive serum. The cells were then harvested at different times after mimosine release. All procedures were performed under level P2 biosafety conditions to minimize the possibility of cross-contamination.

Primary culture of human hepatocytes

Hepatocytes were prepared from a 35-year-old healthy male liver donor according to previously described procedures[28]. Briefly, the liver tissues were cut with scissors into 0.1-0.5 mm^3 pieces and were shattered with a 5-mL syringe into single cells or cell aggregates. Cells were seeded into 12-well culture dishes and incubated with 1 mL of 10% FBS in RPMI-1640 at 37℃ under 50 mL/L CO_2. The medium was changed after the first 48 h with serum-free medium. The serum-free medium was composed of DMEM/F12 (1:1) and 0.01 nmol/L nicotinamide, 0.02 ng/L epidermal growth factor (EGF), 0.02 ng/L basic fibroblast growth factor (bFGF), 0.365 ng/L glutamate, B27 (1:50, Sigma), 0.1 U/L penicillin, 0.1 ng/L streptomycin, and 0.1 ng/L fluconazole. HBV-positive serum was added to the culture medium 2 wk after hepatocyte phenotype cell development, and cells were harvested for immunoblotting assay 3 h later.

Immunoblotting assay

Cell extracts were lysed in ice-cold Tris buffer (50 mmol/L, pH 7.5) containing 5 mmol/L EDTA, 300 mmol/L NaCl, 0.1% Igepal, 0.5 mmol/L NaF, 0.5 mmol/L Na_3VO_4, 0.5 mmol/L PMSF, and antiprotease mixture (Roche Molecular Biochemicals) for 30 min on ice and centrifuged at 13 000 *g* for 10 min. The supernatant protein concentration was determined by the Bradford procedure (BioRad). The proteins were resolved on 15% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked in TBST containing 5% non-fat dried milk (NFDM) and incubated with primary antibodies as follows: antibodies against p21, Mre11, ATR, (Santa Cruz) and tubulin (Sigma) were incubated at room temperature for 1 h. Antibodies against ATM phosphoserine 1981 (ATMp), Chk2 phosphothreonine 68 (Chk2p), Chk1 phosphoserine 345 (Chk1p), p53 phosphoserine 15 (p53p) and H2AX phosphoserine 139 (H2AXp) (Cell Signaling) were incubated at 4℃ overnight. Secondary antibodies were from Jackson Laboratories. Horseradish-peroxidase-based detection was performed using a chemiluminescence reagent (Amersham Biosciences), according to the manufacturer's instructions.

DNA damage sensitivity assays

For radiation sensitivity assays, cells were irradiated with the indicated doses of γ rays from a ^{137}Cs source for indicated times, or 254 nm UV light with complete medium plated in triplicate. HBV-positive serum was added to the cultures before indicated doses of UV or IR radiation. Cells were washed extensively to remove viral inputs 24 h after HBV-positive serum addition, and then treated with different dose of UV or IR radiation. After 48 h of UV and 4 d of IR treatment, cells were collected and surviving cells were counted with Trypan blue staining. The percentage survival was determined by quantization of the relative viable number of treated cells divided by the viable number of untreated cells.

Flow cytometry

For propidium iodide staining, cells were harvested by trypsinization, fixed with ice-cold 70% ethanol, and resuspended in a solution containing 50 mg/L propidium iodide, 0.1% Triton X-100, 50 mg/L RNase A, and 5 mmol/L EDTA at room temperature (RT) for 1 h. Cells were then diluted 1:1 in 1% BSA PBS for cytometric analysis.

Immunofluorescence assay

HL7702 cells were grown on glass coverslips for 12 to 18 h prior to infection. At 0.5 h post-infection, cells were washed briefly in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min, then permeabilized for 10 min in 1% Triton X-100 in PBS. For the visualization of detergent-resistant or chromatin-associated nuclear proteins, an *in situ* extraction method that removed the cytoplasm and nucleosolic proteins was used. Cells were pre-extracted for 5 min on ice with 0.5% Triton X-100 in cytoskeletal buffer as described previously[29] then fixed in 4% PFA for 10 min. After washing with PBS and blocking in 2% fetal bovine serum (FBS) in PBS, cells were incubated with primary antibodies diluted in 2% FBS overnight at 4℃. Anti-Chk1p was used at a concentration of 1:100, while anti-ATR was used at 1:400. After washing with PBS three times, cells were incubated with secondary antibodies diluted 1:200 in 2% FBS for 30 min, then washed again three times with PBS. Next, the nuclei were stained with DAPI diluted in PBS for 10 min. After a final wash in PBS, samples were preserved in glycerol and images were captured by using a Zeiss LSM510 confocal microscope.

RESULTS

HBV infection induced a cellular DNA damage response dependent on ATR

To explore whether acute cellular DNA damage response was induced upon HBV infection, we incubated HL7702 hepatocytes with HBV-positive serum to examine the phosphorylation status of DNA damage response proteins. 10^5 HL7702 monolayer cells in a 6-cm plate were infected with HBV-positive serum containing 10^6 HBV at 37℃ under 50 mL/L CO_2. Serum from uninfected individuals was used as a non-infected control. Cells were washed eight times to remove excess viral inputs before harvesting. Whole-cell lysates from the HBV-infected and non-infected cultures were examined for the status of the DNA damage response proteins. Figure 1A reveals that HBV infection induced an increase in the steady state levels of the ATR protein and in the phosphorylation levels of its downstream substrates Chk1, p53 and H2AX. An increase in Chk1 phosphorylation at Ser-345 was evident at 3 h from the start of HBV-positive serum addition, with further increase from 6 h of infection (hoi) to 48 hoi. The phosphorylation of p53 Ser-15 was elevated beginning at 24 hoi and increased greatly at 48 hoi. Finally, there was a sharp increase in the amount of phosphorylated H2AX Ser-139 beginning at 24 hoi. To confirm these results, primary hepatocytes from a healthy male liver donor were obtained and incubated with HBV-positive serum for 3 h; obvious Chk1 phosphorylation was detected in HBV-infected cells (Figure 1B). We then used immunofluorescence to examine the localization of these proteins early after infection. Figure 1C shows that ATR foci as well as the Chk1 phosphorylation foci in non-infected cells were very faint, as opposed to the HBV infected cells where the foci were larger, more numerous and much brighter.

In contrast to ATR and its targets, the phosphorylated form of ATM at Ser-1981 was not visibly increased upon infection, and phosphorylation of its downstream substrate Chk2 at Thr-68 began to decrease starting from 3 hoi (Figure 1A), with slight recovery at later time points, indicating that the p53-independent pathway was blocked. Phosphorylation of p53 at Ser-15 in response to DNA damage usually correlates with the ability of p53 to trans-activate downstream target genes. Therefore, we examined expression levels of p53 transcriptional targets $p21^{cip1/waf1}$, a cyclin-dependent kinase inhibitory protein. Figure 1A

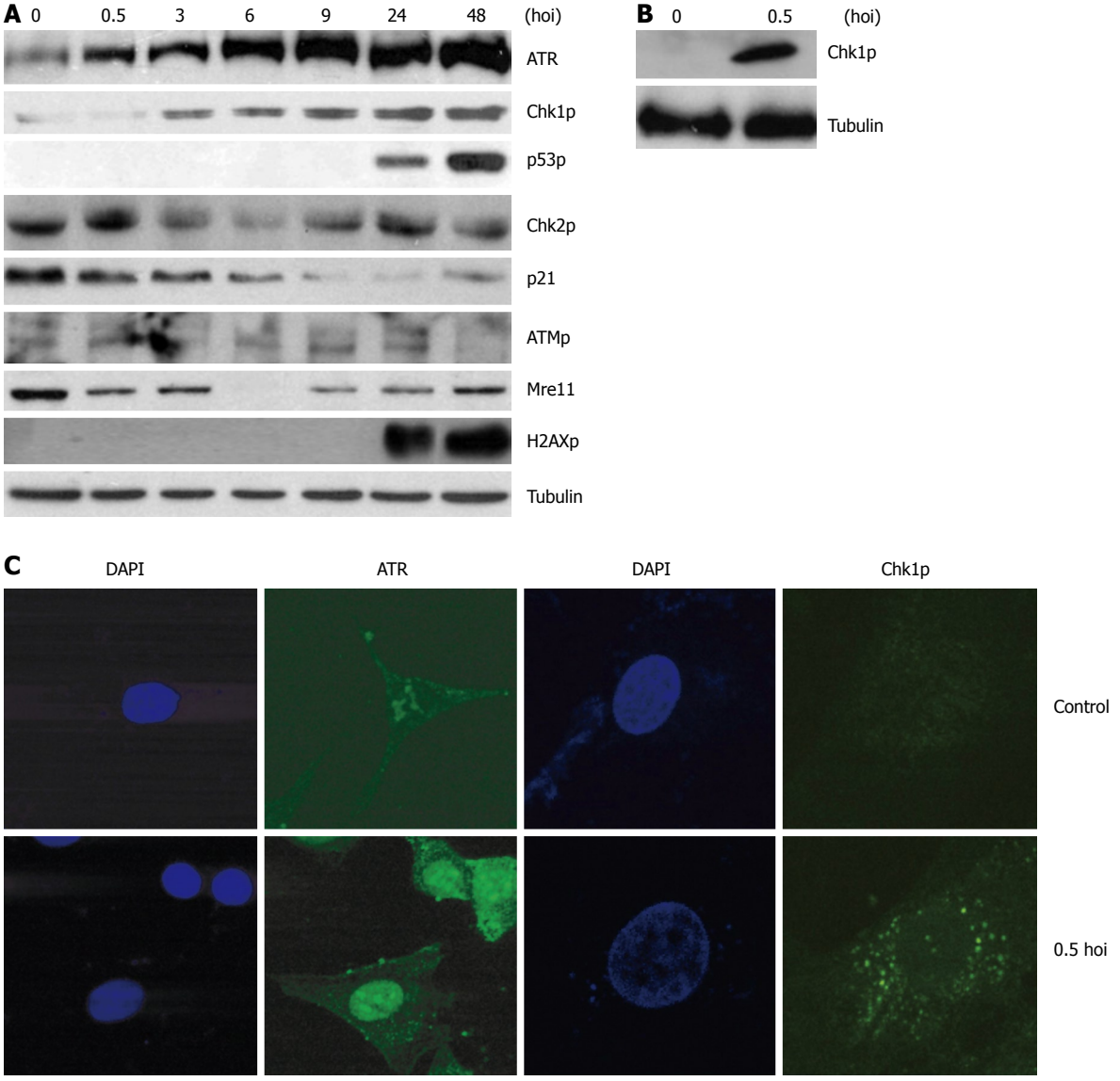

Figure 1 HBV infection activates a cellular checkpoint response dependent on ATR. A: 105 human hepatocyte HL7702 monolayer cells in a 6-cm plate were infected with 106 virus particles from HBV-positive patients at 37℃ under 50 mL/L CO_2; normal serum from healthy individuals was used as a non-infected control. Prior to cell harvesting, the cells were washed eight times thoroughly to remove excess viral input. Whole-cell lysates were prepared at various times of infection (hoi) and subjected to an immunoblotting assay by using antibodies against the indicated proteins. Tubulin was used as the equal loading control; B: Primarily cultured hepatocyte cells were prepared and were harvested 3 h after HBV-positive serum addition. Whole cell lysates were prepared and subjected to immunoblotting assay by using Chk1 phosphorylation antibody; tubulin was used as the equal loading control; C: HL7702 cells were infected with HBV-positive serum for 0.5 h. Normal serum from healthy individuals was used as a non-infected control. Immunofluorescence with antibodies to Chk1 Ser-345 and ATR (green) were monitored. DNA was stained with DAPI (blue).

shows that the amount of p21 decreased substantially with time after infection, suggesting that p53-dependent downstream signaling was blocked during HBV infection, despite the appearance of phosphorylated p53. By 0.5 h after HBV-positive serum addition, downregulation of Mre11 began to be detected. This reduction in Mre11 protein occurs shortly after HBV infection, implying that an incoming virion protein may lead to this degradation. Further investigation is required to explain this phenomenon.

Since HBV infection activated DNA damage checkpoint pathway that responded to replication stress, we asked if HBV infection would effect cell cycle progression and cell death. Cells were synchronized in G1 phase by mimosine, an inhibitor of DNA synthesis initiation, for 24 h. Arrested cells were then washed twice and media replaced with RPMI-1640 containing HBV serum or normal serum. Cells were harvested at different times after mimosine release. The results of fluorescence-activated cell sorting (FACS) analysis showed that approximately 5% of the cells were in early S phase with 95% in G1 after synchronization (Figure 2A), indicating that the synchronization was fulfilled. Control cells treated with normal serum had approximately 44.4% of cells in S phase after 12 h release from mimosine, whereas cells treated with HBV-positive serum still had 56.6% of cells in S phase (Figure 2A and B). The percentage of total cells in S phase indicates the effect of virus in extending the period of DNA synthesis. Toward the end of the cell cycle, there was an accumulation of cells

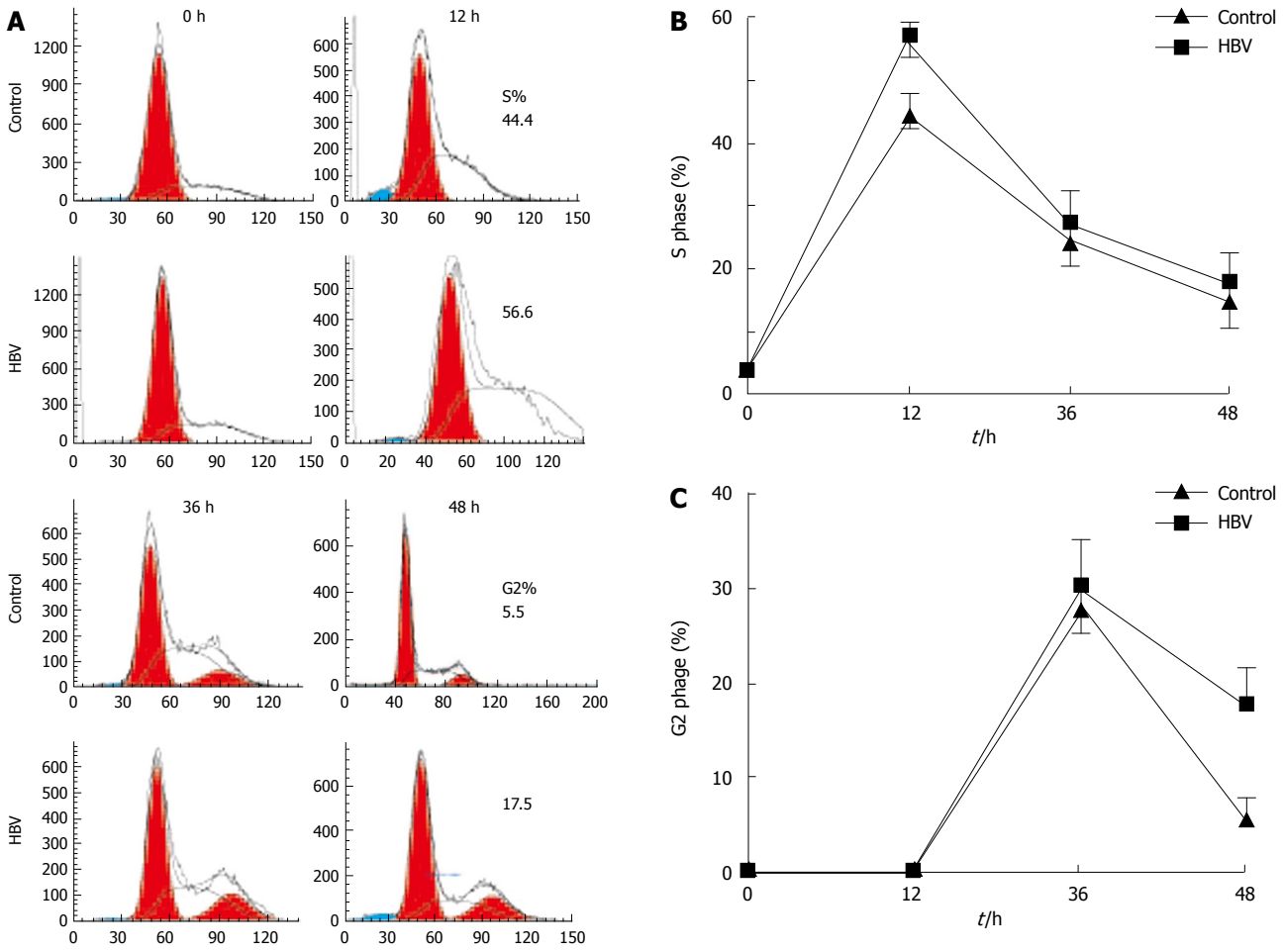

Figure 2 HBV infection caused transient cell cycle arrest in the S and the G2 phase. The duration of the S and G2 phase were measured in HBV-infected or non-infected cells. Cells were synchronized with mimosine for 24 h, and then media were replaced with RPMI-1640 containing 10% HBV serum or normal serum. At indicated times, cells were harvested and examined for cell cycle profile using propidium iodide staining and flow cytometry. A: Cell cycle profile in HBV-infected cells and in non-infected cells; B: Percentages of cells in the S phase; C: Percentages of cells in the G2 phase. Mean and standard error are presented for three independent experiments.

in G2 phase in HBV-infected cells (5.5% for control cells versus 17.5% for HBV infection cells) without the appearance of fragments with less than 2N DNA (Figure 2A and C). These findings indicate that DNA damage pathway responding to replication stress induced by HBV infection will lead to transient cell cycle arrest without accompanying increased apoptosis.

Checkpoint signaling pathway was compromised in HBV-infected cells

Previous results show that HBV infection did not show increased apoptosis even though a cellular DNA damage response dependent on ATR was activated (Figure 2A). To better understand the consequences of the ATR signaling pathway triggered by HBV infection, we examined if HBV infection had an impact on host cell survival after IR and UV radiation. Cells were washed extensively to remove viral inputs 24 h after HBV-positive serum addition and then treated with different doses of UV or IR radiation. After 48 h of UV and 4 d of IR treatment, cells were collected and surviving cells were counted with Trypan blue staining. Figure 3A and B showed that the survival rate of UV-irradiated cells was enhanced by HBV infection, while the survival rate of IR-irradiated cells was reduced by HBV infection. Figure 3C shows that while the cell survival rate after 8 s of UV or 4 Gy of IR was about 30%, the survival rate of HBV-infected cells after 8 s of UV radiation increased from 30% to 49%. By contrast, HBV infection pre-treatment with 4 Gy of IR radiation reduced the cell survival rate from 31% to 21%, suggesting that survival of UV-treated cells was enhanced by HBV infection (Figure 3C). Based on this result, we analyzed the difference in apoptosis between UV radiation only and combined treatment of UV with HBV infection. Decreased apoptosis was seen in combined treatment cells (Figure 4A and B). Twenty-four percent apoptosis was seen in UV-radiation-only cells, while about 8% apoptosis was detected in cells treated with UV radiation followed by HBV infection. It is known that cell cycle checkpoint triggered by DNA damage response induces cell apoptosis if damaged DNA cannot be repaired. The inhibitory effect of HBV on cell survival was reduced in cells treated with UV, indicating that checkpoint signaling controlled by the ATR DNA damage pathway was at least partially compromised in HBV-infected cells; therefore, the ATR signal pathway did not function completely.

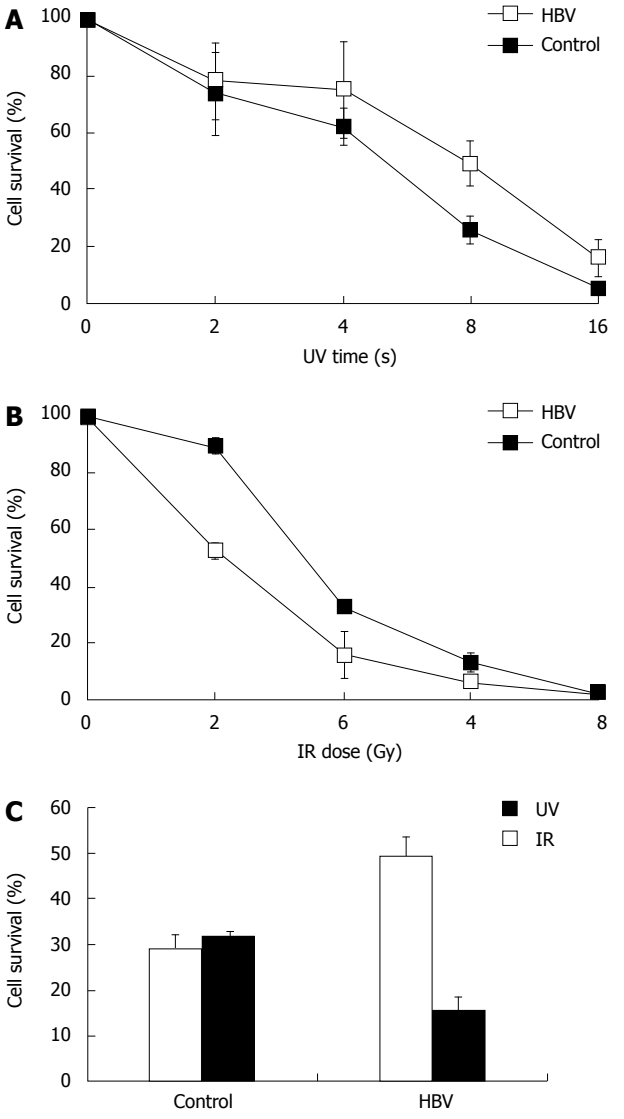

Figure 3 HBV infection hypersensitizes host cells to IR and causes hyper-resistance of host cells to UV. A: HL7702 cells were treated with indicated doses of IR followed HBV positive serum addition for 24 h, and continued in culture for another 4 d, trypan blue staining was used for viable cell counting; B: HL7702 cells were treated with indicated doses of UV followed by HBV-positive serum addition for 24 h, and continued culture for another 48 h, trypan blue staining was used for viable cell counting; C: HL7702 cells were treated with 8 s of UV or 4 Gy of IR followed HBV infection, and viable cells were counted. Percentage survival was determined by the number of treated cells normalized to untreated cells. Mean and standard error are presented for three independent experiments.

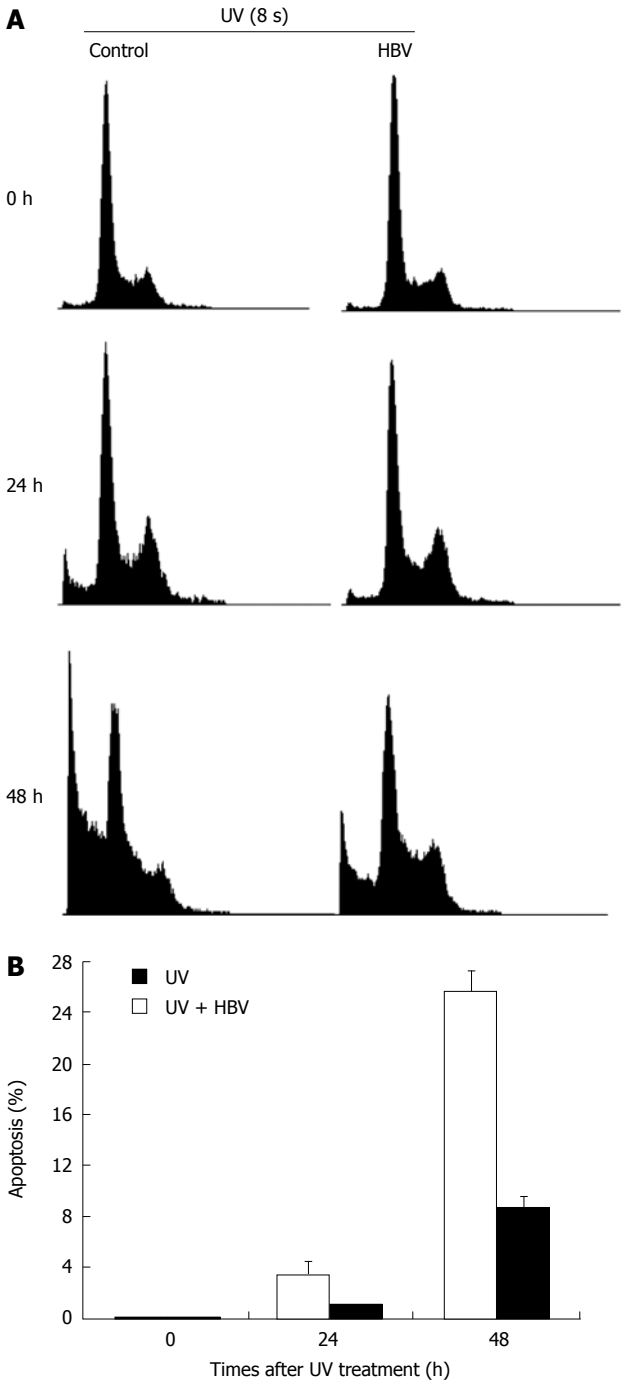

Figure 4 HBV infection followed by UV radiation led to decreased cell apoptosis. A: HBV-positive serum was added to the culture medium before UV radiation treatment. Cells were washed extensively 24 h after HBV-positive serum addition and then treated with 8 s of UV radiation. Cell cycle profile was examined by propidium iodide staining and flow cytometry at indicated times after UV treatment; B: Apoptosis percentage after UV radiation followed HBV infection. Mean and standard error are presented for three independent experiments.

DISCUSSION

The induction of cell cycle checkpoints and activation of the ATM/ATR-dependent pathway have been reported to accompany infection by a number of different viruses. Recent reports have shown that viruses have evolved a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation. In this study, HL7702 cells and primarily cultured hepatocytes were inoculated with HBV-positive serum (10^6 particles per 10^5 cells), mimicking the HBV infection process. Serum from healthy individuals was used as a non-infected control. We propose that HBV infection induces acute cellular DNA damage response dependent on ATR, as demonstrated by ATR protein and increased phosphorylation of Chk1, p53 and H2AX. Since Chk1 phosphorylation, ATR foci formation, and Mre11 and p21 degradation happened shortly after HBV-positive serum addition, we propose that incoming virion protein and genetic materials triggered this response. p53 and

H2AX phosphorylation did not begin to accumulate until 24 h after HBV serum addition, implying HBV replication inside the infected cells may be responsible for these phenomena. Interesting questions are raised and need further investigation.

Although ATM/ATR kinase phosphorylates Chk2 at Thr-68 and p53 at Ser-15, the ATR kinase predominantly targets Chk1 at Ser-345, leading to increased Chk1 activity. Our results indicate that HBV infection preferentially activates ATR DNA damage response signaling, as is the case with human adeno-associated-virus type 2[30-36].

As a latent virus, HBV abrogates checkpoint signaling controlled by ATR, to prevent triggering of signals for apoptosis in multiple ways. The mechanism of regulation of apoptosis by HBV was *via* both p53-dependent and p53-independent pathways. p53-dependent cell cycle checkpoint features p21-mediated inactivation of cdk2/cyclinE; HBV abrogates p53 dependent checkpoint activation by p21 degradation. Chk2 inhibition inhibits cdk2/cyclin E activity by phosphorylation of cdk2 at Tyr-15 in a p53-independent fashion, and the virus-decreased phosphorylation of Chk2 by Mre11 degradation inhibits the p53-independent DNA damage signaling pathway. It is known that ATM and MRN complex function in a common pathway, and the MRN complex can function to activate ATM kinase activity, so degradation of Mre11 protein by virus would inhibit ATM kinase activity, and thus affect phosphorylation of its downstream target Chk2. Therefore, like adenovirus[24,25], HBV appears to have evolved double check mechanisms to block cell cycle checkpoint signaling pathways. Consistent with this, HBV has been reported to express an additional anti-apoptotic gene HBV X that mislocates p53 to the cytoplasm, in order to evade host cellular DNA damage response and modulate apoptosis. HBX has also been reported to sensitize liver cells to environmental carcinogens, including diethylnitrosamine and aflatoxin B and UV. We observed that HBV virus enhanced cell survival upon UV radiation but hypersensitized host cells to IR; this discrepancy may be due to the complex interactions between the virus as a whole and DNA repair machinery, indicating that the virus mainly blocks cellular signaling checkpoint dependent on ATR. The risk of acquiring mutations would be enhanced by compromised cellular DNA repair caused by HBV infection. Accordingly, exposure to other environmental risk factors should act synergistically to favor the carcinogenesis process[37-39].

In summary, HBV induces cellular DNA damage response dependent on ATR, but escapes the consequences of activation of the DNA damage checkpoint by degradation of checkpoint proteins at different levels. The implication of this is that with time, persistent HBV infection may lead to the accumulation of a variety of mutations which would ultimately give rise to hepatocellular carcinoma.

COMMENTS

Background

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA. There are two branches of the checkpoint response pathway, ataxia telangiectasia-mutated (ATM) pathway and ATM-Rad3-related (ATR) pathway. Virus replication presents the host cells with large amounts of exogenous genetic material, including DNA ends and unusual structures. Thus, infected cells recognize viral replication as a DNA damage stress and elicit DNA damage signal transduction, which ultimately induces apoptosis as part of host immune surveillance. However, recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation.

Research frontiers

Epstein-Barr virus (EBV) abrogates p53 checkpoint signaling pathway through the interaction of the BZLF1 protein and p53 to avoid apoptosis. Other viruses such as human immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1) and human cytomegalovirus can activate and exploit a cellular DNA damage response, which aids viral replication. Adenovirus blocks ATM signaling and concatemer formation through targeting the DNA repair complex of MRN for degradation and mislocalization. Thus, under some circumstances, viruses have co-opted endogenous checkpoint regulators to ensure their own efficient replication.

Innovations and breakthroughs

There was no evidence indicating that the ATM/ATR kinase or their downstream pathways were triggered by hepatitis B virus (HBV) infection. The present study was undertaken by culturing normal hepatocyte cell line HL7702 and primary hepatocytes from a healthy liver donor with HBV-positive serum, mimicking natural HBV infection. We showed, perhaps for the first time, that HBV infection elicited acute cellular DNA damage response dependent on ATR. However, the ATR checkpoint signaling was blocked downstream of p53-dependent and p53-independent pathways to evade apoptosis.

Applications

Since DNA damage response is an acute response that happens quickly after virus infection, we assume that early intervention of the DNA damage pathway will function more efficiently and can be used clinically as HBV infection therapy during its early infectious stage or fulminant HBV infection.

Peer review

In this interesting study the authors investigated whether exposure to HBV infection will upregulate DNA damage checkpoint signaling pathways. They show that ATM-Rad3 is upregulated as well as several downstream targets.

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CLINICAL RESEARCH

Does an association exist between chronic pancreatitis and liver cirrhosis in alcoholic subjects?

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Abstract

AIM: To study the possible association between chronic pancreatitis (CP) and liver cirrhosis (LC) of alcoholic etiology, after excluding any other causes.

METHODS: One hundred and forty consecutive alcoholic patients were subdivided into three groups: CP (n = 53), LC (n = 57), and asymptomatic alcoholic (n = 30). Clinical, biochemical and morphological characteristics, Child-Pugh index, indocyanine green test, and fecal pancreatic elastase-1 test were assessed.

RESULTS: In patients with cirrhosis, major clinical manifestations of CP such as pancreatic pain and steatorrhea, as well as imaging alterations of CP such as calcifications, duct dilation and pseudocysts were absent; insulin-dependent diabetes was present in 5.3% of cases, and elastase-1 test was altered in only 7%, and severely altered in none. In patients with CP, clinical characteristics of cirrhosis such as ascites, encephalopathy and gastrointestinal hemorrhage were present in one case, Child-Pugh grade > A in 5.7%, and altered indocyanine green test in 1.9% cases. In asymptomatic alcoholism, there was only a non-coincident alteration of elastase-1 test and indocyanine test in 14.8% and 10%, respectively, but other characteristics of cirrhosis or CP were absent. An inverse correlation (r = -0.746) between elastase-1 test and indocyanine test was found in alcoholic patients.

CONCLUSION: There is a scarce coincidence in clinical and morphological alterations among patients with CP or LC of alcoholic etiology, but an inverse correlation between pancreatic and liver function tests. These findings support that these alcoholic diseases evolve in a different manner and have different etiopathogenesis.

Key words: Alcoholic chronic pancreatitis; Alcoholic liver cirrhosis; Alcoholism; Pancreatic function; Hepatic function

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INTRODUCTION

Chronic alcoholism is a well-known etiologic factor associated with chronic and irreversible pancreatic and liver disorders. Excessive alcohol consumption is the most frequent cause of chronic pancreatitis (CP) in Western countries[1]. There is a correlation between increased ethanol consumption over many years and the risk of developing CP[2,3]. Nevertheless, it has recently been estimated that < 5% of alcoholic subjects develop CP[1,4,5]. This low percentage, together with the absence of adequate experimental models of alcoholic CP, suggest that ethanol is only a cofactor in the development of CP, and therefore other predisposing

factors may be involved[6].

An excessive consumption of alcohol is also associated with liver cirrhosis (LC), again with a correlation between increased ethanol consumption and the risk of LC[7,8]. The development of LC also requires chronic alcoholism over several years[8,9]. For decades, it was considered that around 10%-35% of subjects with chronic alcoholism developed alcoholic LC[10]. However, more recent prospective studies have shown that the prevalence in alcoholics is indeed much lower, around 2%[7,8], once having excluded the confusing cases of hepatitis B or C[11,12]. Experimental models of chronic alcoholism have not provided insights into the pathophysiological mechanisms responsible for the different outcome in CP and LC[13].

There is still controversy about the frequency of coincidence between CP and LC in alcoholic patients. This lack of coincidence may rely on differences in methodology, retrospective *versus* prospective studies[14], and evaluated parameters such as clinical[15], functional[16], imaging[17] or histopathological[18,19]. In addition, there are diagnostic difficulties in the initial stages[20], as well as several confounders hindering this question, such as the presence of hepatitis B or C virus[14,19], and age-related pancreatic alterations[21,22].

It is worth noting that pancreatic function is often increased in patients with alcoholic LC, leading to the hypersecretory status of the pancreas[16,23]. Thus, Hayakawa *et al*[24] have reported that pancreatic secretion, measured with the pancreozymine-secretine test, increases with severity of liver damage in alcoholic liver disease. It has been suggested that this hypersecretory status diminishes protein and calcium concentration in pancreatic juice, protecting against formation of protein plugs and pancreatic stones[16].

In clinical practice, the coincidence of both diseases, LC and CP, is rare[15,24]. Furthermore, these two diseases do not share risk factors apart from alcohol consumption[15]. In alcoholic CP, the duration of chronic alcoholism is shorter than in LC, and liver disease is found at older ages[14]. Nakamura *et al*[15] have recently assessed the genotypes of alcohol dehydrogenase (ADH), ductal anatomy by endoscopic retrograde cholangiopancreatography (ERCP) and the Child-Pugh classification in Japanese alcoholics, and reported a lack of association between the risk of LC and CP. In addition, no clear genetic predisposition has been found so far in studies of genetic polymorphisms in alcoholic CP[25] and alcoholic LC[26].

To the best of our knowledge, there are no previous studies comparing clinical, functional, morphological and biochemical parameters in alcoholic subjects with a definitive diagnosis of CP or LC. In this study, we therefore aimed to assess the association of CP and LC, both with definitive diagnosis, in subjects with chronic alcoholism, based on clinical, functional and morphological parameters.

MATERIALS AND METHODS

Case-control study with two groups of consecutive patients who attended the University Clinic Hospital of Valencia, Spain for 3 years included: (1) alcoholic CP (n = 53); and (2) alcoholic LC (n = 57). Controls were 30 asymptomatic alcoholic (ASA) patients. The diagnosis of CP was based on the Cambridge and Marseille criteria[27,28]. The diagnosis of cirrhosis was histologically proven in 27 patients and based on compatible clinical, laboratory and ultrasonographic finding in the others[29]. Daily ethanol intake was over 40 g for more than 5 years in alcoholic subjects[7,8] and before entering the study a period of 60 d of alcohol abstinence was required. Exclusion criteria for alcoholic LC were the presence of antigens or antibodies against B or C virus, as well as liver diseases not related to chronic alcoholism such as hemochromatosis or any other causes. Exclusion criteria for CP were the presence of toxic-metabolic (other than alcohol), genetic, autoimmune, and obstructive factors considered as confounders[6]. Other exclusion criteria for the study were gastric or liver surgery, pancreatic surgery prior to diagnosis, and gastrointestinal neoplasia. Smoking was considered when more than 10 cigarettes were consumed daily[30,31].

Characteristic manifestations of LC[13] were assessed in all patients, including hemorrhage in the digestive tract secondary to portal hypertension, ascites, hepatic encephalopathy, alterations of the Quick index, and Child-Pugh index (PI)[32]. PI was classified according to the following scoring system: A (5-6 score), B (7-9 score), and C (10-15 score)[32]. The following characteristic manifestations of CP[27,28] were also assessed in all patients: pancreatic pain, non-insulin-dependent diabetes mellitus (NIDDM), insulin-dependent diabetes mellitus (IDDM), and chronic diarrhea/steatorrhea. Liver function was assessed by indocyanine green clearance test (ICG test). Pancreatic function was assessed by determination of fecal pancreatic elastase-1 test (E1 test) and oral glucose tolerance test (OGTT). The presence of esophageal varices, splenomegaly, portal hypertension, pancreatic calcifications, pseudocysts or alterations of the pancreatic duct were assessed by ultrasonography (US), computer tomography (CT), magnetic resonance cholangiopancreatography (MRCP), or ERCP. Splenomegaly was considered when the size of the spleen was $\geqslant$ 135 mm. Portal hypertension was established according to the following criteria: Portal vein diameter > 12 mm and/or presence of esophageal varices and/or splenomegaly. Alterations in the Quick index were established when the international normalized ratio (INR) was higher than 1.2[33].

The ICG test was performed by pulsidensitometry (Limon PC5000, Pusion Medical Systems, Munich RFA). Briefly, ICG was intravenously administered at a dose of 0.5 mg/kg, monitoring the blood concentration of ICG to determine the plasma disappearance rate (PDR, %/min). It was considered that the ICG clearance was altered when PDR was < 15%, and severely altered when PDR was < 10%[34,35].

Pancreatic fecal elastase-E1 activity was determined by ELISA (Pancreatic Elastase stool test, Schebo-Biotech, Giessen RFA). E1 activity was considered altered when it was < 200 μg/g, and severely altered when it was <

Table 1 General characteristic of patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Age at the beginning of the study	51.8 ± 9.7	56.1 ± 9.9	49.9 ± 7.9	CP *vs* LC, CP *vs* ASA, NS; LC *vs* ASA, P = 0.010
Men (%)	98	84	70	CP *vs* LC, P = 0.011; CP *vs* ASA, P = 0.001; LC *vs* ASA, NS
Age at diagnosis	39 ± 9.2	51.5 ± 9.6	-	P = 0.001
Alcohol intake (g/d)	120 ± (75-250)	118 (70-255)	121 (70-400)	NS
Years of alcoholism	20 (10-35)	25 (10-52)	18.5 (9-36)	CP *vs* LC, P = 0.01; LC *vs* ASA, P = 0.006; CP *vs* ASA, NS
Ethanol, kg of total intake	876 (273-2920)	1095 (481-3832)	965 (229-3358)	NS
Smoking	94.3%	80.7%	96.7%	CP *vs* LC, P = 0.030; CP *vs* ASA, NS; LC *vs* ASA, P = 0.035
BMI	23.4 ± 3.6	26.5 ± 4.4	24.8 ± 4.6	CP *vs* LC, P = 0.001

Data are expressed as mean ± SD, percentage or median (minimum-maximum). NS: Not significant.

100 μg/g[36] in at least two consecutive determinations. Fecal fat was determined by the van de Kamer method[37].

The body mass index (BMI) was measured in all subjects as index of nutritional status. BMI values < 20 were considered malnutrition, whereas values > 28 were considered as indicative of being overweight[38].

Statistical analysis

Proportions for qualitative variables and mean ± SD for quantitative variables were calculated. Means were compared by t test or ANOVA and post-hoc Scheffe test. According to the Kolmogorov-Smirnov test, when quantitative variables did not fit a normal distribution, medians and ranges and consequently Kruskal-Wallis test were used. Qualitative variables were analyzed by χ^2 test or Fisher's exact test when appropriate. Odds ratios (ORs) with 95% confidence intervals (95% CI) were also calculated. Spearman's rank correlation coefficient between E1 test and indocyanine green test (ICG test) or PI was calculated. $P < 0.05$ was considered statistically significant.

RESULTS

General characteristics of patients: Alcohol consumption, smoking, and nutritional status

Table 1 shows the general characteristics of each group of subjects. Although the age of patients when entering the study was not different between CP and LC, the age at the moment of diagnosis was significantly lower in CP than LC patients. Most patients were men in all groups, especially in the CP group.

Regarding the alcohol consumption, the daily alcohol intake did not differ among groups. The percentages of heavy drinkers (daily consumption > 150 g) were 30.2%, 33.3%, and 45.0% for CP, LC and ASA groups, respectively, without significant differences among the groups. The duration of alcohol consumption was lower in CP than in LC patients. However, there was no significant difference between CP and LC in the percentage of patients with more than 20 years of alcohol consumption (41.6% and 52.6% for CP and LC subjects, respectively). The percentage of patients with more than 20 years of alcohol consumption was significantly higher in LC than in ASA patients (52.6% *vs* 33.7%). The total amount of ethanol consumed before diagnosis did not differ among groups (Table 1).

Table 2 Potential risk factors for CP or LC: Distribution of frequencies and OR in patients with ACP or ALC n (%)

	ACP 53	ALC 57	OR (95% CI)	P
Gender (female)	1 (1.9)	9 (15.8)	9.7 (1.1-79.8)	0.017
BMI > 28	4 (7.5)	15 (26.8)	4.5 (1.4-14.5)	0.008
BMI < 20	7 (13.2)	4 (7)	0.49 (0.13-1.8)	0.280
Age > 45 yr at diagnosis	14 (26.4)	41 (71.9)	7.1 (3.1-16.6)	0.001
Smoking	50 (94.3)	46 (80.7)	0.25 (0.06-0.95)	0.044
Alcohol > 150 g/d	16 (30.2)	19 (33.3)	1.12 (0.5-2.5)	0.775
Years alcoholism > 20	22 (41.5)	30 (52.6)	1.56 (0.73-3.32)	0.243

The percentage of cigarette smokers was slightly higher in CP than in LC patients. The BMI was higher in LC than in CP patients. The percentage of patients with overweight (BMI > 28) was also higher in LC than in CP group, but it was no different when compared to ASA patients (7.5%, 26.8% and 9% for CP, LC and ASA subjects, respectively) (Table 2). The percentage of patients with malnutrition (BMI < 20) was low in all groups, without significant differences among them (13.3%, 7.0% and 13.3% for CP, LC and ASA subjects, respectively).

Features of alcoholic CP in CP, LC and ASA subjects

It is worth noting that there was absence of the two major clinical manifestations of CP, pancreatic pain and diarrhea/steatorrhea in patients with LC (Table 3). IDDM was frequent in CP (35 cases, 68%), but was found only in three LC cases (5.3%) and in none with ASA. Among these three LC patients with IDDM, one exhibited reduced E1 test (164 μg/g) as the unique altered pancreatic parameter. However, the percentage of patients with NIDDM was higher in the LC group (19.3%) than in the CP group. Diarrhea/steatorrhea was present in many patients with CP (54.7%), but absent in the other two groups (Table 3).

The major morphological alterations such as calcification, dilatation of the pancreatic duct, and pseudocysts were found only in CP patients.

Features of alcoholic LC in CP, LC and ASA subjects

Clinical manifestations characteristic of LC such as ascites, encephalopathy, or upper gastrointestinal

Table 3 Clinical manifestations, functional parameters and imaging characteristics of CP in patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Pancreatic pain	85%	0%	0%	< 0.001
IDDM	68%	5.3%	0%	< 0.001
NIDDM	5.7%	19.3%	10%	CP *vs* LC, P = 0.03; CP *vs* ASA, LC *vs* ASA, NS
Diarrhoea/ steatorrhoea	54.7%	0%	0%	< 0.001
Calcifications	77.4%	0%	0%	< 0.001
Dilatation of pancreatic duct	67.9%	0%	0%	< 0.001
Pancreatic pseudocysts	58.5%	0%	0%	< 0.001

NS: Not significant.

Table 4 Clinical manifestations, functional parameters and imaging characteristics of LC in patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Ascites	0%	66.7%	0%	
Haemorrhage in upper digestive tract	1.9%	45.6%	0%	< 0.001
Child-Pugh index > 5	5.7%	98.2%	0%	< 0.001
INR > 1.2	0%	73.7%	0%	< 0.001
Esophageal varices	1.9%	77.2%	0%	< 0.001
Splenomegaly	1.9%	77.2%	0%	< 0.001
Portal hypertension	3.8%	96.5%	0%	< 0.001

hemorrhage were found in most patients with LC, in one case (1/53) with CP and in none with ASA (Table 4). This particular case with CP exhibited gastrointestinal hemorrhage and portal vein thrombosis secondary to pancreopathy, splenomegaly, a PI of 6, with serum albumin level of 2.9 g/dL, severe steatorrhea, severe diabetes mellitus and malnutrition. Nevertheless, his ICG test was normal (23.8%/min).

The PI was > 5 in almost all cases (56/57) with LC, in three (3/53) with CP and in none (0/30) with ASA (Table 4). According to Child-Pugh classification, 35% (20/57) of LC patients were ascribed to class A, 35% (20/57) belonged to class B, and 30% (17/57) belonged to class C. Three cases of CP had a PI of 6. One was the previously described patient with gastrointestinal hemorrhage. In another case, a liver biopsy was available and ruled out the diagnosis of cirrhosis; however, the patient had an elevated Quick index (1.5 INR) at the moment of inclusion which could be attributed to severe steatorrhea because it normalized after parenteral treatment with vitamin K. The third CP case had a CPI of 6, and at the moment of inclusion exhibited cholestasis (total serum bilirubin = 2.6 mg/dL) due to compression of the common bile duct secondary to pancreopathy. This patient had a normal ICG test (28.9% min) and did not exhibit signs of hepatopathy.

Esophageal varices were found in 77% of LC patients, in one case (1/53) with CP and in none with ASA (Table 4). In this CP patient, there were no manifestations of hepatopathy, and varices were secondary to pancreopathy involving portal vein thrombosis. This patient had a large pseudocyst (50 mm × 50 mm) in the head of the pancreas, which caused obstructive jaundice that required surgical treatment, but the ICG test later at the moment of inclusion was normal (PDR = 28.9%/min).

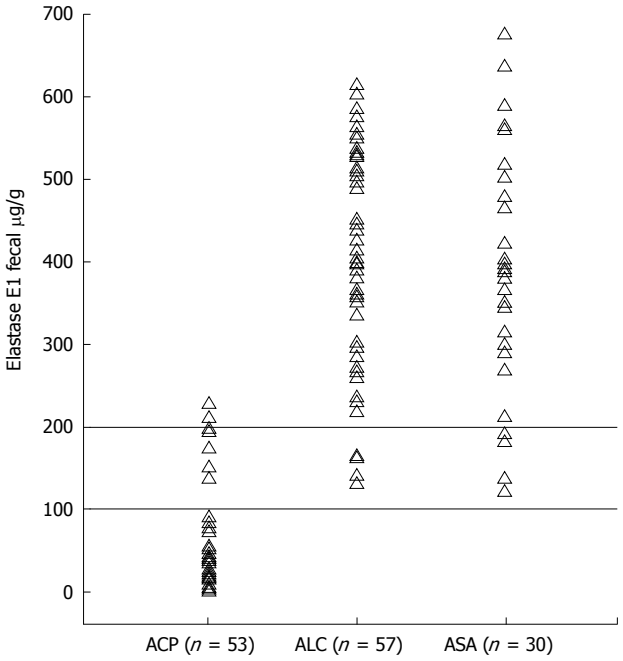

Figure 1 Fecal E1 test (μg/g feces) in patients with ACP, ALC or ASA. Reference lines for altered E1: < 200 μg/g, and severely altered E1: < 100 μg/g. ACP *vs* ALC, P < 0.001; ACP *vs* ASA, P < 0.001; ALC *vs* ASA, P = 0.747.

Splenomegaly was also found in 72.2% of LC patients, in one case with CP and in none with ASA (Table 4). This CP case was the one with upper gastrointestinal hemorrhage, which has been previously described.

Portal hypertension was present in almost all LC patients (96.5%), in two patients with CP and in none with ASA (Table 4). In these two CP cases, portal hypertension was secondary to pancreatic disease, as previously described, with normal ICG test.

Pancreatic fecal elastase E1 in CP, LC and ASA subjects

Fecal E1 test was significantly lower (P < 0.001) in CP patients [9 (0.1-228) μg/g feces] than in LC patients [400.0 (130-614) μg /g feces] and ASA subjects [388 (121-675) μg/g feces] (Figure 1). Moreover, 94.3% of CP patients (50/53) exhibited E1 test lower than the normal limit of 200 μg/g feces, whereas 84.9% (45/53) had very low levels (i.e. < 100 μg/g feces), and among them 29, (64.4%) with diarrhea/steatorrhea. All these percentages were markedly higher (P < 0.001) than those in the LC and ASA groups.

In the LC group, E1 test was < 200 μg/g only in four of 57 cases (7%), and in none of them was it < 100 μg/g. As mentioned before, none of the LC patients exhibited diarrhea/steatorrhea or pancreatic alterations assessed

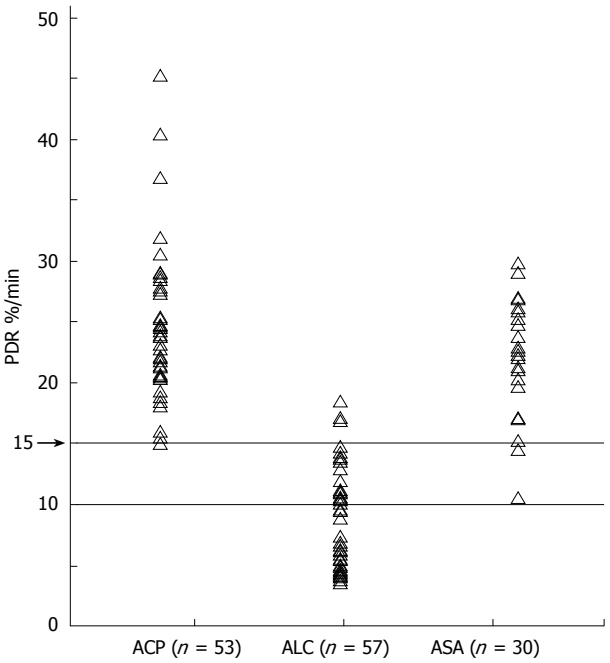

Figure 2 PDR, %/min of ICG in patients with ACP, ALC or ASA. Reference lines for altered PDR: < 15%/min, and severely altered PDR: < 10%/min. ACP *vs* ALC, $P < 0.001$; ACP *vs* ASA, $P = 0.048$; ALC *vs* ASA, $P < 0.001$.

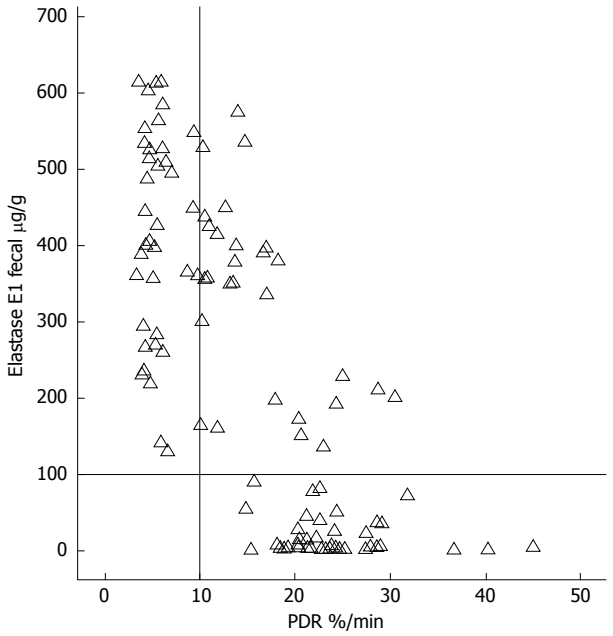

Figure 3 An inverse correlation ($r = -0.746$, $P < 0.001$) between indocyanine clearance (PDR, %/min) and fecal E1 test (μg/g feces) in patients with ACP, ALC or ASA. Reference lines: PDR (%/min) = 15; E1 test = 100. Vertical line for PDR (%/min) = 10, and horizontal line for E1 test = 100 are marked in the figure.

by imaging studies.

Among the 27 ASA subjects, E1 test was reduced only in four (14.8%), but it was never < 100 μg/g. As mentioned, none of the ASA subjects exhibited diarrhea/steatorrhea or pancreatic alterations assessed by imaging studies.

ICG clearance test in CP, LC and ASA subjects

PDR values of the ICG test were markedly more reduced in LC patients [6.1 (3.4-18.2)%/min] than in CP patients [22.6 (14.8-45)%/min] and ASA subjects [22.1 (10.4-30.2)%/min] (Figure 2). Ninety-three percent of LC patients (53/57) had a PDR lower than the normal limit (< 15%/min), and 64% of them exhibited a severe PDR reduction (< 10%/min). However, only one patient with CP (1.9%) showed PDR < 15%/min and none had PDR < 10%/min.

In the ASA group, three of 29 subjects (10%) had reduced PDR (< 15%/min) and none had PDR < 10%/min. These ASA subjects had a normal E1 test, and had no pancreatic or hepatic alterations assessed by imaging studies compatible with CP or LC; all had PI of 5.

It is worth noting that a significant inverse correlation ($r = -0.746$; $P < 0.001$) was found between E1 test levels and PDR values in patients with CP or LC (Figure 3). A significant correlation was also found between E1 test levels and PI ($r = 0.759$; $P < 0.001$). Therefore, in general, the reduction of functional hepatic reserve was associated with normal pancreatic function, and vice versa.

The analysis of potential risks of developing CP or LC showed significantly different values for gender, overweight (BMI > 28), age > 45 years at the time of diagnosis, and smoking (Table 2). Nevertheless, these differences were too low to explain the different evolution towards CP or LC. No significant differences were found regarding malnutrition (BMI < 20), elevated alcohol consumption (> 150 g/d) or long-term chronic alcohol consumption (> 20 years).

DISCUSSION

Chronic ethanol consumption is a common and frequent etiological factor in both CP and LC, and their risk correlates very well with the amount of ethanol daily consumed[2,7]. However, only a minority of alcoholics develop these diseases[1,4,5,7,8]; this together with the requirement of several years of ethanol intake, racial factors[39], and the difficulty of developing adequate experimental models have led to the consideration of other associated etiological factors[26].

The presence of a common etiological factor, i.e. alcoholism, for CP and LC could lead to the assumption that both diseases may coincide in some patients. The study of the presence and coincidence of these two diseases and their risk factors may provide new insights into their etiopathogenesis. Nevertheless, there is controversy about the frequencies of the coincidence between CP and LC in the studies published so far[15-19]. Differences in methodology, and particularly, the presence of hepatitis B or C virus are important confounders which hinder an accurate and reliable interpretation of the data. In fact, more than one-third of patients with alcoholic LC exhibit antibodies against hepatitis C virus[11,12].

In the present study, the frequency of the association between alcoholic CP and LC has been assessed after exclusion of main confounders (hepatitis B or C virus,

non-alcoholic liver or pancreatic diseases, or surgery) and taken into account the major clinical manifestations, the morphological alterations detected by imaging studies, and the alterations of pancreatic and hepatic functions assessed by specific tests.

Our results clearly showed no overlapping between patients with alcoholic CP and alcoholic LC, not only regarding clinical manifestations, but also in imaging studies and functional tests. Furthermore, an inverse relationship was found between pancreatic and liver function in patients with CP or LC.

In our clinical study, the median age of patients at the moment of diagnosis was around a decade higher in LC than in CP (Table 1), due to the different natural history of these diseases[1,2,6,40]. Nevertheless, the age of patients at the time of inclusion in the study was not significantly different. As previously reported in other studies, the percentage of men was higher than that of women in all groups of alcoholics[21,41].

Regarding ethanol consumption, only the duration of ethanol intake was different among groups (20, 25 and 18.5 years for CP, LC and ASA, respectively) (Table 1). However, the mean daily ethanol intake and the total amount of ethanol consumed before diagnosis were not significantly different among groups, which is in agreement with those reported by other authors[2,8,19,42]. Moreover, the percentage of patients with ethanol intake > 150 g/d and the percentage of drinkers for more than 20 years were not significantly different. Therefore, the populations of the three groups in this study were homogeneous regarding ethanol intake.

The percentage of smokers was higher in CP (94.3%) and ASA (96.7%) than in LC (80.7%) (Table 1). Previous studies have reported that smoking is a risk factor for CP in alcoholics, which promotes the progression of the disease[31,43-45]. In addition, smoking has also been considered a risk factor in LC induced by hepatitis C virus[46]. The present study demonstrates that smoking is a risk factor not only for alcoholic CP but also for LC.

The BMI was slightly but significantly lower in CP than in LC, but not when compared with ASA. This slight difference may be ascribed to the presence of steatorrhea and diabetes mellitus in CP. In addition, the percentage of patients with BMI > 28 was significantly higher in LC than in CP and ASA, and these differences may by ascribed to retention of liquids in advanced LC.

The diagnosis of alcoholic CP is hindered in the initial stages of the disease due to the lack of specificity of clinical manifestations. Because of the difficulty in obtaining pancreatic biopsy, the diagnosis is established by combination of clinical manifestations, morphological alterations and impairment of the pancreatic exocrine and endocrine function[27,28].

In our study, 94.3% CP patients showed low E1 levels together with a high percentage of steatorrhea and IDDM, indicating that most patients were at an advanced stage of the disease. However, only four LC patients (7%) had E1 levels between 100 and 200 μg/g (Figure 1), and they neither exhibited steatorrhea nor any clinical or morphological manifestations of CP. In a similar fashion, only three LC patients (5.3%) exhibited IDDM, without the other clinical and morphological manifestations of CP. Only one of these patients exhibited 164 μg/g E-1 level; hence, the presence of CP in this patient could not be ruled out. Eleven LC patients exhibited NIDDM, which is common in LC[47] with normal E-1 level, but not a characteristic manifestation of CP[48]. In the ASA group, three subjects exhibited NIDDM, without symptoms of pancreopathy and normal E-1 and ICG tests.

The former studies that assessed the frequency of pancreatic alterations in patients with LC showed contradictory results. The frequency of pancreatic fibrosis compatible with CP in autopsies of LC patients was between 2% and 20% when the presence or absence of hepatitis B or C was not taken into account[14,16,18,19]. In addition, the age-related changes in morphofunctional parameters of the pancreas may be another confounder when the diagnosis of CP is based only on these parameters[49-53]. In a study using endoscopic ultrasonography and ERCP, Hastier *et al*[17] reported only moderate pancreatic alterations in 5.5% patients with alcoholic LC, without evolution to CP.

Some previous studies[51,54,55] on necropsies of patients with alcoholic LC showed very low frequency of the fibrotic pancreatic alteration characteristic of CP, which is predominantly perilobular fibrosis with heterogeneous distribution. In contrast, these patients exhibited diffuse and intralobular pancreatic fibrosis without calcifications or pseudocysts. Accordingly, these authors suggested that pancreatic morphological lesions in alcoholic LC correspond to a pathological process different from that of alcoholic CP.

It is worth noting that many studies on the association of CP and LC involve patients older than 50 years. It is well known that normal subjects at this age exhibit some morphofunctional pancreatic alterations[21,22,49-51,53] that could be taken for those characteristic of CP, but without evolution towards this disease. Consequently, morphofunctional pancreatic alterations with age may be another confounding factor. These findings might explain some of the discrepancies reported by different authors.

On the other hand, regarding the hepatic alterations, most LC patients in our study were at an advanced stage of the disease and showed ascites, encephalopathy and/or gastrointestinal hemorrhage as well as Child-Pugh B or C and markedly reduced PDR in the ICG test. However, only a few CP patients exhibited these manifestations, which were not ascribed to any hepatic disorder (Table 4).

There is also controversy concerning the frequency of LC in CP patients. Previous studies have reported that the presence of LC in patients with CP was variable, ranging between 5% and 30%[56]. Later, Angelini *et al*[19] found 12.5% of LC cases in CP patients. Although, in this study, cases with hepatitis C virus were not excluded, the authors did not find any relationship between the grade of hepatic histological alteration and the degree of pancreatic functional severity. Recently, Nakamura

et al[15] found no association between liver and pancreatic disease in a population of alcoholics with abdominal pain, after exclusion of patients with viral hepatitis[15]. Accordingly, only 5% of patients who had altered ERCP also exhibited Child-Pugh class B or C[15].

It should be taken into account that many hepatic alterations in patients with alcoholic CP are ascribed to cholestasis secondary to pancreatitis[57,58], and indeed, they are not characteristic of advanced alcoholic liver disease[19,55]. Moreover, most of these cases exhibit regression of liver fibrosis after biliary drainage[59].

In our study, there was no coincidence at all between severe alterations of the pancreatic function assessed by fecal E-1 test and severe alterations of the liver function assessed by ICG test. Furthermore, we found a strong inverse correlation ($r = -0.752$; $P < 0.001$) between these functions (Figure 3). Similarly, we found a significant correlation between PI and fecal E-1 test ($r = 0.759$; $P < 0.001$). These findings support the hypothesis of a different and independent etiopathogenesis of CP and LC associated with alcoholism[25]. Hayakawa *et al*[24] reported a negative correlation between pancreatic and liver functions, but only in patients with alcoholic liver disease. In this regard, a general increase in exocrine pancreatic secretion in LC patients has been reported[19,60]. Thus, Dreiling *et al*[23] have suggested a hypersecretory state of the pancreas in alcoholic LC, with increased volume output[16] and maintaining either normal or elevated bicarbonate and enzyme outputs[16,19,23,24]. This has been considered a washout phenomenon, resulting in a decreased tendency of ductal protein and calcium precipitation in these patients[16].

Hayakawa *et al*[24] have reported that pancreatic secretion increases in patients with different degrees of alcoholic liver disease, assessed by the cholcystokinin-secretin test, ICG test and liver histopathology. Moreover, these authors have found a significant inverse correlation between bicarbonate secretion and ICG clearance[24].

The hypersecretory state of the pancreas in alcoholics with LC has been ascribed to a reduced inactivation of secretin[60] or even to the increased portal pressure[24]. Recently, it has been confirmed by an experimental result that chronic alcoholism impairs the neurohormonal control of the pancreas, both at the central nervous system and acinar levels, promoting the secretory response to feeding or other stimuli[61]. All these findings regarding the inverse correlation between the alterations of liver and pancreatic function tests may be a reflection of some correlation in the etiology of both diseases.

In summary, the present study demonstrates the scarce coincidence in clinical manifestations, morphological alterations and organ function between CP and LC in alcoholic subjects. This finding, together with the inverse correlation between indexes of pancreatic and liver functions in these patients, supports the hypothesis that alcoholic CP and LC evolve in a different manner and have different etiopathogenesis. Accordingly, chronic alcoholism, although a necessary factor, is not a sufficient cause in their etiopathogenesis. Further studies should provide new insights into the different risk factors-other than alcoholism-involved in the etiopathogenesis of alcoholic CP and LC.

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COMMENTS

Background

Despite sharing similar risk factors, medical literature regarding the simultaneous occurrence of chronic pancreatitis (CP) and liver cirrhosis (LC) in patients with chronic alcoholism is scarce and the results are uncertain. This lack of coincidence may rely on differences in methodology such as retrospective *versus* prospective studies, and the evaluated parameters such as clinical, functional, imaging or histopathological. However, it may also reflect the hypothesis that alcoholism is not a sufficient cause, but a cofactor, for the development of such diseases.

Research frontiers

This study has been carefully designed to investigate the coincidence in clinical manifestations, morphological alterations and organ function between CP and LC in alcoholic subjects. The results support the hypothesis that alcoholic CP and alcoholic LC evolve in a different manner and have different etiopathogenesis, despite sharing a common risk factor.

Innovations and breakthroughs

There is a lack of coincidence between CP and LC in alcoholic subjects. There is an inverse correlation between indexes of pancreatic and liver functions in these patients, supporting the hypothesis that chronic alcoholism, although a necessary factor, is not a sufficient cause in their etiopathogenesis.

Applications

This paper suggests the necessity for researching other factors together with chronic alcoholism involved in the development of CP or LC.

Peer review

Authors studied the possible association between CP and LC of alcoholic etiology, after excluding any other causes. This is an interesting study. It was undertaken according to a carefully designed plan and with adequate statistical considerations. The authors successfully provided the evidence, suggesting that CP and LC evolve in a different manner, which will be useful for clinical researchers of these diseases.

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BASIC RESEARCH

Bravo capsule system optimizes intragastric pH monitoring over prolonged time: Effects of ghrelin on gastric acid and hormone secretion in the rat

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Abstract

AIM: To evaluate measurements of intragastric pH with the Bravo capsule system over a prolonged time.
METHODS: A Bravo capsule was placed inside the rat gastric body and pH was studied for periods up to five consecutive days. For comparison, a gastric fistula model was used. Effects of ghrelin and esomeprazole, with or without pentagastrin, on gastric pH were studied. In addition, effects of esomeprazole on plasma ghrelin, gastrin and somatostatin were analyzed.
RESULTS: All rats recovered after surgery. The average 24-h pH during free feeding was 2.3 ± 0.1 (n = 20) with a variation of 18% ± 6% over 5 d. Ghrelin, 2400 pmol/kg, t.i.d. increased pH from 1.7 ± 0.1 to 3.1 ± 0.3 (P < 0.01) as recorded with the Bravo system. After esomeprazole (1 mg/kg, 3 mg/kg and 5 mg/kg) there was a dose-dependent pH increase of maximally 3.4 ± 0.1, with day-to-day variation over the entire period of 8% ± 3%. The fistula and pH studies generated similar results. Acid inhibition with esomeprazole increased plasma ghrelin from 10 ± 2 pmol/L to 65 ± 26 pmol/L (P < 0.001), and somatostatin from 10 ± 2 pmol/L to 67 ± 18 pmol/L (P < 0.001).
CONCLUSION: pH measurements with the Bravo capsule are reliable, and comparable to those of the gastric fistula model. The Bravo system optimizes accurate intragastric pH monitoring over prolonged periods and allows both short- and long-term evaluation of effects of drugs and hormones.

Key words: Gastric acid; Bravo system; Intragastric pH; Ghrelin; Somatostatin

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INTRODUCTION

In the past, different techniques have been employed to study gastric acid secretion in rodents. The main principle for these methods has been collection of gastric juice, and in order to measure acid secretion, pH titration has been carried out. One of the earliest methods was the pylorus ligation technique[1]. The principle of this method is distension of the stomach as a potent stimulus of acid secretion. Later, this method was altered with an esophageal ligature[2,3], after which the stomach of the rat was removed and secretions analyzed. Esophageal ligation in the pylorus-ligated rat has been shown to significantly inhibit acid secretion by inhibition of central vagus function[2]. Since then,

the most reliable method has been the chronic fistula method[4-6] where a gastric fistula is implanted at the greater curvature of the stomach. This technique requires movement restriction of the animal which is in a conscious state during the study. The gastric contents are collected and acid output measured. This technique allows re-use of animals following a recovery period from the experimental procedure. Other methods used today are perfusion of the gastric lumen[7] and isolated perfused, as well as vascularly perfused rat stomach[8-10].

Most of the above studies have the drawback that they do not measure intragastric pH directly and are not very physiological, as the animal is either restrained or anesthetized. The main goal of this study was to test the feasibility of a capsule normally used in the clinical setting in humans to measure gastroesophageal reflux disease (Bravo system) for monitoring intragastric pH in the rat. The Bravo capsule system has primarily been used in humans[11-14], but also in animals[15] for diagnosis of gastroesophageal reflux disease.

The aim of the study was to evaluate the Bravo capsule for pH monitoring in the rat. To validate the method, we compared the data to those of the standard gastric fistula model.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (300-350 g) were purchased from Scanbur B&K AB (Sollentuna, Sweden). The rats were housed in wire-meshed cages at 24℃ with constant humidity and 12:12 h light-dark cycle. The animals were fed *ad libitum* with a commercial rat diet consisting of pellet (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water prior to the studies. The experiments were approved by the Animal Ethics Committee in northern Stockholm.

Surgery

Surgery was performed under anesthesia with pentobarbital sodium (50 mg/kg; Apoteket AB, Stockholm, Sweden) intraperitoneally, and Hypnorm (fentanyl citrate, 0.315 mg/kg and fluanisone 10 mg/kg; Janssen, Oxford, USA) intramuscularly. Marcain (bupivacaine hydrochloride, 2.5 mg/kg; AstraZeneca, Södertälje, Sweden) was given subcutaneously after surgery along the abdominal incision.

For the Bravo system studies, a midline incision was performed, and a small opening created in the proximal greater curvature, and gastric contents were evacuated. An externally pre-calibrated (buffers pH 1.07 and 7.1) Bravo capsule (an electronic sensor encapsulated in PVC-plastic, 25 mm × 5 mm × 5 mm; Synmed Medicinteknik AB, Spånga, Sweden) was placed inside the stomach with the pH sensor pointing distally and anchored with a suture. An indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) was inserted into the external jugular vein.

For the gastric acid fistula studies, rats were provided with a plastic gastric fistula placed immediately proximal to the oxyntic gland area near the greater curvature. The fistula was closed between experimental periods. A silastic catheter was implanted into the external jugular vein for drug administration.

Studies of intragastric pH (Bravo system)

Studies of intragastric pH began in the morning 2 d after surgery. The studies were carried out in conscious rats, one experiment for each rat, under normal conditions, or after a 16-h fasting period in wire-bottom cages with free access to water. The animals gained weight (10 ± 3.4 g during 1 wk) and behaved in a normal fashion, with a normal feeding pattern throughout the experiments. At post-mortem examination, no mucosal lesions, obstruction of the pylorus or gastric distension were seen. Drugs were administered through the external jugular vein in all experiments.

The pH recorded by the Bravo capsule was transmitted to the Bravo receiver placed directly outside the cage. The sampling frequency was 6 Hz. The Bravo system was set for a 48-h registration period, after which the data were downloaded, batteries replaced and recording continued. This procedure was then repeated in two more 48-h periods.

All test compounds were dissolved and diluted in isotonic saline solution (sodium chloride, 9 g/L; 300 mosm/kg H_2O, Fresenius Kabi, Halden, Norway).

The effect of ghrelin on pH: The effect of ghrelin on intragastric pH was studied with ghrelin (2400 pmol/kg) given t.i.d (08:00, 12:00 and 16:00) for 5 d in a row (n = 7).

Evaluation of basal pH: Baseline pH was studied over 24 h under fed (n = 20) and fasting (n = 8) conditions.

Effect of esomeprazole on pH: The effect of increasing bolus doses of esomeprazole (AstraZeneca) (1 mg/kg, 3 mg/kg or 5 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fed rats. Furthermore, the effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fasting rats.

The effect of pentagastrin and esomeprazole on pH: The effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied under pentagastrin (NeoMPS, Strasbourg, France) infusion (90 pmol/kg per min, iv) over 6 h in both fed and fasting rats. In these experiments, the rats were restrained in Bollman cages to mimic the gastric fistula studies and for infusion of pentagastrin.

The effect of a 24-h infusion of pentagastrin (90 pmol/kg per min iv, n = 6), of esomeprazole (9 pmol/kg per min, n = 6), or saline (0.154 mol/L, n = 6) on pH was studied.

Plasma levels of gut hormones: The effect of esomeprazole (3 mg/kg iv) on plasma levels of ghrelin, gastrin and somatostatin was studied. A group of animals (n = 10) was divided into two treatment groups (each n = 5). All animals were treated with esomeprazole

daily during 1 wk. The first group of animals was then euthanized, while the other group was followed for another week without esomeprazole and then euthanaized. Blood was drawn and centrifuged, and plasma assayed for concentrations of ghrelin, gastrin and somatostatin.

For ghrelin measurements, the ghrelin (active) radioimmunoassay kit (Linco Research, St. Charles, MI, USA) was used, which utilizes ^{125}I-labeled ghrelin and ghrelin antiserum to determine the level of active ghrelin in plasma. For the analysis, a Gamma Master 1277 (LKB-Wallac, Perkin-Elmer Inc, Massachusetts, NH, USA) was used. The intra- and interassay coefficients of variation were 7% and 14%, respectively.

Somatostatin was analyzed using an EIA kit (EK-060-03) from Phoenix Pharmaceuticals, Burlingame, CA, USA), which reacts 100% to somatostatin-14 and somatostatin-28. The intra- and interassay coefficients of variation were 5% and 14%, respectively.

Gastrin was analyzed using C-terminal-directed CCK/gastrin antiserum 2609/10 (Rehfeld, 1978). Chloramine-T-labeled and HPLC-purified gastrin-17 (NeoMPS) was used as radioligand and gastrin-17 as calibrator/standard. The intra- and interassay coefficients of variation were 6% and 8%, respectively.

Studies of gastric acid secretion (fistula)

Studies of gastric acid secretion began 7 d after surgery. The animals gained weight (8 ± 2.6 g during 1 wk) and had normal behavior during the experimentation periods. Prior to each experiment, food was withheld for 18 h, but with free access to water. At the start of the experiments, the stomach was rinsed with 10-15 mL luke-warm tap water to evacuate remaining food, followed by a 30-min period before the experiments were started. During the experiments, the conscious rats were placed in Bollman cages. Gastric juice was collected at 30-min intervals, and volumes measured to the nearest 0.1 mL. pH was calculated by back-titration using 0.1 mmol/L sodium hydroxide. Acid output was calculated by multiplying the secretion volumes with hydrogen ion concentrations and expressed as μmol per 30-min period.

Baseline acid secretion was studied for 60 min followed by esomeprazole (3 mg/kg iv), after which acid secretion was studied for another 2 h. During the experiment, saline was administered in the same amount as collected from the gastric fistula to compensate for the volume loss during the experiment. Furthermore, baseline acid secretion was studied for 60 min, followed by an infusion of pentagastrin (90 pmol/kg per min) for 4 h. After 1 h of pentagastrin infusion, a bolus of esomeprazole (3 mg/kg iv) was administered and acid secretion studied for another 3 h.

Data and statistical analysis

The data obtained with the Bravo capsule analyzed using (POLYGRAM NET™ pH Testing Application software, Synmed Medicinteknik) in 48-h periods. Results of studies with esomeprazole were analyzed by calculating changes in pH at various timepoints from baseline (defined as 0.5 h prior to onset of studies). For analysis of the fistula studies, the first 30-min collection was discarded and the second collection used as baseline for comparison with esomeprazole and pentagastrin.

All data are mean ± SE. A Kruskal-Wallis test followed by Mann-Whitney *U* test was used for statistical comparisons using specific time points for pH. $P < 0.05$ was considered statistically significant. For comparison of the variability between the fistula and the Bravo system the Bland-Altman analysis was used[16,17]. The Prism software package 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical comparisons.

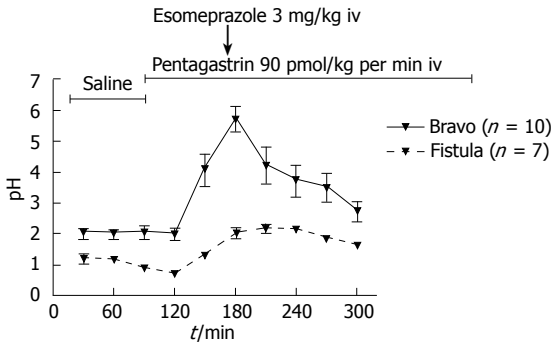

Figure 1 Change in pH ± SE in the Bravo system and gastric fistula model during fasting conditions after iv bolus of esomeprazole (3 mg/kg) and pentagastrin infusion (90 pmol/kg per min) for 2 h.

RESULTS

Comparison between the Bravo system and the fistula model

Pentagastrin resulted in a marked increase, 83 ± 9 mmol/L to 132 ± 8 mmol/L ($P < 0.05$) of acid output in the fistula model, which was not evident as a corresponding decrease in pH with the Bravo system. During esomeprazole treatment, there was a marked increase in pH from 2.0 ± 0.2 to 3.7 ± 0.5, as recorded with the Bravo system and correspondingly, a marked decrease in acid secretion from 105 ± 21 mmol/L to 31 ± 7 mmol/L in the fistula model ($P < 0.05$; Figure 1). Bland-Altman analysis of these conditions showed a high degree of agreement between the Bravo system and the fistula method as shown in Figure 2.

Evaluation of basal pH

A typical 120-h baseline registration including dose of esomeprazole (day 1, 3 and 5) with the Bravo system is shown in Figure 3. The feeding status did not alter the mean pH over 24 h, but increases in pH were observed during afternoon and night-time when animals were fed. The mean 24-h pH was 2.3 ± 0.1 during fed conditions and 2.5 ± 0.3 during fasted conditions, with 18% ± 6% variation during the next four 24-h periods. There was no difference in pH between daytime and night-time (1.4 ± 0.1 and 1.7 ± 0.2, respectively).

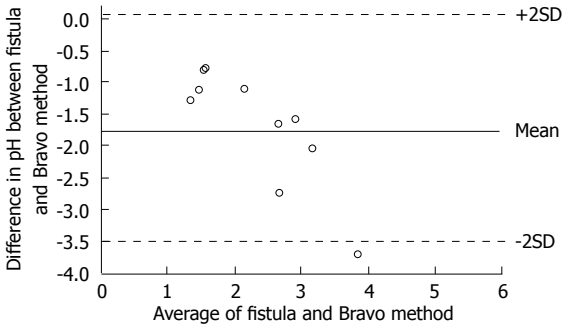

Figure 2 Bland-Altman analysis comparing the Bravo system with the fistula method. Mean value -1.7 with 2SD from -3.5 to 0.12.

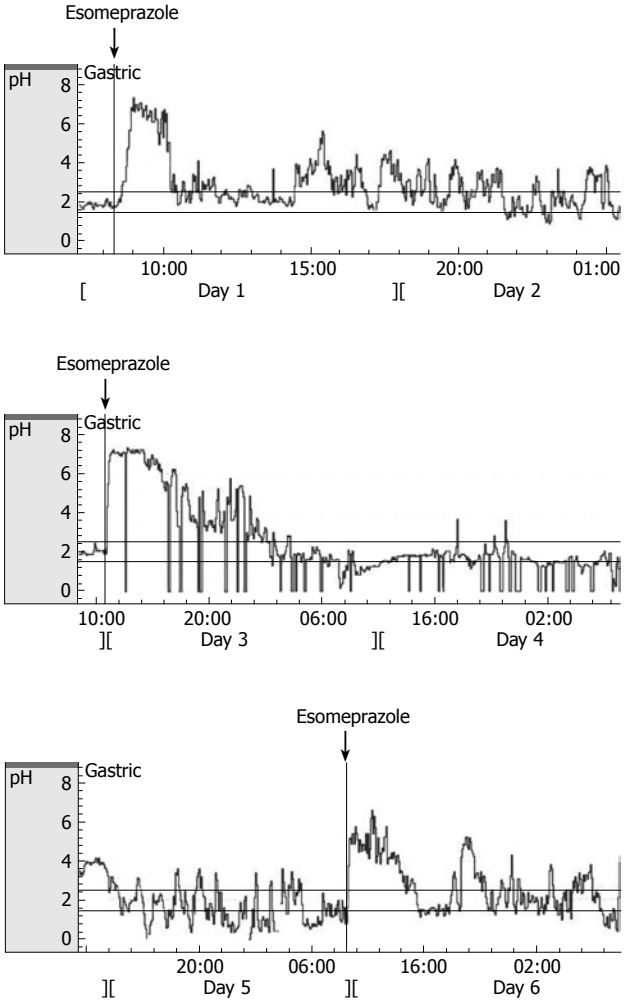

Figure 3 Standard recording with the Bravo system of intragastric pH in a rat during 6 d. The solid line indicates bolus doses of esomeprazole (3 mg/kg) given iv during 3 min under fed conditions.

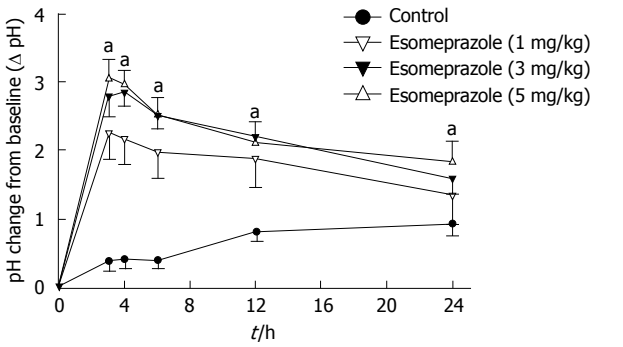

Figure 4 Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole in three different doses and saline studied for 24 h during fed conditions. Mean for all doses *vs* control and for dose 1 mg/kg vs 5 mg/kg ($^aP < 0.05$).

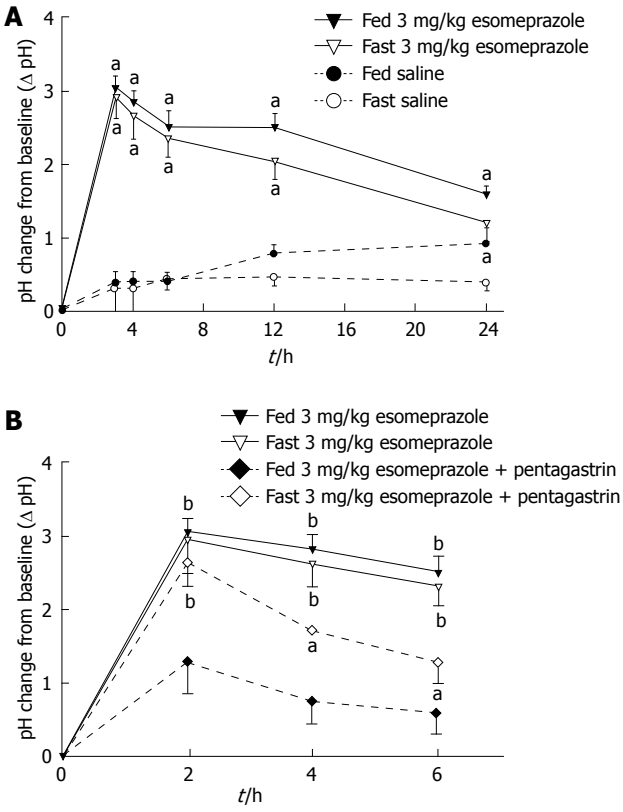

Figure 5 Changes of intragastric after an iv bolus of esomeprazole. A: Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during fed or fasting conditions for 24 h ($^aP < 0.05$); B: Change form baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during pentagastrin (90 pmol/kg per min) infusion during fed or fasting conditions during 6 h. Mean esomeprazole *vs* esomeprazole and pentagastrin for fed ($^bP < 0.01$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^aP < 0.05$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^bP < 0.01$).

The effect of bolus esomeprazole on pH

As studied over 24 h, there was a dose-dependent increase of pH after esomeprazole, 1 mg/kg, 3 mg/kg, and 5 mg/kg, during free roaming conditions (Figure 4). Already 3 h after administration of esomeprazole, pH was significantly higher with 5 mg/kg, 3.1 ± 0.4, than with 1 mg/kg, 2.2 ± 0.4 ($P < 0.05$). Esomeprazole (3 mg/kg) increased intragastric pH during saline infusion over a 6-h period (2.5 ± 0.2) compared to baseline pH (1.6 ± 0.2), whereas saline did not ($P < 0.01$; Figure 5A).

Esomeprazole was equally effective during fed or fasting conditions (Figure 5A). As a control, saline did not change intragastric pH during either fed (baseline pH 1.4 ± 0.1) or fasting (baseline pH 1.6 ± 0.2) conditions (Figure 4, Figure 5A).

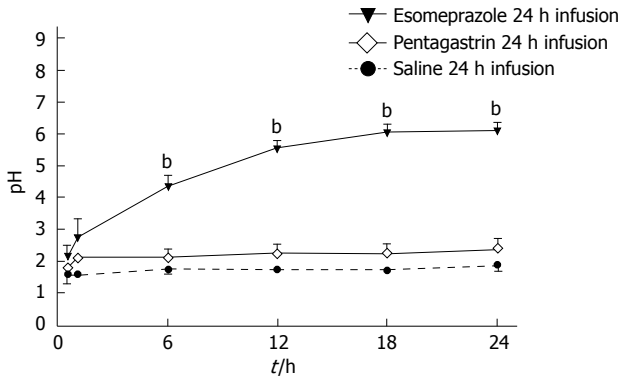

Figure 6 Change in mean pH ± SE over time. Mean esomeprazole (9 pmol/kg per min) 24 h infusion and pentagastrin (90 pmol/kg per min) infusion. ($^bP < 0.01$ *vs* control).

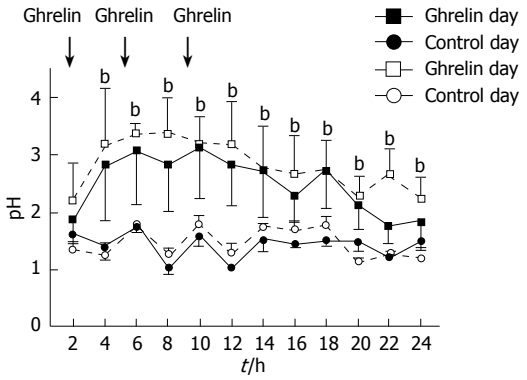

Figure 7 Change in mean pH ± SE during treatment with bolus dose ghrelin t.i.d. (2400 pmol/kg, $^bP < 0.01$ ghrelin *vs* control). There was no day-to-day difference in variation of the ghrelin response during the 5 d.

The effect of pentagastrin on pH

Pentagastrin alone did not change pH over 6 h compared with fed (baseline pH 2.1 ± 0.2) or fasting (baseline pH 2.4 ± 0.2) conditions. After esomeprazole (3 mg/kg), pentagastrin infusion markedly decreased pH from 2.0 ± 0.3 to 1.0 ± 0.2 ($P < 0.05$, Figure 5B). This effect was most marked in fed animals.

The effect of 24-h infusion of esomeprazole on pH

After esomeprazole (9 pmol/kg per min) the average 24-h pH was substantially higher than in the controls, 5.7 ± 0.3 and 2.1 ± 0.2, respectively ($P < 0.01$). Pentagastrin alone did not change pH over the 24-h infusion period as compared to saline (Figure 6).

The effect of ghrelin on pH

Administration of ghrelin, t.i.d markedly increased gastric 24-h pH from day 1 (2.5 ± 0.6) to day 5 (2.8 ± 0.5) compared to control day 1 (1.4 ± 0.1) and day 5 (1.5 ± 0.2) ($P < 0.01$; $n = 7$). There was no significant day-to-day variation of the ghrelin effect during the five days (Figure 7).

Plasma levels of gut hormones

Esomeprazole (3 mg/kg) t.i.d resulted in a marked increase in plasma ghrelin and somatostatin concentrations as shown in Figure 8 ($P < 0.001$). Plasma gastrin, however, remained stable over the same time period (Figure 8).

DISCUSSION

This study demonstrates that the Bravo system can be used for studies of intragastric pH in rats and that the results are comparable to those of a standard fistula model. The system allows for long-term studies during unrestrained living conditions. There are several advantages with the use of the Bravo system. Previous models for studies of gastric acid secretion do not allow measurements of pH over a long time. Furthermore, during these studies, the animals are kept under stressful conditions, which to a certain degree, may influence the responsiveness of the animals to different stimuli. The Bravo system uses a telemetric system that records gastric pH during 24 h for up to five consecutive days. The day-to-day variation was within acceptable limits. The system allows for real-time recordings of intragastric pH with the ability to record from the start of a treatment until a detectable effect is seen. The system is suitable for long-term studies with continuous infusions that are difficult to perform using the fistula model, as the animals do not tolerate being restrained in cages during prolonged studies. The Bravo recording system is also a digital recording system, which means that primary data are logged, and permits detailed measurements as determined by the set sampling frequency.

The data are, however, limited to pH-values as no secretion volumes are obtained. With the gastric fistula model, recordings are made over no less than 15-min periods, which can be a limiting factor as regards rapid changes in pH, i.e. drug effects. However, in the fistula model, secretion volumes are recorded, which permit calculation of a true acid output. The Bravo system has a few drawbacks. It is expensive, the battery life of the capsule is short (5 d) and therefore, the animals can only be used in studies for about a week. This means that experiments must start immediately after the operation (in this case 2 d after the surgical procedure), and the recovery from surgery may influence the results and the comparison with the fistula model. Despite this, the Bravo system seems to be well tolerated, as the stomach of the rats did not show any abnormalities or mucosal lesions upon autopsy. The animals also gain weight and behaved in a normal fashion during the experiments.

From a physiological viewpoint, our results demonstrated expected results; intragastric pH in rodents was stable over time, with a slight increase during the night during fed conditions.

In addition, treatment with esomeprazole and pentagastrin gave expected results. The agreement between the Bravo system and the established fistula method was evaluated employing a Bland-Altman analysis. When the two methods were compared, the pH results obtained with the Bravo system were comparable to those obtained using the fistula model. The differences lie within acceptable limits of agreement approximately 95% of the time, and the variability

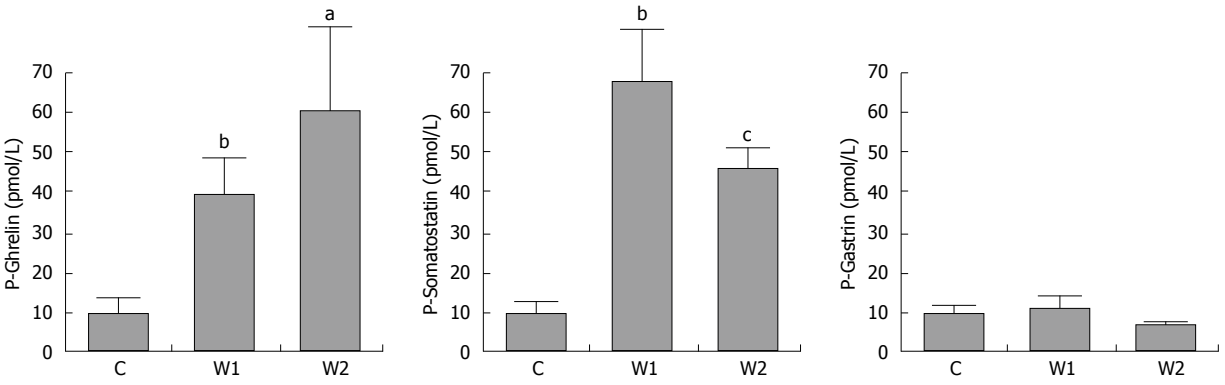

Figure 8 Gut peptide concentrations during treatment week 1 (W1) and week 2 (W2). The two groups of animals (n_{tot} = 10) were treated during one week with esomeprazole (3 mg/kg). After the first treatment week the first group (n = 5) were euthanaized and plasma were taken for peptide measurements. The other group (n = 5) went on for another week without any drug treatment and then euthanaized and plasma taken for analysis of peptides. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ *vs* control.

was consistent across the graph; the scatter around the baseline (mean) did not increase with increasing means.

During comparative studies, the animals were restrained in Bollman cages for infusion of pentagastrin, so the experimental conditions were the same. During esomeprazole treatment, pH rose and gastric acid output decreased accordingly. There seemed to be a slight delay in response to esomeprazole when studied by the fistula method as compared to the Bravo system. The reason for this is probably related to the fact that the secretory response depends on the physical emptying of gastric contents from the fistula until measurements can be done. As judged from our experiments, this causes a delay of the recorded response of about 30 min. Pentagastrin increased acid output, but no change was seen in intragastric pH with the Bravo system. This is explained by the fact that a change in secretion volume does not affect the pH recorded, even though acid output is changed. The fact that pH does not change when introducing pentagastrin may be due to the constantly low basal pH level in the rat stomach.

The gut hormones assayed in this study, ghrelin, gastrin, and somatostatin, are all found in the mucosa of the stomach[18]. They operate in a coherent inhibitory/stimulatory fashion against one another, i.e. increasing levels of somatostatin stimulates ghrelin, while gastrin is inhibited[19,20]. Pentagastrin acts as an agonist on acid secretion and has a stimulatory effect on somatostatin, which in turn down-regulates the release of gastrin so that excessive amounts of acid are not produced[20]. The fact that basal plasma gastrin levels remained stable with the Bravo system indicates that the Bravo capsule by itself does not distend the stomach to such a degree that gastrin levels are affected[21].

Our results using the Bravo system, with an increase of intragastric pH during 1 wk after three times daily, administration of ghrelin, are in accordance with earlier studies[19,22], but at variance with another[23]. This may be explained by the fact that different methods for studying gastric acid secretion have been employed, some of which are dependent on gastric motility for the emptying of gastric secretions through the fistula. By using the Bravo system, we found no desensitization of the pH response to ghrelin. This is at variance with our previous studies on intestinal motility, in which a loss of the ghrelin response was shown[19,24]. This might be due to the fact that motility was stimulated by a continuous infusion of the hormone, whereas the pH effect was brought about by repeated injections of ghrelin, a form of administration that is considered less liable to desensitization effects. As ghrelin not only increases intragastric pH, but also stimulates gastric emptying in rodents[22,23,25,26]. This may be an erroneous factor in determining acid secretion using the fistula method.

With esomeprazole treatment, plasma concentrations of ghrelin and somatostatin were increased. This effect was maintained for 1 wk after esomeprazole treatment. The underlying mechanism for this increase in plasma ghrelin and somatostatin is not yet fully understood, but may be due to a direct effect of esomeprazole on ghrelin and somatostatin, but also by an indirect effect through changes in gastric pH. The counter-balancing effects between pentagastrin (low pH) and esomeprazole (high pH) as regards ghrelin levels point to a physiological role of ghrelin in the control of gastric acid secretion[27,28]. The rise in somatostatin concentration is likely due to a direct effect of the continuous doses of ghrelin, as pH was not affected. The lack of elevated levels of gastrin for the two groups are probably attributed to the increase in somatostatin[29] or, although less likely, low doses of esomeprazole[30,31].

To conclude, the Bravo capsule system is to be used for prolonged studies of gastric pH in free roaming conscious rats over days and is well tolerated, and could serve as a complement to the gastric fistula model, as shown by acid and gut hormone secretion measurements.

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COMMENTS

Background

The pharmacological treatment of gastrointestinal acid-related diseases aims at providing ulcer and mucosal healing, symptom relief and improved quality of life. Gastric acid inhibitory compounds are widely used in the clinical setting in order to treat not only benign gastric and duodenal ulcers, but also gastritis and reflux esophagitis. Over the past two decades, there has been a number of reports on the use of proton pump inhibitors (PPIs) such as omeprazole and the following competitors. The PPIs are activated in the acid environment in the stomach and inhibit the final step of gastric acid secretion. They bind in a non-competitive way to the H^+, K^+ -ATPase and inhibit secretion. Even though the PPIs have many good properties compared to other treatment regims, and are considered the treatment of choice in acid-related gastrointestinal diseases, there are drawbacks with PPI treatment. For instance, the onset of action is slow as compared to that of H2-receptor antagonists, which induce an immediate acid inhibition, and the duration of action may be too short giving room for night-time acid breakthrough. So far, treatments have got around this problem by recommending a two-dose regimen. Pharmaceutical development has been directed against finding a compound with profound acid inhibitory action over prolonged periods of time, not permitting night-time acid breakthrough to take place. The development of such drugs, however, require new methods of studying gastric acid secretion over prolonged periods, up to 120 h over or more.

Research frontiers

Research concerning acid-related diseases has been focused on PPIs targeted against the H^+, K^+ -ATPase of the stomach and H2-receptor antagonists. Recent studies have shown that the proton pump is the most likely candidate for a sustainable therapeutic application in the regulation of acid suppression. One of the hurdles in this field is the possibility to perform long-term measurements of acid secretion in the development of pharmacological treatment of acid diseases. Although PPIs are highly effective as a class, differences in their pharmacokinetics, such as bioavailability, metabolism, and elimination half-life, may translate into differences in clinical outcomes.

Innovations and breakthroughs

Over the latest years, new drugs have emerged on the market, such as being PPIs (3rd generation), new potassium channel blocking agents that inhibit gastric secretion (P-CAP), and even combinations of PPIs and H2-receptor antagonists. A second line to this further development is to be expected and with this new method, developed as a tool for evluation of such long-acting drugs, may become a feasible tool in the clinical setting for treatment of acid-dependent diseases.

Applications

Our research demonstrates stable recordings with the Bravo capsule system in the rat. The animals were given PPI and ghrelin and this resulted in an almost immediate response in pH, sustained during approximately 6 h. The capsule model was compared with the fistula model and showed agreement in compliance between the two methods. This indicates that the capsule model could eventually replace the fistula model. It seems better to use the former method because of less strain on the rats, and easier and more gentle handling and experimental procedures. Furthermore, the Bravo system set-up is easy to manage and the information recorded allows many different analysis variables. The system also records over five consecutive days, which previously has not been possible in this setting.

Terminology

Bravo capsule system: A catheter-free system used to measure esophageal pH (acidity) levels in patients who have or are suspected of having gastroesophageal reflux disease, but has now also been used for intragastric titration of pH.

Peer review

The measurement of intragastric pH with the Bravo capsule system is comparable to that of the gastric fistula model, and is useful for prolonged studies of gastric pH, even in free roaming conscious rats over days, as described. Although further studies are required, this study indicates the novel possibility for investigating the acid and gut hormone secretion under more physiological conditions.

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BASIC RESEARCH

Gardenia jasminoides protects against cerulein-induced acute pancreatitis

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Abstract

AIM: To investigate the effect of *Gardenia jasminoides* (GJ) on cerulein-induced acute pancreatitis (AP) in mice.
METHODS: C57BL/6 mice weighing 18-20 g were divided into three groups. (1) Normal saline-treated group, (2) treatment with GJ at a dose of 0.1 g/kg, (3) treatment with GJ at a dose of 1 g/kg. GJ was administered orally (n = 6 per group) for 1 wk. Three hours later, the mice were given an intraperitoneal injection of cerulein (50 μg/kg), a stable cholecystokinin (CCK) analogue, every hour for a total of 6 h as described previously. The mice were sacrificed at 6 h after completion of cerulein injections. Blood samples were obtained to determine serum amylase, lipase and cytokine levels. The pancreas was rapidly removed for morphologic examination and scoring. A portion of pancreas was stored at -70℃ and prepared for the measurement of tissue myeloperoxidase (MPO) activity, an indicator of neutrophil sequestration, and for reverse-transcriptase PCR (RT-PCR) and real-time PCR measurements.
RESULTS: Treatment with GJ decreased significantly the severity of pancreatitis and pancreatitis-associated lung injury. Treatment with GJ attenuated the severity of AP compared with saline-treated mice, as shown by reduction in pancreatic edema, neutrophil infiltration, serum amylase and lipase levels, serum cytokine levels, and mRNA expression of multiple inflammatory mediators.
CONCLUSION: These results suggest that GJ attenuated the severity of AP as well as pancreatitis-associated lung injury.

Key words: Gardenia jasminoides; Acute pancreatitis; Cerulein

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process of the pancreas that frequently involves peri-pancreatic

tissues and remote organ systems. The severity of the disease varies widely, the clinical course is unpredictable, and specific therapy is limited[1-4]. It is generally believed that the severity of pancreatitis is determined by events that occur after acinar cell injury. Pancreatic acinar cells synthesize and release cytokines and chemokines, resulting in the recruitment of inflammatory cells such as neutrophils and macrophages. This leads to further acinar cell injury, resulting in the elevation of various pro-inflammatory mediators such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α[5,6]. The release of inflammatory mediators such as TNF-α and IL-1β during AP propagates a complex cascade of events between tissue vasculature and inflammatory cells. These inflammatory cells and mediators play a role in the systemic manifestations besides modulating pancreatic acinar cell injury; blocking the cytokine cascade in its early stage, and ameliorating the disease and its systemic complications.

Gardenia jasminoides (GJ) is widely employed in several Asian countries as a natural colorant, and has been used in Chinese traditional medicine for its homeostatic, antiphlogistic, analgesic and antipyretic effects. Its main components include geniposide and crocin[7]. These components exhibit antioxidant, cytotoxic, antitumor and neuroprotective effects[8-10]. However, the impact of GJ and its components on cerulein-induced AP have not been examined.

The present study was designed to confirm the preventive effects of GJ in a mouse model of cerulein-induced AP. In order to gain a better insight into the mechanism of action of the observed anti-inflammatory effects of GJ, we investigated the effects of GJ on (1) pancreas weight/body weight (PW/BW) ratio, (2) pancreatic histology, (3) serum amylase and lipase levels, (4) serum level of pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6, and (5) lung histology.

MATERIALS AND METHODS

Materials

Avidin-peroxidase and 2'-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) tablets, cerulein, Tris-HCl, NaCl, Triton X-100, hexadecyltrimethylammonium bromide and etramethylbenzidine were purchased from Sigma (St. Louis, MO, USA). Anti-mouse TNF-α, IL-6 and IL-1β antibodies, recombinant TNF-α, IL-6 and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of GJ

GJ was prepared by decocting the dried prescription of herbs with boiling distilled water. The decoction time was about 3 h. Their voucher specimens were deposited at the Herbarium at the College of Oriental Medicine, Won-Kwang University.

Animal models

All experiments were performed according to protocols approved by the Animal Care Committee of the university. Female C57BL/6 mice (6-7-wk old, weighting 18-20 g) were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2℃ and a 12-h light-dark cycle for 7 d. Animals were fed standard laboratory chow, given water and randomly assigned to control or experimental groups. The mice were fasted for 18 h before induction of AP. Six mice were included in each experimental group.

Experimental design

AP was induced by supramaximal concentration of cerulein (50 μg/kg), a stable CCK analogue, by administering it intraperitoneally every hour for a total of 6 h as described previously[11]. The mice were fed orally with GJ (1 g/kg, 0.1 g/kg, $n = 6$ each) or normal saline (control group, $n = 6$), followed by intraperitoneal injection of cerulein (50 μg/kg) or saline every hour for a total of 5 h. The mice were sacrificed at 12 h after the completion of cerulein injections. Blood samples were obtained to determine serum amylase, lipase and cytokine levels. The pancreas was rapidly removed for morphologic examination and scoring. A portion of pancreas was stored at -70℃ and prepared for the measurement of tissue myeloperoxidase (MPO) activity, an indicator of neutrophil sequestration, and for reverse-transcriptase PCR (RT-PCR) and real-time PCR measurements.

Histological analyses

The entire pancreas of at least six mice from each treatment group was examined and semi-quantitated based on the degree of necrosis, vacuolization, inflammation, and edema. Using the previously described method of Ethridge *et al*[12], entire sections (a minimum of 100 fields) of pancreas were examined from each sample and scored on a scale of 0-3 (0 being normal and 3 being severe), based on the number of necrotic acinar cells, and the presence of vacuolization, interstitial edema, and interstitial inflammation. The characteristics included were: presence of acinar-cell ghosts, vacuolization and swelling of the acinar cells, and/or the destruction of the histo-architecture of the whole or parts of the acini.

Enzyme-linked immunosorbent assay (ELISA)

ELISA for TNF-α, IL-1β and IL-6 (R&D Systems) was carried out in duplicate in 96-well plates (Nunc, Denmark), coated with each of the following: 100 μL aliquots of anti-mouse IL-6, IL-1β and TNF-α monoclonal antibodies at 1.0 μg/mL in PBS at pH 7.4, and incubated overnight at 4℃. The plates were washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, the standards were added and incubated at 37℃ for 2 h. After incubation for 2 h at 37℃, the wells were washed, and the following were added: 0.2 μg/mL of biotinylated anti-mouse TNF-α, IL-1β and IL-6, and again incubated at 37℃ for 2 h. After the wells were washed, avidin-peroxidase

was added and the plates were incubated for 20 min at 37℃. The wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-1β and IL-6 in serial dilutions.

Measurement of serum amylase and lipase

Arterial blood samples for determination of serum amylase and lipase were obtained 12 h after induction of pancreatitis. The mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood samples were withdrawn from the heart. Serum amylase was measured using ADIVA 1650 (Bayer, USA). Serum lipase was measured using a Cobas-mira (Roche, USA).

mRNA expression

mRNA transcripts were analyzed by RT-PCR in mouse pancreatic tissues. Total RNA was isolated from the mouse pancreas using Qiagen RNeasy kit and subjected to reverse transcription using SuperScript Ⅱ RT (Invitrogen). Taqman quantitative RT-PCR with a 7700 Sequence Detection System was done according to the instructions of the manufacturer (Applied Biosystems). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the gene of interest, and results were normalized to those of the 'housekeeping' HPRT mRNA. Arbitrary expression units were calculated by division of expression of the gene of interest by ribosomal protein HPRT mRNA expression. The forward, reverse and probe oligonucleotide primers for multiplex real-time TaqMan PCR were as follows: for mouse TNF-α (forward, 5'-TCTCTTCAAGGGACAAGGCTG-3'; reverse, 5'-ATAGCAAATCGGCTGACGGT-3'; probe, 5'-CCCGACTACGTGCTCCTCACCCA-3'), for mouse IL-1β (forward, 5'-TTGACGGACCCCAAAAGAT-3'; reverse, 5'-GAAGCTGGATGCTCTCATCTG-3'; universal probe, M15131.1-Roche Applied Science), for mouse IL-6 (forward, 5'-TTCATTCTCTTTGCTCTTGAATTAGA-3'; reverse, 5'-GTCTGACCTTTAGCTTCAAATCCT-3'; universal probe, M20572.1-Roche Applied Science), for mouse HPRT (forward, 5'-GACCGGTCCCGTCATGC-3'; reverse, 5'-CATAACCTGGTTCATCATCGCTAA-3'; probe, 5'-ACCCGCAGTCCCAGCGTCGT-3').

MPO estimation

Neutrophil sequestration in the pancreas was quantified by measuring the tissue MPO activity[11,12]. Tissue samples were thawed, homogenized in 20 mmol/L phosphate buffer (pH 7.4), and centrifuged (10000 × *g*, 10 min, 4℃), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The sample was then centrifuged (10000 × *g*, 5 min, 4℃), and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mmol/L tetramethylbenzidine (Sigma), 80 mmol/L sodium phosphate buffer (pH 5.4), and 0.3 mmol/L hydrogen peroxide. The mixture was incubated at 37℃ for 110 s, the reaction was terminated with 2 mol/L of H_2SO_4, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample (fold increase over control).

Statistical analysis

The results were expressed as means ± SE. The significance of change was evaluated using Student's *t* test. Differences between the experimental groups were evaluated by using analysis of variance. Values of $P < 0.05$ were accepted as statistically significant.

RESULTS

Effect of GJ on PW/BW ratio, serum amylase and lipase activity in cerulein-induced AP

To assess the effect of GJ on the PW/BW ratio, the pancreatic weight was divided by the body weight of the mice. GJ reduced significantly the PW/BW ratio, compared with the normal saline-treated group, in a dose-dependent manner ($P < 0.05$) (Figure 1A). The serum levels of amylase and lipase are commonly used as markers of AP[13,14]. GJ reduced significantly the serum amylase and lipase levels in cerulein-induced AP (Figure 1B and C).

Effect of GJ on serum levels of TNF-α, IL-1β and IL- in cerulein-induced AP

The serum levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were increased in cerulein-induced AP[15-17]. GJ decreased significantly the levels of TNF-α and IL-1β in the cerulein-induced AP. Moreover, GJ was associated with a trend towards suppression of IL-6, although the difference was not statistically significant (Figure 2).

Effect of GJ on mRNA expression of TNF-α, IL-1β and IL-6 in cerulein-induced AP

The GJ-pretreated group showed significant reduction of pancreatic tissue mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, in a dose-dependent manner, compared with the saline pretreated group in cerulean-induced AP (Figure 3).

Effect of GJ on pancreatic histology in cerulein-induced AP

In normal mice, the histological features of the pancreas were typical of a normal architecture. Mice treated with i.p. injections of cerulein developed acute necrotizing pancreatitis. Histological examination of the pancreas (at 12 h after the injection of cerulein) revealed tissue damage characterized by inflammatory cell infiltrate and acinar cell necrosis. GJ pretreatment resulted in significant reduction in pancreatic injury (Figure 4A). The presence

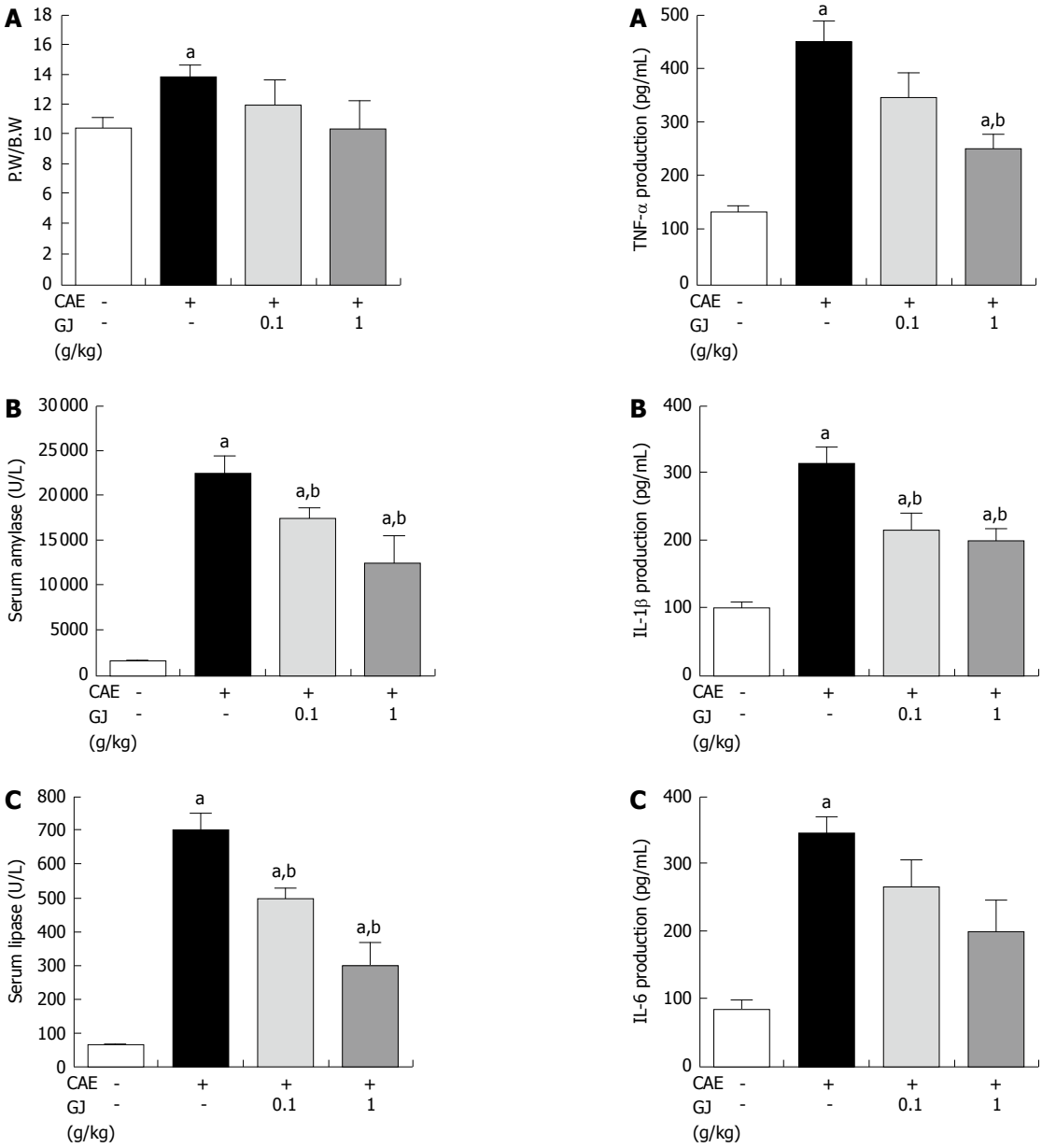

Figure 1 Effects of GJ pretreatment on the (A) PW/BW, (B) serum amylase activity, and (C) serum-lipase activity in cerulein induced AP. The study groups were treated as indicated in the experimental protocol. The mean ± SE of the six animals are shown. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

Figure 2 Effect of GJ on (A) TNF-α, (B) IL-1β and (C) IL-6 secretion in cerulein-induced AP. Mice were treated as indicated in the experimental protocol. The mean ± SE of six animals are shown. The study groups were treated as indicated in the experimental protocol. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

of edema, inflammation, vacuolization, and necrosis were reduced significantly in the GJ-pretreated group compared with the normal saline-pretreated group, in a dose-dependent manner (Figure 4B).

Effect of GJ on lung histology in cerulein induced AP

Lung injury commonly develops early in AP. AP-associated lung injury is characterized by edema and inflammation[18]. In addition to the pancreas, we assessed the lungs after cerulein administration. Histological examination of lung sections (at 12 h after the injection of cerulein) revealed tissue damage characterized by edema and inflammatory cell infiltrate. GJ pretreatment resulted in significant reduction in lung injury. The histological sections were scored for edema and inflammation. The lungs of the GJ-pretreated mice had significantly less edema and inflammation compared with lungs from saline-injected control animals (Figure 5).

Effect of GJ on MPO activity of lung and pancreas in cerulein induced AP

As an additional quantitative assessment of the severity of the inflammatory response, we measured MPO activity, an indicator of neutrophil sequestration, in the pancreas and lung following induction of AP in the GJ-pretreated mice and saline-injected control animals. MPO activity in the pancreas and lung in the GJ-pretreated mice was significantly less compared with the saline-injected control animals (Figure 6).

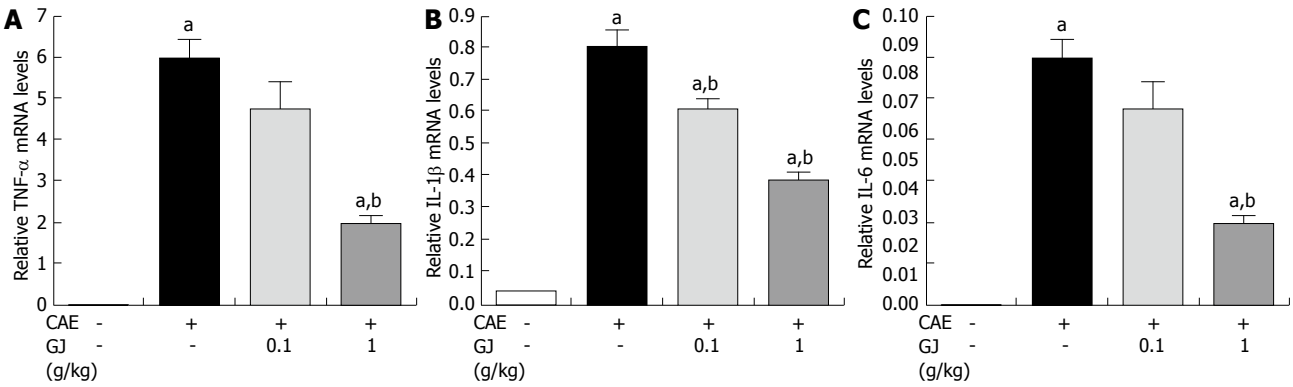

Figure 3 Effect of GJ on TNF-α, IL-6 and IL-1 mRNA levels in cerulein-induced AP. The mice were sacrificed at 1, 3 and 6 h after six injections. Levels of pancreatic mRNA were quantified by real-time PCR for (A) TNF-α, (B) IL-1β, and (C) IL-6. The mean ± SE of six animals are shown. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

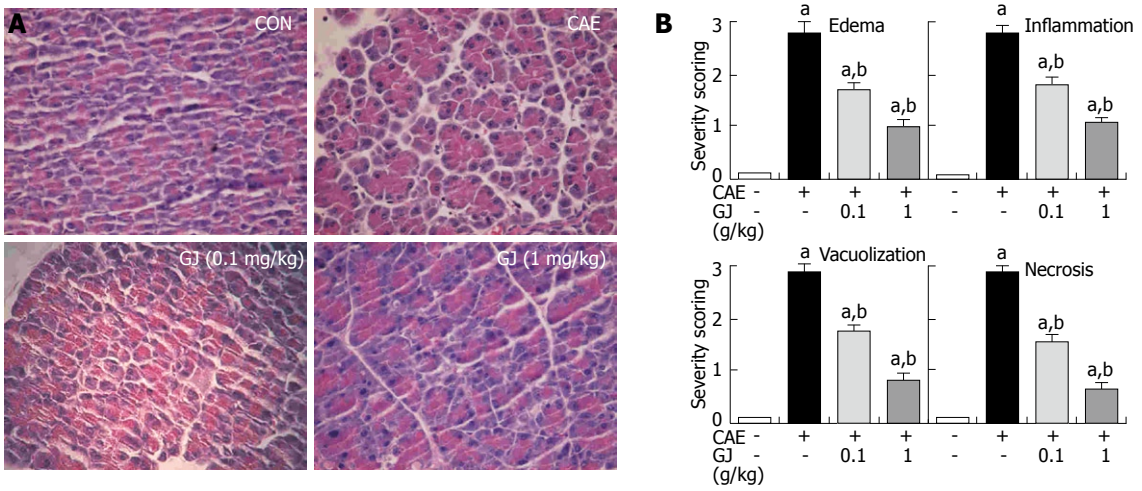

Figure 4 Effects of the GJ on pancreatic inflammatory changes following pancreatitis. A: Representative H&E-stained sections of pancreas in control mice (CON) who were not given cerulein, in mice given cerulein (CAE), and in mice given GJ (1 mg/kg) at the same time as the first cerulein injection; B: Histological sections of pancreas harvested 12 h after injection of saline (CON), cerulein alone, or GJ (1 or 0.1 mg/kg) given at the same time as the first injection of cerulein. The results were scored from 0 (normal) to 3 (severe) for edema, inflammation, vacuolization, and necrosis. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows the results of one experiment in which 4-5 mice were tested per group. The results obtained were similar to those in three additional experiments (x 200).

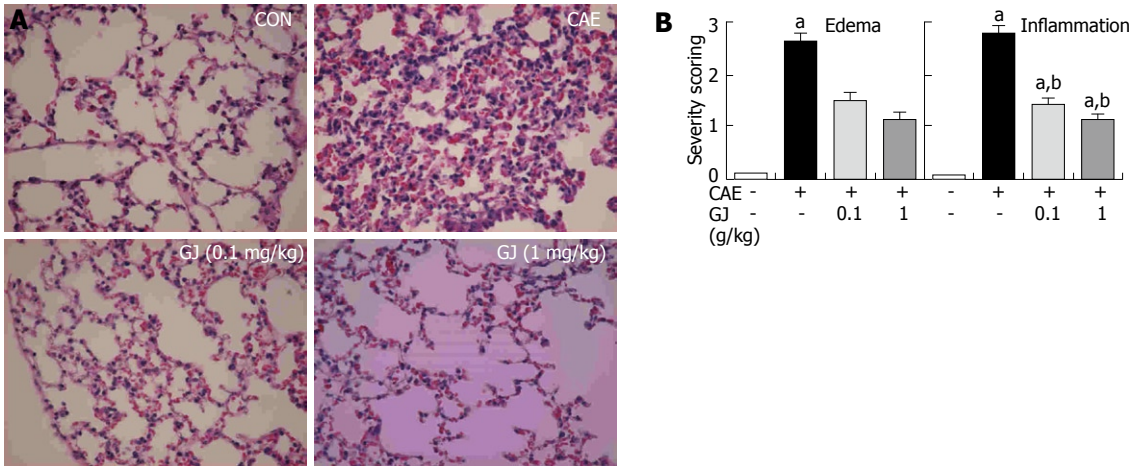

Figure 5 GJ reduced the severity of AP-associated lung injury. A: Representative H&E-stained sections of the pancreas in control mice (CON) not given cerulein, in mice given cerulein (CAE), and in mice given GJ (1 mg/kg) at the same time as the first cerulein injection; B: Histology sections of the lung harvested 12 h after administration of saline (CON), cerulein alone, or GJ (1 or 0.1 mg/kg) given at the same time as the first injection of cerulein. The results were scored from 0 (normal) to 3 (severe) for edema, inflammation, vacuolization, and necrosis. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows one experiment in which 4-5 mice were tested per group. The results obtained were similar to those in three additional experiments (x 200).

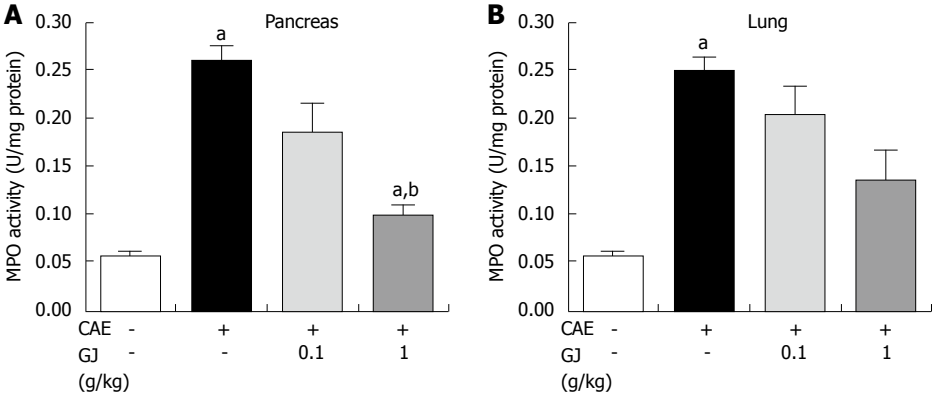

Figure 6 MPO activity was measured in the pancreas 6 h after completion of the cerulein injections and in saline-injected control mice (CON). The data are expressed as MPO activity (U/mg protein). [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows the results of one experiment in which 5-6 mice were tested per group.

DISCUSSION

AP is associated with a high rate of morbidity and mortality. The mortality rate in patients with severe AP is as high as 20% to 30%[1,2]. AP, characterized by interstitial edema, vacuolization, inflammation and acinar cell necrosis, is commonly caused by excessive ethanol consumption, biliary tract disease, certain medications, and invasive procedures of the biliary and pancreatic ducts[19-22]. The pathophysiology of AP is poorly understood, and the clinical course is unpredictable[7].

The fruit of GJ has been used as an oriental herbal medicine in traditional formulations. It has been employed in the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders, and hypertension. In addition, the pigments obtained from the fruit are used as food colorants. The pharmacologic actions of GJ, such as protective effect against oxidative damage, cytotoxic activity, anti-inflammatory actions, and fibrolytic activity have been described in detail[23,24]. The present study was carried out to determine whether GJ could inhibit the severity of cerulein-induced AP.

Several markers of AP such as PW/BW, amylase and lipase activity were reduced (Figure 1). There is much evidence to implicate the involvement of inflammatory mediators, such as cytokines, TNF-α, IL-1β, and IL-6 in the development of pancreatitis[25]. In experimental pancreatitis, the serum levels of TNF-α and IL-1β are elevated and their blockade attenuates the disease process[18,25]. IL-6 is one of the principal cytokine mediators of the acute-phase response, and has been suggested as a marker for predicting the severity of AP[25]. As shown in Figures 2 and 3, GJ may inhibit these cytokines in AP.

The results of the present study indicate that cerulein caused significant morphological abnormalities in the pancreas, as demonstrated by the appearance of vacuolization, inflammatory infiltration and changes in histo-architecture of the pancreatic acini. Pretreatment with GJ inhibited acinar cell death as well as infiltration by inflammatory cells in cerulein-induced AP. To rule out interference by the binding of cerulein to CCK receptors on pancreatic acinar cells, we examined the effect of GJ in pancreatic acinar cells. GJ itself did not have any cytoxicity at 6 h. However, GJ inhibited cerulein-induced aciniar cell death in a dose-dependent manner (data not shown).

Lung injury commonly develops early in AP. AP-associated lung injury is characterized by edema and inflammation[12]. Therefore, we also assessed the lungs after cerulein administration. Histological examination of lung sections (at 12 h after the injection of cerulein) revealed tissue damage characterized by edema and inflammatory cell infiltrate. GJ pretreatment resulted in a significant reduction in lung injury. The histology sections were scored for edema and inflammation and, as shown in Figure 5, the lungs of the GJ pretreated mice had significantly less edema and inflammation compared with lungs from the saline treated control animals.

In conclusion, the present study shows that GJ pretreatment ameliorated the severity of cerulein-induced AP in rats. Additionally GJ pretreatment ameliorated many of the laboratory and biochemical parameters of the disease. Our findings suggest that GJ may be beneficial in the treatment of acute pancreatitis.

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RAPID COMMUNICATION

Patient education improves adherence to peg-interferon and ribavirin in chronic genotype 2 or 3 hepatitis C virus infection: A prospective, real-life, observational study

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Abstract

AIM: To evaluate the impact of therapeutic education on adherence to antiviral treatment and sustained virological response (SVR) in a real-life setting in genotype 2/3 hepatitis C, as there are few adherence data in genotype 2/3 infection, even from randomized trials.

METHODS: This prospective survey included genotype 2/3 patients who received peg-interferon alfa-2b and ribavirin. There was no intervention. Adherence was self-reported over the past 4 wk (peg-interferon) or 7 d (ribavirin). Adherence to bitherapy was defined as adherence to the two drugs for ⩾ 20 wk. SVR was defined as undetectable RNA ⩾ 12 wk after the end of treatment.

RESULTS: 370/674 patients received education during the first 3 mo of treatment. After 6 mo, adherence to bitherapy was higher in educated patients (61% *vs* 47%, $P = 0.01$). Adherence to peg-interferon was 78% *vs* 69% ($P = 0.06$). Adherence to ribavirin was 70% *vs* 56% ($P = 0.006$). The SVR (77% *vs* 70%, $P = 0.05$) and relapse (10% *vs* 16%, $P = 0.09$) rates tended to be improved. After adjustment for baseline differences, education improved adherence [Odds ratio (OR) 1.58, $P = 0.04$] but not the SVR (OR 1.54, $P = 0.06$).

CONCLUSION: In genotype 2/3 patients, therapeutic education helped maintain real-life adherence to bitherapy.

Key words: Viral hepatitis; Adherence; Therapeutic education; Real life; Peg-interferon; Ribavirin

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INTRODUCTION

As pointed out by the WHO[1], poor adherence to treatment is a worldwide issue in all chronic conditions, which results in poor health outcomes and increased health care costs[2,3]. Even in clinical trials, mean adherence rates are low (43%-78%) in chronic conditions[4-6]. In clinical practice, adherence rates of

about 50% are usually reported[7].

Adherence to therapy is critical in the treatment of chronic hepatitis C virus (HCV) infection. The current gold standard therapy is a combination of peg-interferon alfa and ribavirin[8,9]. Patients with genotype 1 infection have a 42%-51% likelihood of achieving a sustained virological response (SVR) after 48 wk of therapy; 78%-82% of patients with genotype 2 or 3 infection respond to 24 wk of treatment, whereas patients with genotype 3 infection and high viral load are difficult to treat (< 70% responders)[10,11]. Non-responders to prior standard bitherapy respond to retreatment in 13% of the cases (29% in non-1 genotype), and relapsers in 58.5% of the cases[12]. Therapy requires weekly subcutaneous injections, twice-daily oral dosing and frequent visits, with blood tests. Side effects occur in nearly all patients. As a result, 15%-20% of patients in clinical trials and > 25% in clinical practice discontinue therapy.

In clinical trials, SVR was significantly improved in those patients with HCV genotype 1 infection who received > 80% of their total peg-interferon dose and > 80% of their ribavirin dose for > 80% of the scheduled treatment duration, in comparison with those who failed these adherence criteria[13]. A review of the 2002-2007 literature confirmed that treatment response is influenced not only by HCV genotype and viral load, but also by patient-related factors including adherence[14]. Moreover, optimal HCV healthcare requires further efforts from providers in communicating with patients, as advocated in France by hepatitis C experts and the Health Ministry[15,16], and shown in studies using patient questionnaires in North America[17,18].

We carried out a large survey named CheObs to evaluate adherence to chronic hepatitis C treatment in the real-life setting in France. We observed that some patients received therapeutic education by a third party (other than the investigator), at the discretion of the investigators, during the study period. According to the consensus that efforts to boost treatment adherence improve SVR rates, we performed the present analysis to evaluate the impact of patient education on real-life adherence and response to treatment with peg-interferon alfa-2b and ribavirin. This analysis was carried out in patients with genotype 2/3 HCV infection (one third of the CheObs cohort), as there are few adherence data for these patients even from randomized trials, and because their data were available before those of patients with other genotypes, due to shorter treatment duration.

MATERIALS AND METHODS

The prospective, multicenter, CheObs survey was carried out in teaching hospitals, non-teaching hospitals, and private practice offices highly involved in the management of hepatitis C in France, and supervised by a Scientific Committee. Consecutive patients aged ⩾ 18 years with chronic hepatitis C were enrolled if initiation of bitherapy with peg-interferon alfa-2b and ribavirin was scheduled. They could be naive for any chronic hepatitis C therapy or non-responders/relapsers to previous therapy. In accordance with French law, the Ethics Committee's approval was not required as the protocol was strictly observational and usual practice was unchanged. However, all patients gave informed consent to participate.

Included patients saw their physician at a frequency corresponding to the usual practice in the center. The investigator and the patient completed a questionnaire each at inclusion, at the visits occurring approximately every 3 mo during treatment, and at the visit occurring approximately 6 mo after the end of treatment. Patients filled in their questionnaires in the waiting room and either gave it back to the investigator in a sealed envelope or returned it using a prepaid envelope.

The investigators recorded socio-demographic data, history of HCV infection (including previous treatments), risk factors, comorbidities, patient therapeutic education (provided or not), planned/prescribed hepatitis C treatment, modification of treatment during follow-up, concomitant medications, and adverse events. The virological status, documented by qualitative PCR (Amplicor™, Roche) and test date, was recorded at the last visit.

The patient questionnaires concerned adherence to peg-interferon and ribavirin, and the persons involved in the management of their disease (e.g. health professionals of any discipline, patient associations). The following parameters relating to the past 4 wk were recorded to evaluate adherence to peg-interferon: date of injections (or reason for not having an injection), frequency of and reason for taking peg-interferon at a higher/lower dose than prescribed. The following parameters relating to the past 7 d were recorded to evaluate adherence to ribavirin: number of (200 mg) capsules prescribed morning and evening, date of dosing and number of capsules taken in the morning and evening, reasons for missing doses, reasons for and frequency of taking more/less capsules than prescribed.

As for any survey, there was no protocol-specific intervention. Therapeutic education was defined by intervention of a third party (healthcare professionals other than the prescribing physician) and distribution of support documents and educational material during individual sessions. It was provided at the discretion of the physician. No instruction was given related to which patient should be considered or how education should be provided.

Adherence to bitherapy was defined as adherence to both peg-interferon and ribavirin over a sufficient time period, as self-reported by the patients. According to the "80/80/80" criteria previously defined[13], patients were considered to have adhered to peg-interferon if they had received three or four injections during the past 4 wk, and to have adhered to ribavirin if they had taken at least 22 (200 mg) capsules over the past week. Patients were considered to have adhered to bitherapy if they had adhered to the two drugs for at least 20 wk. These rules were defined according to the recommended number of peg-interferon injections (one per week), the recommended ribavirin daily dose (at least 800 mg/d),

and the recommended duration of bitherapy in genotype 2/3 HCV infection (24 wk).

SVR was defined as undetectable HCV RNA in the serum 12 wk after the end of treatment or later. This time interval was considered to be sufficient for this evaluation, since relapse after 12 wk of follow-up is rarely (2%) observed whatever the HCV genotype[19], and the dates of the visits and laboratory tests could not be forced in this observational protocol. Non-response was defined as detectable RNA at the end of treatment, and relapse as undetectable RNA at the end of treatment but detectable at a later time point.

Statistical analysis

Due to the lack of data in the literature, the CheObs sample size calculation was based on real-life adherence observed in chronic conditions other than chronic HCV infection, such as HIV infection[20], diabetes[21], or hypertension[22]. A total of 1537 patients were required to estimate a 50% adherence rate, with a precision of 2.5% and a type I error of 0.05. Assuming 25%-30% of patients were lost to follow-up or discontinued treatment early, approximately 2000 patients were included overall.

The present analysis was carried out in the subset of patients with genotype 2/3 HCV infection from the CheObs cohort. Statistical analysis was conducted using SAS 8.2 (SAS Institute Inc, Cary, NC, USA). Tests were two-sided and type I error was set at 0.05. Descriptive statistics were performed using all available data. Group comparisons were carried out using Kruskal-Wallis or Fisher's exact tests. The relationships between adherence or virological response and a set of potential explanatory variables were analysed by forward stepwise logistic regressions. These variables included not only those for which groups differed significantly at baseline ($P < 0.05$), but also those expected to have a significant impact according to the literature.

RESULTS

Patients' characteristics

Between 2002 and 2006, 184 investigators enrolled 2001 HCV patients in the CheObs survey, including 705 patients infected with the genotype 2/3 HCV (Figure 1). Of these, 674 patients were analyzed. We observed that 370/674 (55%) patients received therapeutic education during the first 3 mo of treatment and 304 (45%) did not. Among the 82 centers which included the analyzed population, 24 (29%) did not educate any patient, 18 (22%) educated > 0 to 50% of their patients, 19 (23%) educated > 50% to < 100% of their patients, and 21 (26%) educated 100% of their patients. The 31 patients excluded from analysis were similar to the analyzed population for the therapeutic education rate (58%) and all other baseline variables, except for the Metavir activity score, which was more frequently A2/A3 (78% *vs* 50%, $P = 0.009$).

In the analyzed population, educated patients had a higher body weight (70.5 ± 14.0 kg *vs* 67.9 ± 14.3 kg, $P = 0.02$) than patients without therapeutic education,

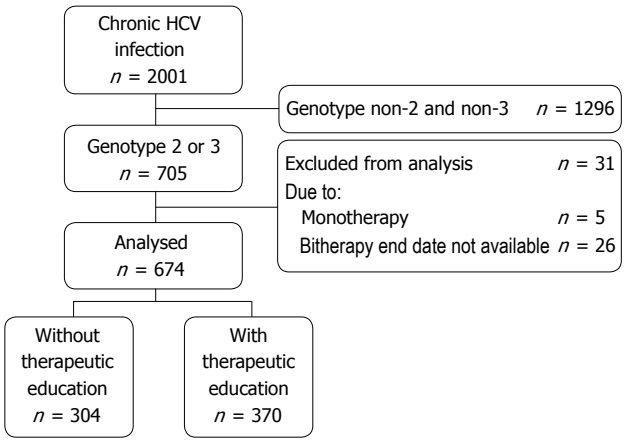

Figure 1 Patient flow.

but a similar body mass index (BMI) (Table 1). They more frequently had a past history of depression ($P = 0.01$) and current psychiatric disorders ($P = 0.04$, mainly depression and anxiety), though there was no difference for the nature or proportion of each disorder. Educated patients were also more frequently psychoactive drug users ($P = 0.02$, mainly cocaine and heroin), but the profile of consumption (injecting behavior and frequency of abuse) was similar. Regarding HCV infection, although educated patients had more frequently significant liver fibrosis than those without therapeutic education ($P = 0.04$), the proportion of cirrhotic patients was similar (13% and 15%).

Treatment

A 24-wk bitherapy was scheduled in most patients (571/667, 86%), with no marked difference between groups (Table 2). Treatment for 48 wk was more frequently planned in non-responders/relapsers to previous therapy (43/125, 34%) than in naive patients (49/542, 9%, $P < 0.001$). The planned weekly dose of peg-interferon was higher in educated patients ($P = 0.01$), at 1.5 μg/kg per week in most cases (82% *vs* 75%) whereas patients without therapeutic education were more frequently prescribed lower doses. However there was no difference between the groups for the proportion of retreated patients (Table 1) or that of relapsers/non-responders to previous treatment ($P = 0.95$). The ribavirin dose prescribed was similar in both groups. It was 800 mg/d in half of the patients (344/669, 51%) and < 800 mg/d in 18 (3%) patients.

The actual duration of bitherapy was shorter than 20 wk in 85/674 (13%) patients ($P = 0.20$ between groups, Table 2), with an average of 11.1 ± 4.9 wk (median 12) in these patients. Reasons for premature discontinuation were: lost to follow-up ($n = 28$), safety ($n = 27$), patient's request ($n = 14$), virological (HCV RNA detectable or decrease < 2 log, $n = 7$), unknown ($n = 3$), investigator's request ($n = 2$), and other ($n = 4$). They were similar in nature and occurrence ($P = 0.79$) in both groups. These patients, who were not included in the analysis of virological response, did not differ significantly from those who received a longer bitherapy

Table 1 Baseline characteristics of the patients (n = 674): Univariate analysis

	Therapeutic education		
	No (n = 304)	Yes (n = 370)	P
Socio-demography			
Men	170/303 (56)	229/370 (62)	0.14
Age (yr)	44.1 ± 11.4	44.9 ± 11.4	0.16
Body mass index (kg/m^2)	23.5 ± 4.1	24.1 ± 4.2	0.06
Employment status			
Professional activity	190/303 (63)	220/370 (59)	0.59
Unemployed	48/303 (16)	69/370 (19)	
Other	65/303 (22)	81/370 (22)	
Educational level			
Low	182/300 (61)	223/365 (61)	0.94
High	118/300 (39)	142/365 (39)	
Origin of incomes			
Paid employment	166/298 (56)	183/368 (50)	0.24
Unemployment incomes	48/298 (16)	60/368 (16)	
Other	84/298 (28)	125/368 (34)	
Debts			
Difficult to manage	8/232 (4)	27/315 (9)	0.02
None or easily managed	224/232 (97)	288/315 (91)	
Comorbidities			
Past psychiatric history			
Depression	67/303 (22)	113/370 (31)	0.01
Suicide attempt	23/302 (8)	29/368 (8)	0.81
Hospitalisation for mental disease	25/301 (8)	36/369 (10)	0.59
Psychiatric disorder	60/300 (20)	98/367 (27)	0.04
Chronic disease	64/298 (22)	89/366 (24)	0.41
Risk factors			
Alcohol consumption > 20 g/d	10/70 (14)	24/100(24)	0.17
Tobacco consumption	167/299 (56)	187/366 (51)	0.24
Drug abuse			
None	139/303 (46)	172/368 (47)	0.02
Former	158/303 (52)	174/368 (47)	
Current	6/303 (2)	22/368 (6)	
HCV infection			
Source of HCV infection[1]			
Transfusion	62/304 (20)	76/370 (21)	1.00
Injection or intranasal drug abuse	157/304 (52)	193/370 (52)	0.94
Other	86/304 (29)	100/370 (27)	0.86
Duration of HCV infection (year)	20.4 ± 8.4	20.0 ± 8.9	0.85
Serum HCV-RNA			
⩽ 800 000 IU/mL or equivalent	121/193 (63)	162/265 (61)	0.77
> 800 000 IU/mL or equivalent	72/193 (37)	103/265 (39)	
HCV genotype			
2	85/304 (28)	117/370 (32)	0.31
3	219/304 (72)	253/370 (68)	0.31
Coinfection			
Human immunodeficiency virus	12/303 (4)	14/369 (4)	1.00
Hepatitis B virus[2]	3/301 (1)	6/369 (2)	0.63
Metavir activity grade or equivalent			
A0 or A1	119/226 (53)	131/272 (48)	0.32
A2 or A3	107/226 (47)	141/272 (52)	
Metavir fibrosis stage or equivalent			
F0 or F1	110/227 (49)	109/272 (40)	0.04
F2 or F3	82/227 (36)	129/272 (47)	
F4	35/227 (15)	34/272 (13)	
Knodell score	7.9 ± 3.0	8.4 ± 3.5	0.19
Previous anti-HCV treatment course			
None	242/303 (80)	303/370 (82)	0.74
One or more	61/303 (20)	67/370 (18)	

Data are expressed as mean ± SD, or proportions of patients. [1]One patient could have more than one presumed source of infection. [2]Hepatitis B antigen positivity.

Table 2 Treatment planned and actually received (n = 674): Univariate analysis

	Therapeutic education		
	No (n = 304)	Yes (n = 370)	P
Duration of bitherapy (wk)[1]			
Planned	27.7 ± 8.9	27.1 ± 8.2	0.49
Actual	28.4 ± 12.7	30.3 ± 14.2	0.25
Premature discontinuation (< 20 wk)	44/304 (15)	41/370 (11)	0.20
Peginterferon weekly dose (μg/kg)			
Planned	1.35 ± 0.29	1.41 ± 0.22	0.01
Actual at 3 mo[2]	1.31 ± 0.30	1.38 ± 0.25	0.006
Actual at 6 mo[2]	1.18 ± 0.35	1.25 ± 0.31	0.02
Ribavirin daily dose (mg)			
Planned	897 ± 147	906 ± 154	0.67
Actual at 3 mo[2]	871 ± 166	885 ± 175	0.25
Actual at 6 mo[2]	771 ± 201	803 ± 209	0.06

Data are expressed as mean ± SD, or proportions of patients. [1]Date of end of bitherapy minus date of inclusion; [2]Cumulated over the past 3 mo (AUC, investigator report).

with regards to the main variables.

Physicians modified the peg-interferon dose less frequently in educated patients (16% *vs* 22% without therapeutic education, P = 0.046), whereas the ribavirin dose was changed in similar proportions of patients in both groups (17% *vs* 18%, P = 0.28) nearly always because of adverse effects and weight loss and depression in particular. The difference in the dose between groups remained constant for peg-interferon whereas it increased over time for ribavirin, in particular after the third month of treatment (Table 2). The occurrence of adverse events over the whole study was 82% of patients in each group (P = 0.92).

Adherence to bitherapy

Overall adherence to bitherapy was 64% (301/468) at 3 mo and 55% (209/383) at 6 mo of treatment. Adherence to peg-interferon (80% and 74% at 3 and 6 mo, respectively) was higher than to ribavirin (72% and 64%, respectively). Patients prescribed high doses of ribavirin (⩾ 1000 mg/d) did not differ significantly from those with lower doses with respect to premature treatment discontinuation and adherence to peg-interferon and/or ribavirin.

At 3 mo, the proportion of adherents to both drugs was 66% with therapeutic education and 63% without therapeutic education (non-significant difference) (Figure 2 and Table 3). At 6 mo, this proportion was still 61% in educated patients, whereas it dropped down to 47% without therapeutic education (P = 0.01). Multivariate analysis showed that, after adjustment, therapeutic education increased the probability of adhering to bitherapy at 6 mo by a factor of 1.58 (95% CI: 1.02 to 2.46).

At 6 mo, the adherence rate was still 78% for peg-interferon and 70% for ribavirin in educated patients, whereas it was reduced to 69% (P = 0.06) and 56% (P = 0.006), respectively, in patients without therapeutic

Table 3 Adherence to treatment, virological response (univariate analysis) and their association with therapeutic education (multivariate analysis)

	Therapeutic education					
	Univariate analysis			Multivariate analysis		
	No (n = 304)	Yes (n = 370)	P	OR	95% CI	P
Adherence[1] at 3 mo						
Bitherapy	137/218 (63)	164/250 (66)	0.56	1.04	0.69 to 1.56	0.87
Peginterferon	175/218 (80)	201/250 (80)	1.00	0.94	0.57 to 1.53	0.79
Ribavirin	152/218 (70)	186/250 (74)	0.30	1.13	0.72 to 1.77	0.59
Adherence[1] at 6 mo						
Bitherapy	83/175 (47)	126/208 (61)	0.01	1.58	1.02 to 2.46	0.04
Peginterferon	121/175 (69)	162/208 (78)	0.06	1.78	1.07 to 2.96	0.03
Ribavirin	98/175 (56)	145/208 (70)	0.006	1.67	1.05 to 2.65	0.03
Virological response[2]						
SVR	171/246 (70)	230/298 (77)	0.05	1.54[3]	0.99 to 2.40	0.06
Nonresponse	37/246 (15)	37/298 (12)	0.38			
Relapse	38/246 (16)	31/298 (10)	0.09			

Data are expressed as proportions of patients. Multivariate analyses were adjusted for sex, weight, BMI, educational level (adherence only), history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, HCV genotype (SVR only), and peginterferon dose prescribed at inclusion. [1]≥ 3 peginterferon injections during the last 4 wk and/or ≥ 22 ribavirin capsules of 200 mg each during the last 7 d (self-report); [2]≥ 12 wk after the end of treatment; [3]The rate of SVR was used as dependent variable.

education. After adjustment, therapeutic education increased the probability of adherence by a factor of 1.78 for peg-interferon and 1.67 for ribavirin after 6 mo of bitherapy.

Educated patients more frequently reported contacting persons in the hospital for management of their disease, with a median of three persons *vs* two for patients without therapeutic education during the first 3 mo of treatment (P=0.016), and three persons *vs* one during the next 3 mo (P = 0.003). Conversely, patients without therapeutic education reported more frequent contacts with office-based persons, with a median of two persons *vs* one in educated patients during the first 3 mo (P = 0.012), and two persons *vs* none during the next 3 mo (P < 0.001).

Virological response

The overall SVR rate was 74% (401/544). There were 13.6% of non-responders and 12.7% of relapsers. The virological response was better, though not significantly, in educated patients (Figure 2 and Table 3). The SVR rate was higher (77% *vs* 70%, P = 0.05) and the rate of relapse was lower (10% *vs* 16%, P = 0.09) in these patients than in patients without therapeutic education. Multivariate analysis confirmed that, after adjustment, the relationship between SVR and therapeutic education was borderline significant (P = 0.06). Response was better in patients whose ribavirin dose was not reduced during the first 3 mo of treatment (P < 0.001), mostly due to an increased SVR rate (362/473, 77% *vs* 37/67, 55%, P = 0.001) and a decreased rate of non-response (51/473, 11% *vs* 21/67, 31%, P < 0.001).

The virological response is shown taking into account genotype and baseline viral load (> or ≤ 800 000 IU/mL) in Figure 3. Though this effect was not significant, therapeutic education was found to have a beneficial impact in all patients. In those with genotype 2 and low viral load, an impact on the SVR (P = 0.038) and relapse (P = 0.047) rates was observed, but the sample size was small (n = 80).

Moreover, although favorable trends were observed, therapeutic education had no significant impact on the virological response in patients treated for the first time (P = 0.27) or in non-responders/relapsers to previous therapy (P = 0.22).

DISCUSSION

We evaluated the impact of patient therapeutic education by a third party on adherence and virological response to peg-interferon alfa-2b and ribavirin in 674 patients with chronic genotype 2/3 HCV infection. This is the first time that adherence has been evaluated in patients carrying these genotypes, and also the first time that adherence has been assessed in a real-life setting.

Therapeutic education given during the first 3 mo of treatment significantly improved the proportion of patients adhering to bitherapy at 6 mo (OR 1.58, P = 0.01 after adjustment for sex, weight, BMI, educational level, history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, and peg-interferon dose prescribed at inclusion). The proportion of adherents was stable above 60% until the sixth month of treatment in educated patients, whereas it dropped by more than 10% between the third and sixth month of treatment when there was no therapeutic education. The virological response was also improved in educated patients, with an increased SVR rate (77% *vs* 70%) and a lower relapse rate (10% *vs* 16%), though this effect was statistically not significant (P = 0.06 after adjustment for sex, weight, BMI, history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, HCV genotype, and peg-interferon dose prescribed at inclusion). Since a 12-wk treatment may be very effective

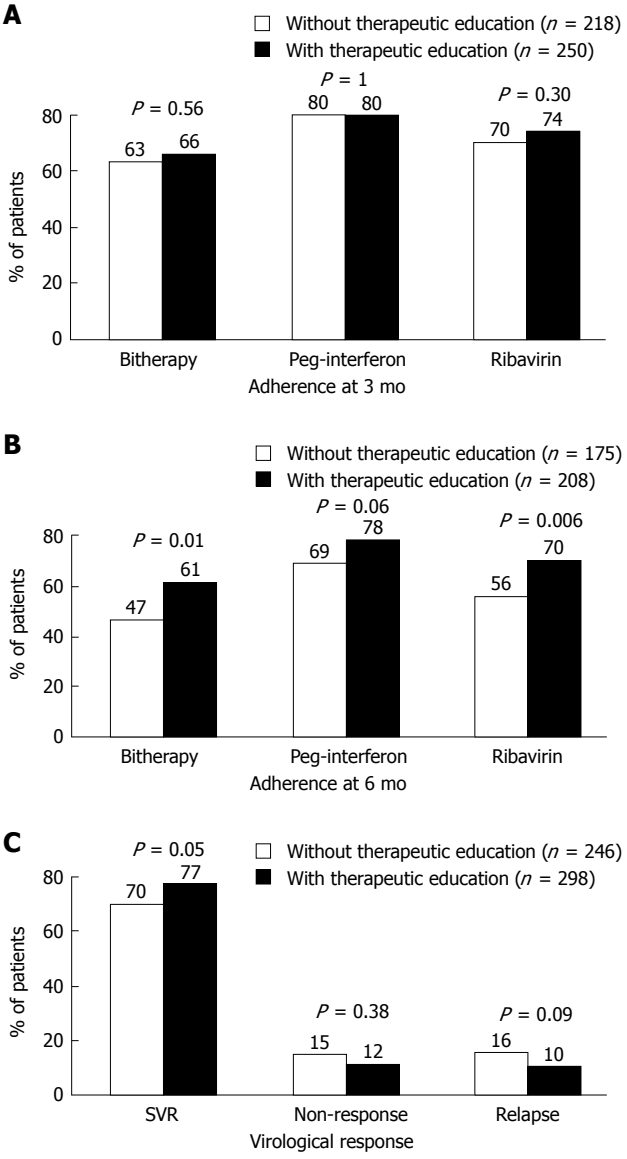

Figure 2 Impact of patient therapeutic education on adherence to treatment (A and B) and virological response (C). At 3 mo of treatment, the proportion of patients adhering to treatment (patient self-report) was similar in the two groups. At 6 mo, the proportion of adherents dropped in patients without therapeutic education only. The virological response was better in educated patients, with an increased rate of SVR and a lower relapse rate.

in naive genotype 2/3 patients, the fact that adherence was the same over 12 wk may explain why adherence in the later part did not translate to significantly higher SVR rates. As suggested by the borderline *P* value, this might also be due to the fact that inclusion of one-third of the CheObs cohort in this analysis limited the power to detect statistical significance.

The overall adherence rate to ribavirin (64%-72%) was consistently lower than that to peg-interferon (74%-80%), as expected from their regimen. The ribavirin regimen is somewhat complex (twice-daily oral dosing) whereas peg-interferon alfa-2b adherence is facilitated by the low frequency of administration (weekly injections) and availability of an injecting pen device. Therapeutic education significantly enhanced adherence to ribavirin, which is particularly important for obtaining an SVR[23]. A positive impact was also observed on adherence to peg-interferon, showing that an improvement can still be obtained.

Our results are consistent with the general consensus that patient therapeutic education in clinical practice effectively improves adherence to treatment in chronic disease[2,15,16] and that adherence, even to placebo, is essential to achieve health outcome goals[3]. It should be noted however, that education required the cooperation of specialists and nursing staff, and therefore probably affected both patient-related and health care team-related factors, which are recognized to improve adherence.

The adherence rate to bitherapy (64%) was low compared to the rates of > 70% reported in clinical trials[13]. The SVR rate observed here (72%) was however, in line with that reported in clinical trials (80%) in genotype 2/3 HCV patients treated with similar therapy[24]. Apart from the fact that results are usually more marked in randomized trials, our population included a high proportion of patients with psychiatric disorders and/or drug addicts. This suggests that in real-life situations such comorbidities and high-risk behavior do not have an impact on adherence or response to treatment, so that they may not be as difficult to treat as usually believed.

Unlike during clinical trials, patients in this observational study were not selected or obliged to follow specific procedures and physicians were not instructed on which patient should be considered for therapeutic education. This may be considered to be a weakness of the study, as comparisons were performed on non-randomized groups. The CheObs study was not designed to perform comparisons, but aimed to provide a picture of the real-life setting. The use of multivariate analysis in the present evaluation reduced such bias by taking into account the differences between groups observed at baseline.

The method used to assess adherence may also be criticized. When the study was designed, we chose to use data reported by patients rather than by investigators, to best reflect the real-life situation. However, asking patients to fill a diary each day would have influenced their behavior and led to an unquantifiable overestimation of our primary endpoint. Adherence data were therefore collected over limited time periods. We also chose not to take into account body weight when calculating adherence to ribavirin. Theoretically, we overestimated adherence to ribavirin in patients over 65 kg body weight, i.e. in approximately half the study population at baseline. This bias was however reduced, as weight is known to decrease markedly over time in most treated patients and, as expected, dose reductions occurred for safety reasons in a large proportion of patients (17%-18%) in both groups.

Our results demonstrate that, although patients with HCV genotype 2/3 are those who usually show the best response rates, further efforts may be made to improve outcomes. There is more than one barrier preventing patients from optimal compliance to their treatment regimen[1]. Five interacting dimensions affect adherence: social and economic factors, and factors related to the

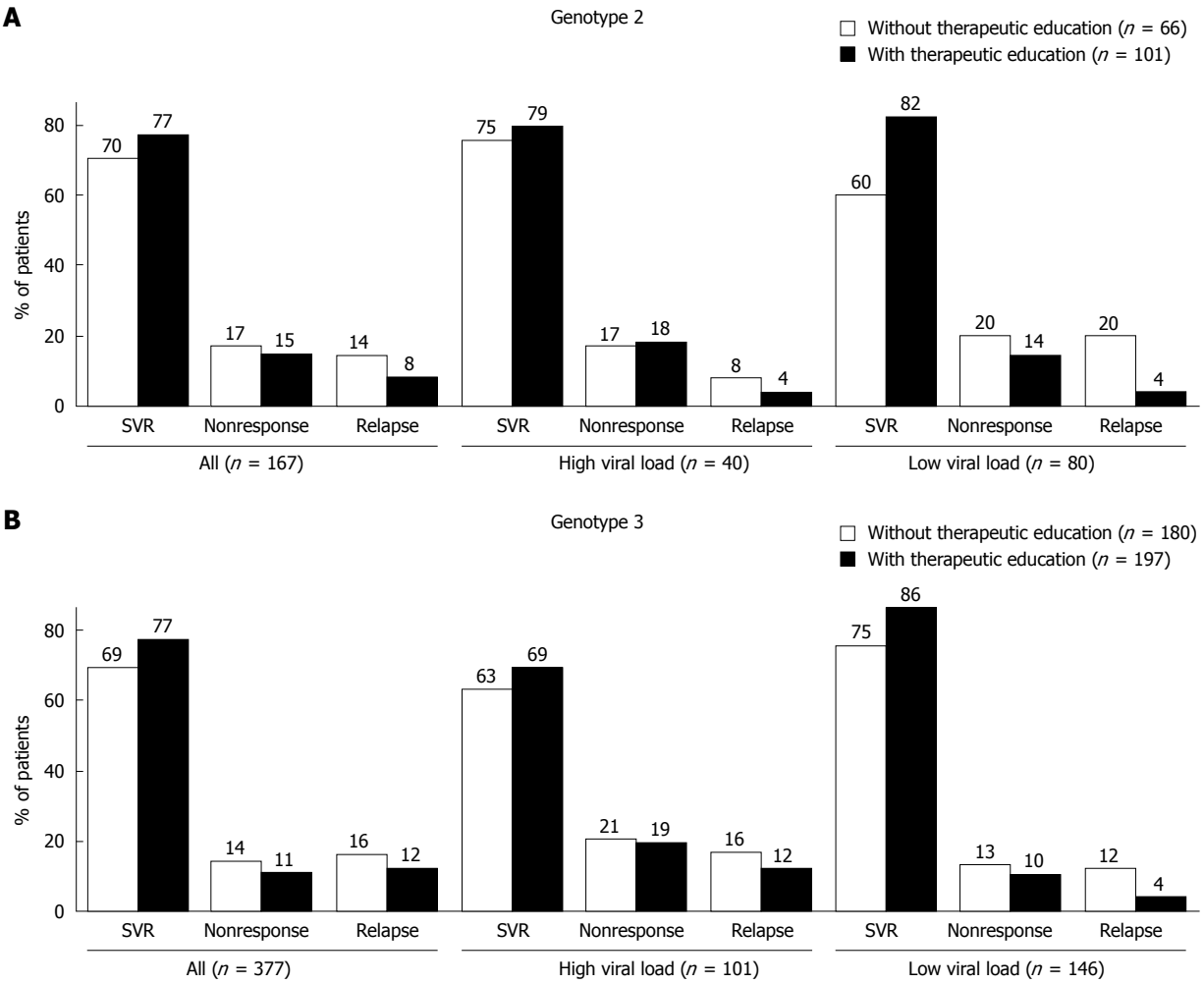

Figure 3 Virological response in patients infected with genotype 2 (A) or genotype 3 (B), and according to viral load (> or ⩽ 800 000 IU/mL). Although not statistically significant, patient therapeutic education was beneficial in all patient subgroups, especially in those with genotype 2 and low viral load, with a marked impact on the SVR (SVR, $P = 0.038$) and relapse ($P = 0.047$) rates.

health care team and system, the condition, treatment, and the patient[2,15,16]. Increasing the impact of interventions aimed at patient-related factors and/or health care team-related factors is essential. Methods that have been shown to be effective in improving therapy include: educational interventions involving patients[25,26]; strategies to improve dosing schedules[6]; interventions that enlist ancillary health care providers such as pharmacists, behavioral specialists, and nursing staff[27,28]; and enhancing communication between physicians and patients[29-31].

To conclude, in the real-life setting, therapeutic education helped maintain adherence to bitherapy in patients with genotype 2/3 infection. There was a trend for a benefit on virological response. This analysis of the real-life impact of patient education by a third party on health outcomes may help to further improve patient quality of life and outcome. The importance of adherence and the role of education should now be studied in a randomized controlled trial in genotype 1/4 infection.

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COMMENTS

Background

Adherence to therapy is critical in the treatment of chronic hepatitis C virus (HCV) infection. The current gold standard therapy is a combination of peg-interferon alfa and ribavirin. Patients with genotype 2 or 3 infection have a 78%-82% likelihood of achieving a sustained virological response (SVR) after 24 wk of therapy, whereas patients with genotype 3 infection and high viral load are difficult to treat (< 70% responders). Therapy requires weekly subcutaneous injections, twice-daily oral dosing and frequent visits, with blood tests. Side effects occur in nearly all patients. As a result, 15%-20% of patients in clinical trials and over 25% in clinical practice discontinue therapy.

Research frontiers

In clinical trials, the SVR rate was significantly improved in those patients with HCV genotype 1 infection who received > 80% of their total peg-interferon dose and > 80% of their ribavirin dose for > 80% of the scheduled treatment duration. A review of the 2002-2007 literature confirmed that treatment response is influenced not only by HCV genotype and viral load, but also by patient-related factors including adherence.

Innovations and breakthroughs

The authors evaluated, perhaps for the first time, the impact of patient therapeutic education by a third party on adherence and virological response to peg-interferon alfa-2b and ribavirin. The analysis was carried out in the 674 patients with chronic genotype 2/3 HCV infection from the CheObs cohort. Therapeutic education given during the first 3 mo of treatment significantly improved the proportion of patients adhering to bitherapy at 6 mo (odds ratio 1.58). Though not significantly, the virological response was also improved in educated patients, with an increased SVR rate (77% *vs* 70%) and a lower relapse rate (10% *vs* 16%). Therapeutic education significantly enhanced adherence to ribavirin, which is particularly important for obtaining a SVR. A positive impact was also observed on adherence to peg-interferon, showing that an improvement can still be obtained.

Applications

This analysis of the real-life impact of patient therapeutic education by a third party on health outcomes may help to further improve patient quality of life and outcome. Five interacting dimensions affect adherence: social and economic factors, and factors related to the health care team and system, the condition, treatment, and the patient. Methods that have been shown to be effective in improving therapy include: educational interventions involving patients; strategies to improve dosing schedules; interventions that enlist ancillary health care providers such as pharmacists, behavioral specialists, and nursing staff; and enhancing communication between physicians and patients.

Peer review

This is a subgroup analysis of a bigger project. Since the a priori power calculation was based on 2000 subjects for meaningful statistical analysis, the inclusion of only 630 or so patients in this study clearly limits the power to detect statistical significance.

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RAPID COMMUNICATION

Hydrogen breath test for the diagnosis of lactose intolerance, is the routine sugar load the best one?

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Abstract

AIM: To evaluate the prevalence of lactose intolerance (LI) following a load of 12.5 g in patients diagnosed as high-grade malabsorbers using the hydrogen breath test (HBT)-25.

METHODS: Ninety patients showing high-grade malabsorption at HBT-25 were submitted to a second HBT with a lactose load of 12.5 g. Peak hydrogen production, area under the curve of hydrogen excretion and occurrence of symptoms were recorded.

RESULTS: Only 16 patients (17.77%) with positive HBT-25 proved positive at HBT-12.5. Hydrogen production was lower as compared to HBT-25 (peak value 21.55 parts per million (ppm) ± 29.54 SD *vs* 99.43 ppm ± 40.01 SD; $P < 0.001$). Symptoms were present in only 13 patients. The absence of symptoms during the high-dose test has a high negative predictive value (0.84) for a negative low-dose test. The presence of symptoms during the first test was not useful for predicting a positive low-dose test (positive predictive value 0.06-0.31).

CONCLUSION: Most patients with a positive HBT-25 normally absorb a lower dose of lactose and a strict lactose restriction on the basis of a "standard" HBT is, in most instances, unnecessary. Thus, the 25 g lactose tolerance test should probably be substituted by the 12.5 g test in the diagnosis of LI, and in providing dietary guidelines to patients with suspected lactose malabsorption/intolerance.

Key words: Lactase deficiency; Lactose malabsorption; Lactose intolerance; Hydrogen breath test

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INTRODUCTION

The enzyme lactase phlorizin hydrolase, located at the intestinal brush border, is necessary for the hydrolysis of lactose, the main sugar in milk. Due to the genetically programmed decrease in intestinal lactase activity that occurs post-weaning (lactase non-persistence), a large proportion of the human population loses, in adult age, the possibility to digest and absorb lactose[1-3]. In Europe, its prevalence increases with a north-south and west-east gradient. Thus, about 50% of adult Italians cannot digest and absorb lactose normally[4,5].

Lactose malabsorption (LM) may be asymptomatic or induce symptoms similar to those of functional bowel disorders and irritable bowel syndrome, consisting of abdominal pain, gaseousness, flatulence and diarrhea. LM is not necessarily a predictor of the occurrence of symptoms, and the term "lactose intolerance (LI)" refers to a condition in which abdominal symptoms are experienced after the ingestion of lactose, in milk or dairy food.

The test for identifying the genotype responsible for lactase deficiency[6-8] is not widely available and its use for the diagnosis of LM is debatable. Thus, the diagnosis of LM is usually based on a positive hydrogen breath test (HBT) with an oral load of 25 g lactose (HBT-25) and is often followed by the institution of a lactose-free diet, also in those patients who do not experience abdominal symptoms. This approach is debatable, as a reduction of calcium intake below the recommended daily allowance (RDA) may ensue. Moreover, also in those cases in which

symptoms are triggered by the 25 g of lactose ingested during the test, such a strict reduction of milk and dairy products is often unnecessary, as the amount of lactose administered during the test considerably exceeds the amount of lactose ingested daily, by most adults.

The present study was aimed at evaluating whether a reduction in the daily intake of milk and lactose-containing food is really necessary in subjects with LM during a standard HBT-25. To this end, we performed the HBT with an oral load of 12.5 g lactose (HBT-12.5) in a group of patients with marked LM documented by means of HBT-25. Positivity of the test, occurrence and type of symptoms during the two tests were compared.

MATERIALS AND METHODS

During the period January, 2001 to May, 2004, 913 outpatients underwent a lactose tolerance test in our laboratory. The HBT was performed after 24 h on a low-fiber diet and a 12-h fasting period with an oral load of lactose at a dose of 0.5 g/kg body weight, up to a maximum of 25 g. End-alveolar air samples were collected in syringes using a modified Haldane-Priestly tube[9], prior to the administration of lactose, and thereafter every 30 min for 4 h. Hydrogen (H_2) and methane (CH_4) concentrations were measured in parts per million (ppm) by means of a Quintron Model DP Microlyzer gas chromatograph (Quintron Instruments, Milwaukee, WI, USA). The test was defined as "positive" when a H_2 peak exceeding 20 ppm over baseline values was observed in two or more samples. Tests not fulfilling the above-mentioned criteria were defined as negative. Those patients with a negative HBT, who did not excrete increased amounts of H_2 after oral administration of 20 g lactulose in a subsequent HBT (24 patients), were defined as hydrogen non-producers. A positive test identified patients with LM, irrespective of the presence or absence of abdominal symptoms. Positivity of LM was arbitrarily defined as "high-grade" when H_2 excretion exceeded 70 ppm in at least two samples, and "low-grade" in all other instances.

Of the 353 patients with positive HBT-25, 147 fulfilled the above-mentioned criteria for high-grade LM. Of these, 50 were excluded from the study due to the presence of small bowel diseases, such as Crohn's disease and celiac disease, in which medical treatment or dietary modifications could result in variations in lactase activity. The remaining 97 patients were considered eligible for entry to the study and were required to undergo a further lactose tolerance test, with a lactose load of 12.5 g. Only seven refused to enter the study (compliance 92.78%) and the test was performed, 4-12 wk after the first test, in 90 patients (12 male, 78 female, mean age 41.81 ± 15 SD years). Of these, 65 had experienced symptoms during HBT-25 and 25 had not. The excretion of gas during HBT-12.5 was quantified as: (1) peak H_2 concentration; (2) area under the curve (AUC) of H_2 concentration from 60 to 240 min, calculated with the triangular rule and expressed in arbitrary units of ppm/h. During the test, occurrence and type of symptoms were recorded.

Statistical analysis

Data were analyzed using the χ^2 test, the inference between proportions and the *t*-test for paired data, when appropriate.

RESULTS

Positivity of the HBT-12.5 g

Only 16 (17.7%) of the 90 patients enrolled still had a positive test during the HBT-12.5 while the remaining 74 (82.3%) were negative. The difference between HBT-12.5 and HBT-25, evaluated by means of the inference between proportions, was highly significant ($P < 0.001$). Of the 65 LI patients, only five experienced symptoms during the HBT-12.5, while another seven had a positive test, but reported no symptoms. Of the 25 patients with LM, only four had a positive test after an oral load of 12.5 g lactose. None reported symptoms.

Peak H_2 excretion

Considering all 90 patients together, the mean value of peak H_2 excretion during HBT-12.5 was 21.55 ppm ± 29.54 SD, whereas in HBT-25, the mean peak H_2 excretion was 99.43 ppm ± 40.01 SD. As expected, the difference from HBT-25 was highly significant ($P < 0.001$). Considering only the data from the 16 patients who proved positive in both tests, the peak H_2 excretion was 97.68 ppm ± 27.37 during HBT-25 and 69 ppm ± 36.53 SD during HBT-12.5. Thus, even in those patients who had LM during HBT-12.5, the amount of hydrogen excretion was significantly lower as compared to the first test ($P < 0.01$). No difference was found between patients who had symptoms during the test (LI) and those who were LM, but did not experience symptoms, as far as concerning the peak H_2 excretion.

Amount of H_2 excreted (AUC)

The amount of H_2 excreted by the entire population of 90 patients was 18 ppm/h ± 27.12 SD in the HBT-12.5 compared to 97.08 ppm/h ± 40.56 SD in the HBT-25 ($P < 0.001$). Again, taking into account only data from the 16 patients who proved positive in both tests, the amount of H_2 excreted during HBT-12.5, the AUC was significantly lower compared to that of the HBT-25 (54.29 ppm/h ± 41.23 SD *vs* 99.21 ppm/h ± 35.58 SD, respectively; $P < 0.01$). Again no difference was observed between LI and LM.

Symptoms

During the test with 25 g lactose in the 65 patients with LI, gaseousness was present in 36 (55.3%), abdominal pain in 34 (52.3%), flatulence in 16 (24.6%) and diarrhea in eight (13.8%), with some patients reporting more than one symptom (Figure 1). Only five patients with a positive HBT-12.5 experienced symptoms, namely gaseousness in three, flatulence in three and abdominal pain in one. None experienced diarrhea. Eight patients with a negative HBT-12.5 reported experiencing minor symptoms, consisting of gaseousness in five and abdominal pain in five. The relationship between

Table 1 Occurrence of symptoms during positive or negative test with a lactose load of 12.5 g, in relation to the outcome of the test with 25 g lactose

	LI (HBT 25) 65 patients		LM (HBT 25) 25 patients	
	Symptoms +	Symptoms -	Symptoms +	Symptoms -
HBT 12.5 +	5	7	0	4
HBT 12.5 -	8	45	0	21

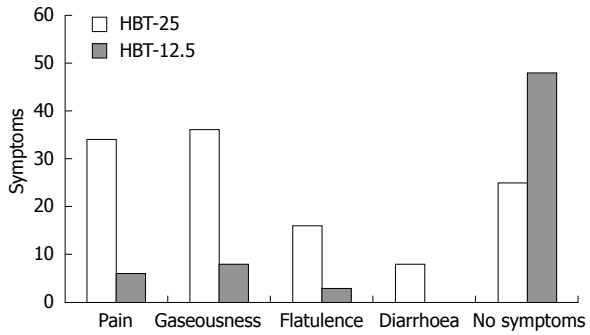

Figure 1 Occurrence of symptoms during the HBT using 25 g lactose (HBT-25) and 12.5 g (HBT-12.5). Some patients reported more than one symptom. It should be noted that, in HBT-12.5, minor symptoms, such as mild abdominal discomfort and abdominal distension, were reported not only by five patients with positive tests, but also by eight patients with a negative one.

positivity/negativity of the low-dose test (HBT-12.5) and occurrence of symptoms during HBT 12.5 is reported in Table 1.

Probability of predicting a positive HBT-12.5

Due to the small number of patients who proved positive during the low-dose test, positivity of HBT-25 does not help predict a positive HBT-12.5 (positive predictive value: 0.17). The occurrence of any symptom during HBT-25 showed only a slightly better positive predictive value for a positive HBT-12.5 (positive predictive value: 0.41). Taking into consideration the occurrence of individual symptoms during HBT-25, the positive predictive value was 0.06 for abdominal pain, 0.25 for gaseousness, 0.31 for flatulence and 0.12 for diarrhea. On the other hand, the absence of abdominal symptoms during HBT-25 had a negative predictive value of 0.84.

DISCUSSION

The diagnosis of LM is usually based upon the positivity of HBT after an oral load of lactose[10-15]. The most commonly used load of lactose is 20-25 g, corresponding to an intake of 400-500 mL of milk, which is rarely ingested in a single dose. Indeed, 400-500 mL of milk exceeds in most instances, the total daily intake of milk and diary products. As HBT has been found to correlate with lactase activity in duodenal biopsies, the HBT-25 is, indeed, useful for population studies[16,17]. HBT, however, is used in the clinical setting with the primary aim of diagnosing LM and LI, the rate of positive tests depending not only upon the degree of hypolactasia, but also the amount in the oral load used for the test. Moreover, the prevalence of symptoms, which is clinically relevant and of great importance for the patient, is dose-related. Thus, the traditional test with 25 g lactose likely overestimates the prevalence of LI. This may lead to unnecessary restrictions in the intake of foods that represent the main source of dietary calcium[18,19]. The present study was aimed at evaluating whether, and to what extent, the use of an oral load of 12.5 g lactose, instead of 25 g, could influence the prevalence of positive tests for diagnosing LI and LM. The present data confirm, in a large series of patients, previous observations showing that high loads of lactose (50 g, corresponding to 1 L of milk) induce abdominal pain and diarrhea in most lactose malabsorbers[20]. Conversely, small amounts of the sugar were usually well tolerated[21-24]. The present data indicates that the absence of abdominal symptoms during an HBT-25 is, in most instances, associated with a negative HBT-12.5. Unexpectedly, the presence of symptoms during HBT-25 was not useful for predicting a positive HBT-12.5. Less than 50% of the patients with abdominal symptoms (LI) display malabsorption of lactose in detectable amounts when the sugar load is reduced and the occurrence of symptoms are relatively rare. Thus, a moderate intake of lactose during a standard HBT-25 may prove harmless in the large majority of patients diagnosed as LI or LM[25].

Interestingly, during the HBT-12.5, eight patients reported symptoms despite a negative test with prevalence similar to that observed in a previous study performed in normal subjects and in patients with irritable bowel syndrome[26-29]. In the present series, symptoms consisted of gaseousness and mild abdominal pain, whereas none of the patients had diarrhea. As patients were asked to report even minor symptoms, a "nocebo", or "inverse placebo", effect may have been elicited by the investigators.

Finally, false-negative results cannot be completely ruled out in these patients, due to a better sensitivity of late (> 240 min) increases in hydrogen excretion, as suggested by Di Stefano *et al*[30]. These data, however, are debatable as these authors, using different hydrogen cutoff levels, considered definitely as lactose intolerant with a false-negative breath test those patients reporting symptoms during the HBT, irrespective of the test results. This is unlikely, as negative expectations often induce non-specific abdominal symptoms not only during lactose HBT, but also after a sham lactose load (personal unpublished data).

In conclusion, these data reaffirm that LI is dose-dependent. Considering the daily mean lactose intake in the general population, 50 or 25 g lactose tolerance breath tests may prove useful for epidemiological studies, looking for lactose deficiency. The widespread availability of genetic testing for lactase polymorphism may render obsolete this technique. Conversely, in the clinical setting, the use of the 12.5 g lactose tolerance test should be probably preferred to the 25 g test, at least in Caucasians and in the populations of the Mediterranean basin, as it may help to identify those patients who would profit from dietary restriction of lactose-containing food, minimizing the risk of inappropriately reducing calcium intake to those who do not need it.

COMMENTS

Background

Hydrogen breath test (HBT), after a lactose load of 25-50 g is widely used in the clinical setting for diagnosing lactose malabsorption (LM) and, when abdominal symptoms are present, of lactose intolerance (LI). The positivity of the test often induces dietary modifications, leading to the reduction of calcium intake.

Research frontiers

The authors confirmed in a large series of patients previous findings suggesting that most patients with LM, documented by HBT, tolerate well small amounts of lactose.

Innovations and breakthroughs

The present data indicates that an oral load of 12.5 of lactose, corresponding to about 250 mL milk, is well tolerated by the majority of patients unable to completely digest and absorb 25 g lactose. Moreover, in the majority of them, an increased excretion of hydrogen was not documented after ingesting an isoosmolar solution of 12.5 g of lactose, indicating that they can normally digest lactose at least up to a dose corresponding to 250 mL milk.

Applications

The authors suggest that 12.5 g lactose HBT should be preferred to the usual oral load of 25-50 g, in order to identify those patients who could really profit from a reduction of lactose-containing food, and minimize the risk of unnecessary reductions of calcium intake.

Terminology

HBTs are indicated by HBT. The positivity of the test defines a subject's LM, irrespective of the occurrence of abdominal symptoms. The coincident occurrence of symptoms is required for defining LI patients.

Peer review

This is an interesting study in which the authors argue that the use of 25 g of lactose to test for LM may be inappropriate, as this is higher than the average dietary intake, and the removal of lactose from the diet may have other deleterious consequences such as reduced calcium intake.

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RAPID COMMUNICATION

Sildenafil does not influence hepatic venous pressure gradient in patients with cirrhosis

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Abstract

AIM: To investigate if sildenafil increases splanchnic blood flow and changes the hepatic venous pressure gradient (HVPG) in patients with cirrhosis. Phosphodiesterase type-5 inhibitors are valuable in the treatment of erectile dysfunction and pulmonary hypertension in patients with end-stage liver disease. However, the effect of phosphodiesterase type-5 inhibitors on splanchnic blood flow and portal hypertension remains essentially unknown.

METHODS: Ten patients with biopsy proven cirrhosis (five females/five males, mean age 54 ± 8 years) and an HVPG above 12 mmHg were studied after informed consent. Measurement of splanchnic blood flow and the HVPG during liver vein catheterization were done before and 80 min after oral administration of 50 mg sildenafil. Blood flow was estimated by use of indocyanine green clearance technique and Fick's principle, with correction for non-steady state.

RESULTS: The plasma concentration of sildenafil was 222 ± 136 ng/mL 80 min after administration. Mean arterial blood pressure decreased from 77 ± 7 mmHg to 66 ± 12 mmHg, P = 0.003, while the splanchnic blood flow and oxygen consumption remained unchanged at 1.14 ± 0.71 L/min and 2.3 ± 0.6 mmol/min, respectively. Also the HVPG remained unchanged (18 ± 2 mmHg *vs* 16 ± 2 mmHg) with individual changes ranging from -8 mmHg to +2 mmHg. In seven patients, HVPG decreased and in three it increased.

CONCLUSION: In spite of arterial blood pressure decreases 80 min after administration of the phosphodiesterase type-5 inhibitor sildenafil, the present study could not demonstrate any clinical relevant influence on splanchnic blood flow, oxygen consumption or the HVPG.

Key words: Cirrhosis; Sildenafil; Portal hypertension; Portal hemodynamics; Hepatic blood flow; Erectile dysfunction; Hepatic venous pressure gradient

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INTRODUCTION

Erectile dysfunction is a common problem affecting about half of all patients with end-stage liver disease[1,2]. The systematic use of beta-adrenergic blockade[3,4], initiated in order to reduce the hepatic venous pressure gradient (HVPG), may also per se induce impotence[5]. Selective phosphodiesterase type-5 inhibitors, such as sildenafil, represent an important advance in management of erectile dysfunction[6,7]. Inhibition of phosphodiesterase type-5, the prominent isoform in corpora cavernosa, leads to diminished degradation of cyclic guanosine monophosphate, relaxation of smooth muscle and blood filling of corpora cavernosa[8].

In rats, phosphodiesterase type-5 is also present in the superior mesenteric artery[9]. Administration of sildenafil

results in a dose-dependent increase in mesenteric blood flow and a minor increase in portal venous pressure, in both the bile-duct-ligated rat, as well as in control rats[10]. It has been suggested that phosphodiesterase type-5 is also present in human mesenteric arteries[11]. The use of sildenafil could, therefore, be hazardous in patients with cirrhosis and portal hypertension. Indeed, acute variceal bleeding has been described after intake of 25 mg sildenafil[12,13]. On the other hand, a more recent study in patients with Child A liver cirrhosis has shown a decrease in portal and sinusoidal resistance after inhibition of phosphodiesterase-5[14], which was introduced as a new potential treatment modality of portal hypertension[14].

Since sildenafil seems to be valuable in the treatment erectile dysfunction[14] and portopulmonary hypertension[15] in patients with cirrhosis, the aim of the present study was to determine if splanchnic blood flow and HVPG are influenced by sildenafil in patients with cirrhosis.

MATERIALS AND METHODS

Twelve patients with biopsy-proven cirrhosis were included in the study during diagnostic work-up and/or evaluation for liver transplantation. None of the patients were treated with nitrates or beta-adrenergic drugs at the time of the study. Furthermore, only patients with an HVPG above 12 mmHg were considered for inclusion in this study. Two patients were excluded due to this last criterion. Thus, 10 patients participated (five females/five males, mean age 54 ± 8 years). The reason for referral to our tertiary liver failure unit was chronic hepatic insufficiency in five, recurrent bleeding from esophageal varices in four, and therapy-resistant ascites in one patient. None of the patients had suffered from variceal bleeding within 14 d of the study. Participation in the study included measurement of the HVPG and the estimation of splanchnic blood flow before and 80 min after oral administration of 50 mg of sildenafil. All patients were included after written informed consent, and after the local scientific-ethical committee had approved the study.

Hepatic venous catheterization (Cordis, MP-A1, open end catheter, Miami, USA) was performed through the right or left intermedian cubital vein. During the advance of the catheter, blood pressure in the right atrium was registered. After location of a hepatic vein, the catheter was advanced to the wedge position. Pressure was measured *via* a pressure monitoring set and was continuously recorded (Hewlett Packard, 78354C) and could be printed (Hewlett Packard, M1125A). The pressure transducer was calibrated to zero pressure at the level of the right atrium. The wedge pressure was only accepted if the wedge position afterwards could be confirmed by visualization of a characteristic wedge pattern on the fluoroscope by use of X-ray contrast media (Omnipaque 240 mg/mL, Amersham Health). Free hepatic venous pressure was measured as close as possible to the inferior caval vein. Pressures, free and wedged, were measured in three different hepatic veins and the mean was used. The catheter was then positioned in a hepatic vein half way between wedge position and the caval vein and used for blood sampling.

Splanchnic blood flow was estimated by use of indocyanine green as previously described[16]. In short, indocyanine green was continuously infused and simultaneously, five paired samples of arterial and hepatic venous blood were drawn at intervals of 5 min. Calculation of hepatic blood flow was based on Fick's principle with correction for non-steady state[16]. Splanchnic oxygen consumption was calculated as the arterio-venous oxygen content difference times splanchnic blood flow.

Patients were given 50 mg sildenafil with 100 mL of tap water. From 60 to 80 min thereafter, blood was again sampled for estimation of hepatic blood flow. HVPG was measured again, as described above, in three different veins. Pressure in the right atrium was recorded during withdrawal of the catheter.

Indocyanine green was determined by use of HPLC as earlier described[17]. To ensure absorption of sildenafil, blood was sampled 80 min after administration and plasma sildenafil concentration was later analyzed using automated sequential trace enrichment of dialyzates and HPLC[18].

Statistical analysis

Data are shown as mean ± SD. Paired *t* test was used for comparison, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics and clinical data from the 10 participating patients showed that the majority had advanced cirrhosis (Table 1). None of the patients had allergy to sildenafil. The concentration of sildenafil in plasma was 222 ± 136 ng/mL, 80 min after administration of the drug.

There was a statistically significant decrease in mean arterial pressure from 77 ± 7 mmHg to 66 ± 12 mmHg, $P = 0.003$, after sildenafil administration (Table 2). No statistically significant changes were observed in other hemodynamic variables or in splanchnic oxygen consumption (Table 2). In particular, the HVPG remained statistically unchanged, i.e. 18 ± 2 mmHg before *vs* 16 ± 2 mmHg, after administration of sildenafil.

The plasma concentration of sildenafil at 80 min was not statistically significantly correlated to the changes in HVPG, splanchnic blood flow, mean arterial pressure, or right atrial pressure.

Four patients had a decrease in HVPG i.e. 3, 3, 3 and, 8 mmHg, respectively. Two of these where Child class A/B and all four had alcoholic cirrhosis.

DISCUSSION

This study reports the effects of sildenafil on the splanchnic hemodynamics in patients with biopsy proven

Table 1 Patient characteristics and clinical data (mean ± SD)

Characteristics	Data
Age (yr)	54 ± 8
Weight (kg)	75 ± 16
Height (cm)	175 ± 7
Female/Male	5/5
Aetiology (ALC/AIH/PBC)	8/1/1
Varices, present	9
Bleeding, earlier	5
Ascites, present	8
Child Pugh (A/B/C)	1/2/7
GEC (μmol/kg per min)	20 ± 4
INR	1.7 ± 0.5
Bilirubin (mmol/L)	64 ± 50
Albumin (g/L)	24 ± 5

ALC: Alcoholic cirrhosis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis. INR: International normalization ratio; GEC: Galactose elimination capacity, normal > 32 μmol/kg per min.

Table 2 Effect of 50 mg sildenafil (mean ± SD, *n* = 10)

	Baseline	80 min after
MAP (mmHg)	77 ± 7	66 ± 12[1]
HF (beats/min)	83 ± 13	85 ± 11
RA (mmHg)	5 ± 1	6 ± 2
Hepatic blood flow (L/min)	1.14 ± 0.71	1.14 ± 0.94
VO_2 (mmol/min)	2.3 ± 0.6	2.5 ± 1.2
Wedged pressure (mmHg)	29 ± 6	27 ± 6
Free pressure (mmHg)	12 ± 6	11 ± 5
Gradient (mmHg)	18 ± 2	16 ± 2

[1]P = 0.003, *vs* baseline. VO_2: Splanchnic oxygen consumption. MAP: Mean arterial pressure; RA: Right atrial pressure.

cirrhosis and manifest portal hypertension. Despite a small decrease in mean arterial pressure, the main finding in this study was that the splanchnic blood flow, oxygen consumption and the mean HVPG did not change 80 min after administration of sildenafil, i.e. at a time-point when a therapeutic plasma concentration of sildenafil[19] could be documented. These data appear to be in accordance with preliminary results testing the effect of an oral dose of 25 mg of sildenafil with which no effect on portal pressure was reported, in spite a decrease in arterial pressure[20]. However, our results appear to be in contrast to an earlier study reported by Deibert *et al*[14], in which vardenafil (another phosphodiesterase type-5 inhibitor) was found to lower portal pressure in four of five patients with Child A cirrhosis. The reason for this discrepancy is not clear, but three of the five patients in that latter study[14] had HVPG < 12 mmHg before administration of vardenafil.

As illustrated in Figure 1, the individual HVPG changes ranged from -8 to +2 mmHg. In an animal study with bile-duct-ligated rats, a statistically significant increase in the HVPG of 3% ± 1% after intramesenteric (10 mg/kg) administration of sildenafil has been reported[10]. Thus, our finding suggests that the effect of sildenafil on splanchnic hemodynamics in humans with cirrhosis is different from that in rats. This could be due to the uncertainty concerning which phosphodiesterase

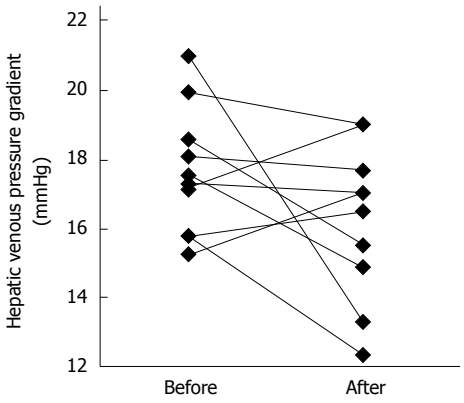

Figure 1 HVPG in ten patients with cirrhosis of the liver before and 80 min after oral intake of 50 mg sildenafil.

isoform, PDE5 and/or PDE1 is present in the human mesenteric artery[11]. It can be argued that even a 10%-15% increase in HVPG, as seen in one out of 10 patients in our study, might be problematic in the individual. However, small changes in the gradient probably take place during everyday life. For example, digestion of food increases hepatic blood flow by almost 100%. In particular, a major increase is seen in the superior mesenteric artery and thus portal blood flow[21]. Moreover, it has been demonstrated in patients with cirrhosis that a meal increases the mean HVPG from 16 to 20 mmHg, corresponding to 25%[22]. This could be even higher in the individual. The risk of experiencing a bleeding episode after intake of sildenafil should, therefore, be interpreted in the light of such everyday events.

The splanchnic hemodynamic data were collected during rest in the present study. However, supposedly the user of sildenafil is rarely in a resting position, but is more likely to increase the level of physical activity, which is known to influence the splanchnic hemodynamics[23]. In fact, physical activity, with an exercise level of 30% of peak workload, appears to increase HVPG from 16.7 to 19.2 mmHg[23]. Working at 50% of peak workload will not increase this HVPG gradient any further. Thus, the physical activity, which often is required to engage in sexual activity, may itself slightly increase the gradient. However, and of importance, the opposite has been observed in patients with cirrhosis during exercise and beta-adrenergic blockade[24]. In these patients, exercise at 30% of peak workload was associated with a decrease in HVPG from 16.3 to 12.9 mmHg[19]. This was explained by a lesser increase in cardiac output and a larger decrease in hepatic blood flow in the propranolol group compared to the non-beta-blocker-treated group of patients.

The possible effect on splanchninc hemodynamics after longer use of sildenafil is elusive and has not been examined. One concern about chronic use may be the possible renal effects. In patients with cirrhosis, administration of a single dose of sildenafil was followed by a decrease in sodium excretion[25]. However, the long-term renal effects may be insignificant as chronic

administration of sildenafil prevented the decrease in sodium excretion seen in bile-duct-ligated rats[26].

The present study shows that sildenafil does not induce any profound clinically relevant changes in splanchnic blood flow, oxygen consumption and HVPG. This indicates that phosphodiesterase type-5 inhibition is of no use as a therapeutic agent for alleviating portal hypertension in patients with chronic end-stage liver disease. On the other hand, the use of sildenafil in such patients, initiated because of erectile dysfunction, appears to be safe per se. However,, clearly more studies on safety are needed, as the use of sildenafil is usually associated with physical activity and often also the use of beta blockers.

COMMENTS

Background

Erectile dysfunction is a common problem affecting about half of all patients with end-stage liver disease. Selective phosphodiesterase type-5 inhibitors, such as sildenafil, represent an important advance in management of erectile dysfunction. In rats, administration of sildenafil results in a dose-dependent increase in mesenteric blood flow and a minor increase in portal venous pressure. The use of sildenafil could, therefore, be hazardous in patients with cirrhosis and portal hypertension and acute variceal bleeding has been described after intake of 25 mg sildenafil.

Research frontiers

Bleeding from esophageal varices is a major contributor to death in patients with end-stage liver disease and it could be hazardous to recommend treatment of erectile dysfunction with sildenafil if this treatment, in turn, leads to an increase in hepatic venous pressure gradient (HVPG).

Innovations and breakthroughs

Another human study addressing the presented problem has been published in abstract form, but the patients investigated all had Child class A cirrhosis whereas, erectile dysfunction as a "quality of life problem" tends to increase with increasing severity of liver disease i.e. Child class B and C, and such patients where investigated in the present study.

Applications

The present study does not support a restrictive use of sildenafil in patients with cirrhosis. Future research should be done with larger patient groups in order to determine the long-term risk of bleeding (if any) during treatment with sildenafil.

Terminology

Liver disease leads to accumulation of fibrous tissue in the liver, and in turn, this leads to increased resistance to the blood flow through the portal vein. The resistance is estimated by the HVPG and the risk of bleeding from esophageal varices in cirrhosis increases with this gradient.

Peer review

This is an interesting and informative study. The authors investigated if sildenafil increases splanchnic blood flow and changes the HVPG in patients with cirrhosis. Phosphodiesterase type-5 inhibitors are valuable in the treatment of erectile dysfunction and pulmonary hypertension in patients with end-stage liver disease.

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RAPID COMMUNICATION

Cholangiocarcinoma: A 7-year experience at a single center in Greece

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Abstract

AIM: To evaluate survival rate and clinical outcome of cholangiocarcinoma.

METHODS: The medical records of 34 patients with cholangiocarcinoma, seen at a single hospital between the years 1999-2006, were retrospectively reviewed.

RESULTS: Thirty-four patients with a median age of 75 years were included. Seventeen (50%) had painless jaundice at presentation. Sixteen (47.1%) were perihilar, 15 (44.1%) extrahepatic and three (8.8%) intrahepatic. Endoscopic retrograde cholangiography (ERCP) and/or magnetic resonance cholangiography (MRCP) were used for the diagnosis. Pathologic confirmation was obtained in seven and positive cytological examination in three. Thirteen patients had co-morbidities (38.2%). Four cases were managed with complete surgical resection. All the rest of the cases (30) were characterized as non-resectable due to advanced stage of the disease. Palliative biliary drainage was performed in 26/30 (86.6%). The mean follow-up was 32 mo (95% CI, 20-43 mo). Overall median survival was 8.7 mo (95% CI, 2-16 mo). The probability of 1-year, 2-year and 3-year survival was 46%, 20% and 7%, respectively. The survival was slightly longer in patients who underwent resection compared to those who did not, but this difference failed to reach statistical significance. Patients who underwent biliary drainage had an advantage in survival compared to those who did not (probability of survival 53% *vs* 0% at 1 year, respectively, $P = 0.038$).

CONCLUSION: Patients with cholangiocarcinoma were usually elderly with co-morbidities and/or advanced disease at presentation. Even though a slight amelioration in survival with palliative biliary drainage was observed, patients had dismal outcome without resection of the tumor.

Key words: Cholangiocarcinoma; Surgical resection; Palliative biliary drainage; Survival

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INTRODUCTION

Cholangiocarcinoma is the second commonest primary hepatic malignant disease, after hepatocellular carcinoma[1]. Several studies have shown that the incidence and mortality of the disease are rising worldwide[1,2]. The high fatality rate has been attributed to the poor knowledge of the tumor pathogenesis and the paucity of effective methods of diagnosis and management. First, the diagnosis of perihilar and extrahepatic cholangiocarcinoma still remains a clinical challenge, particularly in the presence of primary sclerosing cholangitis. Second, surgical resection is the only curative option for cholangiocarcinoma, but only a minority of patients are suitable for resection[3,4]. Factors that have been considered as contraindications for resectability include, among others, metastatic disease, multiple comorbidities, invasion of the hepatic artery or portal vein and extension of cholangiocarcinoma to involve segmental bile ducts on both liver lobes[3,4,5]. Sometimes the lack of available surgical expertise renders the surgical approach difficult to apply.

We aimed at evaluating the clinical features, diagnostic modalities, therapeutic options and survival rates in a series of 34 patients with cholangiocarcinoma hospitalized

at the Hippokration University Hospital, Athens, Greece. We also attempted to investigate whether there were differences in survival, compared to data published in the literature, and to identify factors associated with survival.

MATERIALS AND METHODS

The medical records of 34 patients diagnosed with cholangiocarcinoma at the Hippokration University Hospital between January 1999 and December 2006 were retrospectively reviewed. The study was approved by the local Ethics Committee. Our hospital has a liver unit and several patients with cholangiocarcinoma are referred from other hospitals. Diagnosis of cholangiocarcinoma was based upon clinical, imaging, cytologic and histopathologic findings. Medical records were scrutinized for epidemiologic characteristics, predisposing factors, initial manifestations of the disease, method of diagnosis, laboratory findings, surgical or palliative therapy, and overall morbidity and mortality.

Cholangiocarcinoma was classified as intrahepatic, perihilar and distal extrahepatic type[3,6]. The staging of the tumor was based on the tumor-node-metastasis system[7]. The perihilar tumors were classified according to the Bismuth classification[8] and the resectability was evaluated according to T-stage criteria[4]. All patients had ultrasound of the liver and gallbladder as the first diagnostic imaging procedure. Metastatic disease was evaluated by imaging of the chest, abdomen and pelvis by helical computed tomography (CT) or magnetic resonance imaging (MRI). No laparoscopic staging was performed.

Statistical data were analyzed using SPSS 13.0 for Windows. Descriptive statistics including mean, ranges and standard deviation values were calculated for all the continuous baseline demographic and laboratory characteristics. We used the Kruskal-Wallis non-parametric test to compare continuous data. The results are presented as means ± 95% confidence intervals (CI). The χ^2 test was used to compare categorical data; the results are presented as counts with percentages. All reported *P* values are based on two-tailed tests of significance. Comparisons were considered significantly different if $P < 0.05$. Overall survival was estimated from the admission of the patient to the hospital until death or last follow-up visit. Survival probabilities were estimated using the Kaplan-Meier method and compared by the log-rank test. Univariate analysis using the Cox regression test was used to determine factors associated with survival.

RESULTS

The study group included 18 men and 16 women, all of Greek origin. Sixteen of 34 (47.1%) were perihilar, 15 (44.1%) extrahepatic and three (8.8%) intrahepatic tumors. Demographic characteristics and laboratory data on presentation are shown in Table 1. Initial manifestations were painless jaundice in 17 patients (50%), whereas 12 (35.3%) presented with abdominal pain and weight loss.

Table 1 Demographic and biochemical characteristics of all patients at baseline (mean ± SD)

Patient characteristics	
Gender (M %)	18/34 (53%)
Age (years)	71.7 ± 13.3
Bilirubin (mg/dL)	13.3 ± 9.1
AST (U/mL)	117 ± 80
ALP (U/mL)	472 ± 237
Albumin (g/dL)	3.6 ± 0.5
Predisposing factor (%)	4/34 (11.7%)
Tobacco use (%)	10/34 (29.4%)

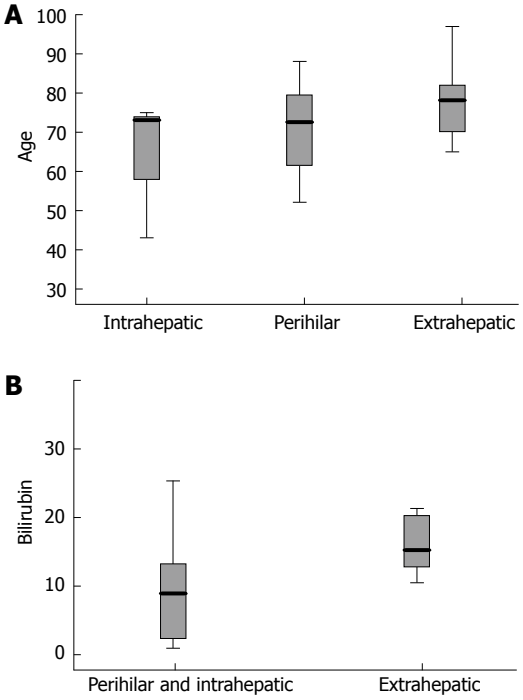

Figure 1 Comparison of baseline characteristics of the patients with respect to site. A: Age distribution with respect to site (intrahepatic, perihilar and extrahepatic) is significantly not different; B: Bilirubin values at presentation with respect to site (intrahepatic, and perihilar *vs* extrahepatic; $P = 0.027$).

Patients with extrahepatic type of cholangiocarcinoma were older than those with perihilar and intrahepatic type, but the difference was not statistically significant (79 ± 8.4 years *vs* 72.5 ± 11 years and 63.6 ± 17.9 years respectively, $P = 0.181$, Figure 1A). Mean values of total bilirubin were higher in the extrahepatic than in perihilar and intrahepatic type (17 ± 9 mg/dL *vs* 8.9 ± 7.5 mg/dL, $P = 0.027$, Figure 1B).

A history of a predisposing factor was recognized in four patients, two with primary sclerosing cholangitis and two with chronic hepatitis B and cirrhosis. No case of Caroli's syndrome, congenital hepatic fibrosis, choledochal cyst or occupation in the chemical industry was found. Moderate consumption of alcohol and use of tobacco were present in four (11.7%) and 10 (29.4%) patients, respectively. Thirteen (38.2%) patients presented with co-morbidities, mostly diabetes mellitus with complications and/or coronary disease.

Intrahepatic tumors were histologically proven using

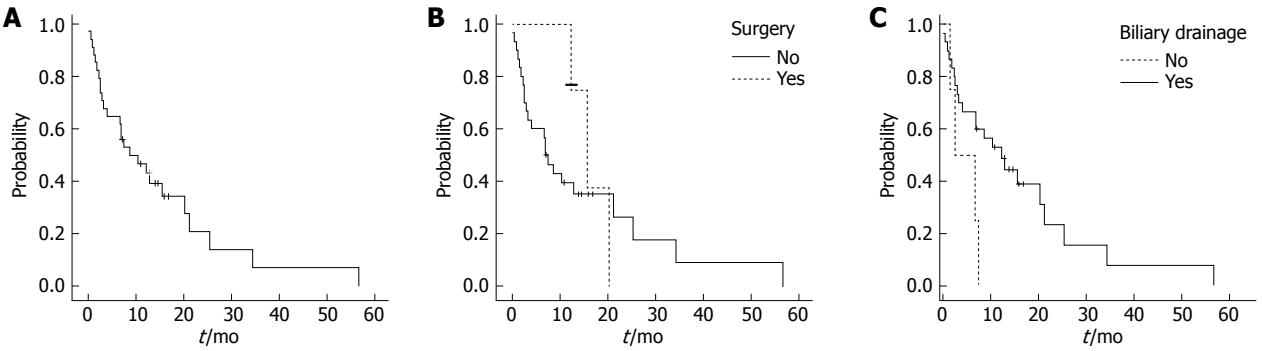

Figure 2 Survival rates in different groups of patients according to treatment. A: Survival of all the 34 patients with cholangiocarcinoma; B: Survival rates of patients with cholangiocarcinoma, stratified by surgical (n = 4) or not surgical resection (n = 30) were not significantly different; C: Survival of patients with unresected cholangiocarcinoma among those who had any kind of biliary decompression (n = 26) and those who had no interventional therapy (n = 4, P = 0.038).

a CT-guided biopsy. Eight of 16 (50%) perihilar tumors were diagnosed by magnetic resonance cholangiography (MRCP) and magnetic resonance imaging (MRI), 4/16 (25%) by endoscopic retrograde cholangiography (ERCP) and 4/16 (25%) by histology (biopsies were obtained at laparoscopy in three cases and at endoscopic ultrasound in one case). Five of 15 (33%) extrahepatic tumors were diagnosed by MRCP/MRI, and 10/15 (66%) by ERCP. Overall, tissue for pathologic confirmation was obtained from seven patients, three with intrahepatic and four with perihilar tumors. A positive cytologic examination was obtained in three further cases.

A local surgical team evaluated the cases as resectable or non-resectable. Four cases were managed with complete surgical resection aiming at histologically negative resection margins (two extrahepatic, one perihilar and one intrahepatic type). All of the remaining cases were characterized as non-resectable. Among the 15 unresectable perihilar tumors, three (20%) were classified as T2-T3 stage, and extension of the hepatectomy was considered too risky compared to the poor patients' performance status; nine others (60%) had evidence of either metastases or extensive local lymphadenopathy or portal vein involvement; and the three remaining patients (20%) had multiple co-morbidities. Two of three intrahepatic tumors were multifocal and were assessed as non-resectable. Three of 13 (23%) cases with unresectable extrahepatic tumors had evidence of either metastases or extensive local lymphadenopathy or portal vein involvement, and 10 (77%) had multiple co-morbidities, including cardiopulmonary disease or diabetes with complications, poor performance status and advanced age (> 80 years) and thus were considered as unsuitable for curative resection.

Palliative biliary drainage to relieve symptoms was performed in the vast majority of cases who did not undergo surgical curative therapy (12/15 perihilar, 13/13 extrahepatic, 1/2 intrahepatic). More specifically, among the perihilar-type cholangiocarcinoma cases who were not resected, eight were managed by stent placement (seven biliary stents were inserted by endoscopic and one by percutaneous routes), three by palliative percutaneous biliary drainage, and one by palliative surgical biliary drainage. No biliary decompression was decided in three cases. Among the two intrahepatic cases not resected, one was managed with palliative percutaneous biliary drainage and no biliary drainage was decided in one case. Among the 13 extrahepatic cases not resected, 12 were managed by stent placement endoscopically and one by palliative percutaneous biliary drainage.

A high number of patients who underwent a palliative biliary drainage (11/26, 42%) were managed with two or more endoscopic or percutaneous sessions for biliary decompression because of stent occlusion (median number of procedures for each patient was two, range 1-7). Metal stents were placed in the vast majority of patients.

Four cases (one intrahepatic, one extrahepatic and two perihilar type) were managed with chemotherapy without surgical resection (two in combination with biliary drainage and two without any other intervention). The causes of death in two-thirds of the patients were infective complications (acute cholangitis) following an occluded biliary stent or acute pancreatitis. Other causes of death were hepatic failure and acute myocardial infarction.

The mean follow-up for all patients was 32 mo, 95% CI 20-43 mo. Overall median survival was 8.7 mo (events, 26/34; 95% CI, 2-16 mo). The probability of 1-year, 2-year and 3-year survival was 46%, 20% and 7%, respectively (Figure 2A). The survival was longer in patients who underwent surgical resection (n = 4) compared to those who did not (n = 30), but the difference failed to reach statistical significance. The probability of survival for the former was significantly higher than for the latter (75% *vs* 39% and 37% *vs* 26% at 1 and 2 years respectively, P = 0.6, Figure 2B). The median survival was 15.7 mo, (95% CI 11-20.6 mo) in the former and 7 mo, (95% CI, 4.4-9.6 mo) in the latter group.

Patients who received any interventional treatment for biliary drainage (either stent replacement or percutaneous biliary drainage or palliative surgical procedure, n = 26) had an advantage in survival in comparison to those who did not (n = 4). The probability of survival for the former was significantly higher than for the latter (53% *vs* 0% at 1 year respectively; median survival, 12.3 mo; 95% CI, 6-18.7 mo and 2.5 mo, 95% CI, 0-7.6 mo, respectively; P = 0.038; Figure 2C). None of the four patients who did not undergo biliary drainage survived beyond 6 mo. None of the following

factors were associated with a statistically significant difference in survival: age, gender, bilirubin, site of the tumour, albumin, stent placement, and chemotherapy.

DISCUSSION

Cholangiocarcinoma is a relatively rare disease accounting for less than 2% of all human malignancies[9]. The specific features of cholangiocarcinoma depend on the anatomical location of the tumor, which are useful to optimize the appropriate therapy. The most common location of these tumors (60%-70%) is the bifurcation of the hepatic ducts (perihilar or Klatskin tumours), while 20%-30% are extrahepatic, arising from the distal common bile duct, and 5%-10% are peripheral or intrahepatic tumors, originating from the small bile ducts of the liver parenchyma[10]. In our series, the perihilar and extrahepatic tumors had the same incidence (47% and 44%, respectively) while intrahepatic tumors accounted for a small minority (8.8%).

The distribution of age among our patients was different in the three types of tumor, with the extrahepatic cases being older, in agreement with the literature[7]. Co-morbidities were common in the extrahepatic type, as older individuals have more illnesses than younger ones. The clinical presentation and risk factors were rather similar with respect to the site of the malignancy. Painless jaundice, abdominal pain, weight loss, use of tobacco and consumption of alcohol did not show predilection for any site of the tumor. It appeared however that jaundice at presentation was more profound in the extrahepatic type of disease than in the perihilar cases. Only a few cases of cholangiocarcinoma were associated with a predisposing factor such as primary sclerosing cholangitis and chronic hepatitis B, a rather low rate compared with other series[11]. Other chronic inflammatory diseases of biliary epithelium such as parasitic infections or intrahepatic biliary stones are not endemic in the Greek population.

At present, only surgical excision of all detectable tumors is associated with improvement in survival. The median survival time for patients with distal bile duct cancers who undergo resection has been reported to be about 38 mo[12,13]. The median survival time for patients with perihilar cholangiocarcinoma varies from 12 to 46 mo[4,14,15,16]. However, factors associated with both the patient and the tumor may preclude surgical resection. It is generally accepted that many patients are not considered surgical candidates because of co-morbidities and advanced age, despite evidence of resectable disease. On the other hand, more than half of cholangiocarcinoma cases usually present with advanced unresectable malignancy[4,17]. In our series, 50% of the patients were considered to have advanced disease and the remaining had multiple co-morbidities and/or advanced age. In the literature, neither a T2 or T3 stage nor portal involvement are considered absolute contraindications for resection for perihilar tumours[4,5]. Similarly, local lymphadenopathy is not a contraindication to resection of extrahepatic tumors[17]. Co-morbidities and advanced age along with the available local surgical expertise determined the low rate of resectability in our series. In an old series from Mayo Clinic, only 22 of 125 patients (18%) underwent curative resection, whereas 82% were candidates for palliative intervention[18]. In another more recent series of 225 patients with hilar cholangiocarcinoma, 80 (35%) underwent resection[4] while resectability rates were higher for intrahepatic cholangiocarcinoma ranging from 45% to 90%[19,20].

No significant survival advantage was found in patients who underwent surgical resection in our series. Curative surgical resection was attempted in only four patients and the disease recurred after resection in three of the four. It is noteworthy that no free-of-cancer surgical margins were found at histology among those three surgical specimens. The natural history of resected cholangiocarcinoma with no disease-free surgical margins is comparable to unresected cholangiocarcinoma receiving palliative therapy[4,21,22]. The overall median survival in our series was 8.7 mo, with a survival rate comparable to that reported for unresected cholangiocarcinoma from previous investigators[18,23,24].

Palliative treatment to relieve symptoms and resolve obstructive jaundice has an important role in the management of cholangiocarcinoma, since the majority of cases are not suitable for resection, as stated above, or they recur after resection[4,25]. Palliation and relief of jaundice can be accomplished by either endoscopic, percutaneous, or operative means. We found that the single factor that may provide a survival benefit in unresected cases is successful biliary decompression. It is noteworthy that of our four patients with cholangiocarcinoma with no interventional procedure for biliary decompression, none survived beyond 6 mo. Similarly, median survival for non-resectable cholangiocarcinoma has been considered as favourable in cases in which biliary drainage is performed, since the median survival has been reported to be 3 mo without and 6 mo with biliary drainage[18,26]. Our attempt to identify other factors associated with survival, e.g. gender or additional palliative therapy (i.e. chemotherapy), failed to show any survival advantage for any of them, even if some were shown to be important in previous studies[18].

Despite the benefit in survival afforded by biliary drainage, the enhancement in quality of life with this kind of intervention seemed to be minimal, since the majority of patients had repeat procedures to ensure the patency of the stents. Patency rates of self-expanding metal stents are higher than those of plastic ones[27]. Even if metal stents remain patent longer, a high percentage of our patients needed the replacement of two or more stents in repeat procedures for resolution of jaundice.

The major limitation of our study was that it involved only a single center and the number of patients was small, and thus the results may not be generalizable.

In conclusion, the present study confirms that cholangiocarcinoma is a tumor with high incidence among the elderly with multiple co-morbidities, which precludes aggressive curative resection. Moreover, survival of patients with unresected cholangiocarcinoma

is short and the benefit in survival, but not in quality of life, from endoscopic or percutaneous biliary drainage, although evident, is rather weak. Therefore, early diagnosis and surgical resection of the tumor are crucial in the management of these patients.

COMMENTS

Background

Cholangiocarcinoma is difficult to diagnose and its prognosis is dismal due to late stage at presentation. The incidence and mortality of the disease are rising worldwide.

Research frontiers

The high fatality rate of cholangiocarcinoma is attributed to the poor knowledge of disease pathogenesis and the paucity of effective methods of diagnosis and management. Surgical resection is the only curative option for cholangiocarcinoma, but only a minority of patients are suitable for resection.

Innovations and breakthroughs

This study confirms that cholangiocarcinoma is diagnosed at an advanced stage, with high incidence among the elderly with multiple co-morbidities, which precludes aggressive curative resection. Survival of patients with unresected cholangiocarcinoma is short and the benefit in survival, but not in quality of life, from endoscopic or percutaneous biliary drainage, although evident, is rather weak.

Applications

The study suggested that early diagnosis and surgical resection of the tumor are crucial in the management of these patients.

Peer review

This is a retrospective study of the outcome of cholangiocarcinoma in a group of 34 patients seen at a single hospital, characterized by a homogeneous ethnic population of Greek origin. The aims are not ambitious and the originality of study is not high. However, the study is still relevant due to the severity of the disease and the scarce information available.

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RAPID COMMUNICATION

Bcl-2 expression significantly correlates with thymidylate synthase expression in colorectal cancer patients

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Abstract

AIM: To examine the expression of thymidylate synthase (TS) and oncoprotein Bcl-2 in advanced colorectal cancer (CRC) patients, and to determine their mutual relationship, association to therapeutic response and impact on disease outcome.

METHODS: Tumor samples from 67 patients with CRC, who were treated at advanced stage with either irinotecan alone or in combination with 5-fluorouracil/leucovorin, were analyzed for expression of TS and Bcl-2 using immunohistochemistry.

RESULTS: A significant linear correlation between lower expression levels of Bcl-2 and lower levels of TS expression was found (P = 0.033). Patients with high levels of both TS and Bcl-2 expression had a significantly longer disease-free survival (DFS) (42.6 mo *vs* 5.4 mo, n = 25) than those with low TS/Bcl-2 index (P = 0.001). Tumors with low levels of both TS and Bcl-2 were associated with a longer survival with metastasis (WMS) interval in the whole patients group (n = 67, P = 0.035). TS/Bcl-2 index was not significantly related to disease-specific survival.

CONCLUSION: The present data suggest that CRC patients with low TS/Bcl-2 demonstrate a significantly shorter DFS and longer WMS.

Key words: Thymidylate synthase; Bcl-2; Colorectal cancer; Disease-free survival; Survival with metastases

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INTRODUCTION

Colorectal cancer (CRC) is the second most frequent cancer in Europe in 2004, responsible for 13% (376 400) of all incident cancer cases. It is also the second most frequent cause of cancer mortality in Europe, with an annual mortality of 11.9%, 203 700 annual deaths[1]. In the early stages, CRC is often a curable disease, but the overall prognosis is determined by the extent of local and particularly metastatic tumor spread. However, disease outlook is relatively poor for advanced disease and thus is a significant cause of worldwide cancer-related mortality[1]. For locally advanced and metastatic CRC, fluoropyrimidine, 5-fluorouracil (5-FU) has been the standard cytostatic drug for the last 50 years, in recent years used as modulated by leucovorin and in combination with irinotecan or oxaliplatin.

Fluoropyrimidine metabolites form a covalent complex with thymidylate synthase (TS). Formation of this complex prevents biosynthesis of intracellular thymidylate, which is essential for DNA biosynthesis. TS expression has been shown to be an independent prognostic factor in several other cancers. Higher TS levels in hepatic metastases and resection margin are independent predictors of disease progression and survival in patients with metastatic CRC[2]. Comparable results have been reported in other tumors e.g. gastric[3], cervical[4], ovarian, and head and neck cancers, for which TS+ tumors have demonstrated significantly worse outcome as compared to TS-negative tumors.

Increased expression of the proto-oncogene Bcl-2, a 24-kDa intracellular membrane protein that is able to inhibit programmed cell death without affecting cell proliferation, has been reported in gastrointestinal adenocarcinoma and its precursor lesions[5,6]. Bcl-2

has been shown to prolong cell survival by inhibiting apoptosis in several cell types[7,8]. Abnormal activation of the Bcl-2 gene appears to be an early event in colorectal tumorigenesis[6].

In this study, we examined the expression of TS and the oncoprotein Bcl-2 in locally advanced and metastatic CRC and determined their inter-relationships, as well as their impact on patient survival.

MATERIALS AND METHODS

Patients, treatment and follow-up

A series of 67 patients were diagnosed and treated for Stage Ⅱ, Ⅲ, Ⅳ CRC at the Department of Oncology and Radiotherapy, Turku University Hospital (TUH) and six other hospitals in the same hospital district, between January 1996 and August 2003. The key clinical characteristics of the patients are summarized in Table 1.

At the time of diagnosis, 11 patients had stage Ⅱ, 14 had stage Ⅲ and 42 had stage Ⅳ disease. When patients developed metastases or inoperable local recurrence, they were entered into the chemotherapy protocol. In the protocol, patients received one of two treatment regimens; 18 received irinotecan alone and 49 received a combination of irinotecan, 5-FU and folinic acid (FA) as first line treatment for metastatic disease. Irinotecan (350 mg/m^2) was administered as a 60-90 min intravenous (i.v.) infusion every 3 wk. In the combination regimen, irinotecan (180-210 mg/m^2) was administered as 60-90 min intravenous infusion and 5-FU (500 mg/m^2, i.v. bolus) modulated with folinic acid (FA) (60 mg/m^2, i.v. bolus). The 5-FU/FA administrations were repeated again on the following day. The cycle was repeated every 2 wk[9]. The mean duration of chemotherapy was 6.3 mo (SD, 3.4 mo). Treatment was continued until disease progression, or occurrence of unacceptable toxicity.

The patients were prospectively followed-up until the end of March 2007; mean follow-up time from diagnosis was 34.4 mo (± 26.2 mo). We used three endpoints to calculate the patient survival: (1) disease-free survival (DFS), which was calculated in 26 patients with stage Ⅱ or Ⅲ diseaseat diagnosis; (2) overall disease-specific survival (DSS); and (3) survival with metastases (WMS). DFS is the time from diagnosis to the appearance of metastatic disease and relevant only for those patients with radically operated stage Ⅱ and Ⅲ patients at the time of diagnosis (n = 25). DSS is the time from diagnosis to death or to the time point when last seen alive at the clinic, and was calculable for all patients in the study. WMS was calculated from the date of recording the appearance of disease recurrence/metastases at the clinical visit, until to death or to the time point when last seen alive.

The study was approved by the Ethical Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

Immunohistochemical detection of TS and Bcl-2 expression

Sixty-seven formalin-fixed, paraffin-embedded primary tumors were obtained from 67 patients. Sections were cut serially at 5 μm for routine hematoxylin and eosin staining and for immunohistochemical analysis. An experienced pathologist confirmed all histological diagnoses.

TS expression was studied immunohistochemically using monoclonal antibody (Mouse Clone TS 106) from Zymed Laboratory. Bcl-2 protein expression was studied using anti-Bcl-2 monoclonal mouse antibody, which recognizes a peptide comprising amino acids 41-54 of the human Bcl-2 protein (Clone 124, DAKO A/S, Glostrup, Denmark). Signal detection was performed using the streptavidin-biotin method (Vectastain ABC kit). Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene, rehydrated in graded alcohol, immersed in 0.01 mol/L citrate buffer (pH 6.0), heated in a domestic microwave oven at full power for 2 × 5 min, and left in the buffer to cool to room temperature. The sections were incubated in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Incubation with the primary antibody diluted in 1%

Table 1 Characteristics of the patients and their tumors at diagnosis

Variable	Total patient group n (%)
Patients	67 (100)
Female	24 (36)
Male	43 (64)
Age (yr)	
Mean (SD)	57.6 (24-80)
Tumor localization	
Rectum	14 (20.9)
Left colon	31 (46.3)
Right colon	15 (22.4)
Transverse colon	7 (10.4)
Primary tumor status[1]	
T1	0 (0)
T2	5 (7.5)
T3	48 (71.6)
T4	14 (20.9)
Primary nodal status[1]	
N0	18 (26.9)
N1	35 (52.2)
No data	14 (20.9)
Histological tumor grade	
Grade Ⅰ	10 (14.9)
Grade Ⅱ	45 (67.2)
Grade Ⅲ	12 (17.9)
Stage[1]	
Ⅱ	11 (16.4)
Ⅲ	14 (20.9)
Ⅳ	42 (62.7)
Localization of metastases	
Liver	28 (41.8)
Lung	1 (1.5)
Multiple	34 (50.7)
Local	4 (6.0)
Disease-specific survival (months)	
Mean (SD)	34.4 (26.2)
Disease-specific outcome	
Alive	3 (4.5)
Died of disease	64 (95.5)

[1]TNM classification.

bovine serum albumin/Tris-buffered saline, TS (1:25) and Bcl-2 (1:50), were carried out overnight in a humid chamber at 4℃. The following day, the slides were washed and incubated first with the biotinylated secondary antibody (30 min, 20℃), then with avidin-biotin-peroxidase complex (30 min, 20℃). Positive staining was visualized with 3,3' diaminobenzidine (DAB) substrate solution and the sections were counterstained with Mayer's hematoxylin. As negative controls, slides were processed with the omission of the primary antibody.

Evaluation of TS and Bcl-2 expression

Expression of TS and Bcl-2 was assessed by an observer blinded to the clinical data. The slides were first screened for an overview of the general staining pattern. Four pictures of each slide, covering most of the tumor area, were taken with a light microscope (4 × magnification) connected to a camera and AnalySIS v 3.00 software (Soft Imaging System GmbH, Munster, Germany). Expression of TS in the four pictures was analyzed using Imaging Research Inc., St. Catharine's, Ontario, Canada), which detected the brown color of the positively stained tumor cells and counted the area of those cells in pixels, and also counted in pixels the total tumor area. The percentage of positively stained tumor cells from the whole tumor area was counted and used in further analysis. This method of evaluating TS expression was able to distinguish between the presence of many cells expressing low amounts and a few cells expressing high amounts of TS, such that the percentage of TS expression reflected total TS expression in the tumor, which may be more relevant biologically.

For statistical purposes, expression profiles of each marker were treated as dichotomous variables, where tumors with negative or weak expression of Bcl-2 was one category (reduced expression), and all those with moderate or strong expression were grouped into the second category. For TS, we used median values as cut-off to build up the dichotomous variable of low- and high TS expression. In addition, combined TS-Bcl-2 indices were created, using the dichotomous Bcl-2 variables and TS variables (median cut-offs), resulting in four possible combinations of TS/Bcl-2: low/low (L/L); low/high (L/H); high/low (H/L); and high/high (H/H). Finally, these were converted to a 3-class index as follows: class 1, TS/Bcl-2, L/L; class 2, TS/Bcl-2, L/H or H/L; and class 3, TS/Bcl-2 H/H.

Statistical analysis

Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, IL, USA) and STATA (Stata Corp., TX, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 10.1). Frequency tables were analyzed using the χ^2 test, with likelihood ratio (LR) or Fisher's exact test being used to assess the significance of the correlation between categorical variables. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis tests) for two and multiple independent samples, respectively. ANOVA was only used for deriving the mean values in each stratum. Univariate survival (life-table) analysis for the outcome measure, DSS, DFS and WMS was based on Kaplan-Meier method, and the groups were compared with the log-rank (Mantel-Cox) test. In all tests, $P < 0.05$ was regarded as statistically significant.

RESULTS

Expression of Bcl-2 and staining of TS are shown in Figure 1. Bcl-2 index and TS index (absolute values) were significantly correlated ($r = 0.286$, Spearman rho, $P = 0.019$). Similarly, using the median-cut off values, there was a significant correlation between Bcl-2 and TS expression profile ($P = 0.039$, Spearman rho or $P = 0.037$, χ^2). In pair-wise comparison, individual samples did not significantly deviate in their Bcl-2 and TS expression profile (median cut-off; $P = 0.841$, Wilcoxon signed ranks test), indicating that in individual tumors, co-detection of H/H and L/L of both markers was a frequent occurrence.

The combined TS-Bcl-2 index was evaluated in relation to all clinical variables recorded, including the response to treatment and survival (DSS, DFS and WMS). Interestingly, for the first time, a significant correlation between TS/Bcl- 2 index and DFS was observed. Among stage Ⅱ and Ⅲ disease ($n = 25$), tumors with H/H profile of TS and Bcl-2 (class 3) were associated with substantially longer DFS (42.6 mo) than those with L/L (5.4 mo) or those with H/L or L/H profile (20.7 mo; $P = 0.031$, Kruskal-Wallis), and this was even more evident in life-table analysis ($P = 0.001$, Mantel-Cox; Figure 2A).

There was a close correlation between TS/Bcl-2 expression and WMS; tumors with L/L profile of TS and Bcl-2 (class 1) were associated with a longer WMS (31.9 mo) than those with H/H (25.7 mo) or H/L and L/H (18.9 mo) in the whole series ($n = 67$, $P = 0.076$, Kruskal-Wallis and $P = 0.092$ Mantel-Cox) (Figure 2B). There was no such difference in DSS among the three TS/Bcl-2 expression profiles, however. TS/Bcl-2 expression was not significantly associated with any other clinicopathological variables, including age, sex, TNM status, grade, stage or carcinoembryonic antigen (CEA) levels.

DISCUSSION

The treatment of CRC has become increasingly complex over recent years. With the emergence of new chemotherapy drugs and targeted agents, there has been a major improvement in the prognosis of patients with metastatic CRC. The identification of prognostic and predictive markers is clinically important, because CRC is a heterogeneous disease with various biological and clinical characteristics.

The present study analyzed the combined TS and Bcl-2 expression in CRC, with particular reference to disease outlook. An increase in TS levels has been

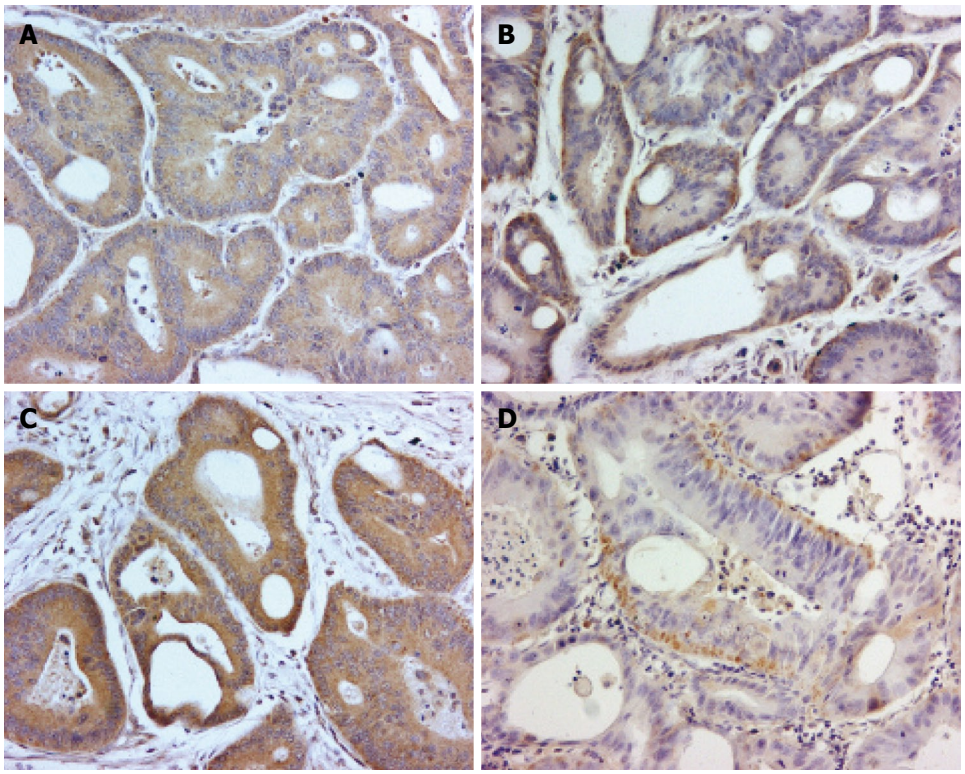

Figure 1 Examples of expression of Bcl-2 and staining of TS. A: High expression of Bcl-2; B: Low expression of Bcl-2; C: High staining of TS expression; D: Low staining of TS expression.

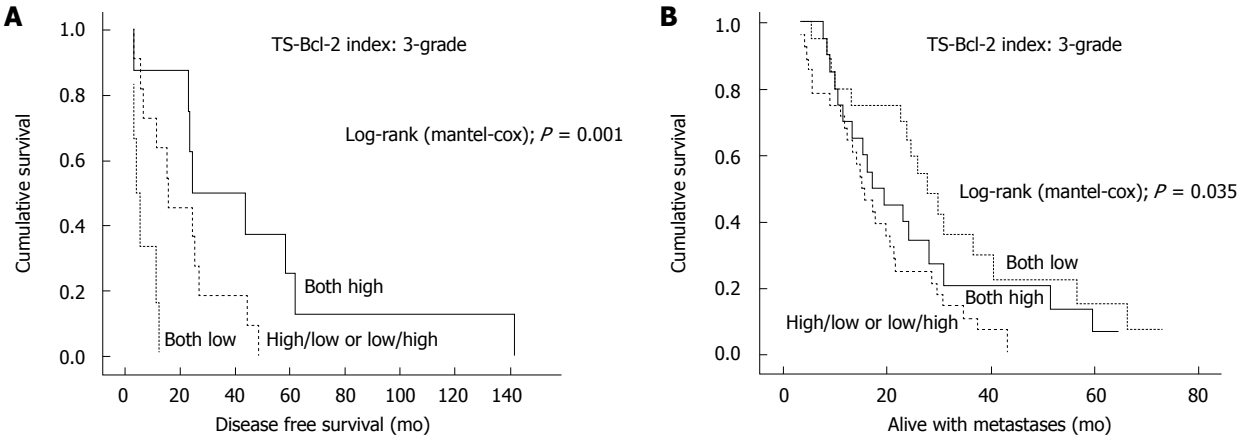

Figure 2 Univariate (Kaplan-Meier) analysis. A:TS/Bcl-2 expression and disease free survival (DFS); B:TS/Bcl-2 expression and survival with metastases (WMS).

suggested to be an important mechanism of resistance to fluoropyrimidine-based chemotherapy[10]. In several studies, TS is reported to be a predictor of both survival in CRC[11-14] and response to 5-FU therapy, with higher levels being associated with poorer prognosis and response to therapy[11,12,15,16]. This suggests that TS levels are not only of importance in predicting the natural course of the disease, but may also predict the sensitivity of cancer cells to 5-FU, which is widely used both in the adjuvant setting and in the treatment of metastases[17,18].

The role of the Bcl-2 family of proteins in chemoresponse has been evaluated extensively in *in vitro* models. Over-expression of Bcl-2 and Bcl-XL has been shown to induce drug resistance[19-21]. In relation to treatment, it has been demonstrated that elevated levels of Bcl-2 protein confers cytotoxic drug resistance to tumor cell lines[7,22]. As Bcl-2 blocks apoptosis *in vitro* and thus contributes to malignant cell accumulation, its over-expression is expected to be associated with more aggressive tumor biology. Indeed, genetic alteration of the Bcl-2 gene located on chromosome 18 is considered to be a key process in the pathogenesis and chemoresistance of human tumors, such as follicular lymphoma[23].

In our previous analysis of Bcl-2 expression in a subset of these tumors (n = 49), we found a weak association of lower levels of Bcl-2 expression with longer overall survival[24]. In another small series (n = 28)[25], we described that lower levels of Bcl-2 expression were significantly associated with lower levels of TS expression. The present study clearly confirmed this observation in a larger series of CRC patients (n = 67), for which a significant linear correlation was established between the two markers (P = 0.039, Spearman). High expression of

TS (above the median) significantly correlated with higher Bcl-2 expression levels. In our previous study, some evidence has suggested that higher levels of TS and Bcl-2 are associated with shorter overall survival[24,25], implicating that elevated levels of TS and Bcl-2 may confer cytotoxic drug resistance among these patients.

To the best of our knowledge, our study shows for the first time the relationship between these two key molecules in a group of patients with locally advanced or metastatic CRC receiving similar treatment. As to the patient survival, there was a significant correlation of TS/Bcl-2 expression with DFS, in that the patients with high TS/Bcl-2 index had a longer DFS (Figure 2A). No such effect was shown for DSS, which was not significantly different among the patients with low- and high TS/Bcl-2 indices.

To conclude, the present data suggest that patients with CRC whose tumors have high TS and Bcl-2 demonstrate a significantly longer DFS and shorter WMS, as compared to patients with low expression of these markers.

COMMENTS

Background

Thymidylate synthase (TS) is reported to be a predictor of both survival in colorectal cancer (CRC) and response to 5-FU therapy, with higher levels being associated with poorer prognosis and response to therapy. Elevated levels of Bcl-2 protein also confer cytotoxic drug resistance to tumor cell lines. We here examined the expression of TS and oncoprotein Bcl-2 in advanced CRC patients, and determined their mutual relationship, association to therapeutic response and impact on disease outcome.

Research frontiers

This study represents a translational study in which a clinical series of CRC samples were analyzed for two important molecular markers: TS and Bcl-2. Accordingly, this study represents a combined molecular analysis and a clinical study, whereby some key molecular pathways were analyzed and their relevance to clinical data was assessed in a series of 67 CRC patients with well-characterized treatment history and long-term follow-up data.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to show the relationship between these two key molecules in a group of patients with locally advanced or metastatic CRC receiving similar treatment. As to the patient survival, there was a significant correlation of TS/Bcl-2 expression with DFS, in that the patients with high TS/Bcl-2 index had a longer DFS.

Applications

The present data suggest that patients with CRC whose tumors have high TS and Bcl-2 demonstrate a significantly longer DFS and shorter WMS, as compared to patients with low expression of these markers. This information should have potential clinical implications in the management of these patients.

Peer review

The importance of this paper to the reader resides in the fact that this study is believed to be the first to suggest that CRC patients with low TS/Bcl-2 demonstrate a significantly shorter DFS, but keep alive longer with a metastatic disease. In relevant cases, one might consider utilizing this type of molecular marker data in more individualized tailoring of the appropriate therapies.

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RAPID COMMUNICATION

Quadruple therapy with furazolidone for retreatment in patients with peptic ulcer disease

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Abstract

AIM: To establish the efficacy and safety of a 7-d therapeutic regimen using omeprazole, bismuth subcitrate, furazolidone and amoxicillin in patients with peptic ulcer disease who had been previously treated with other therapeutic regimens without success.

METHODS: Open cohort study which included patients with peptic ulcer who had previously been treated unsuccessfully with one or more eradication regimens. The therapeutic regimen consisted of 20 mg omeprazole, 240 mg colloidal bismuth subcitrate, 1000 mg amoxicillin, and 200 mg furazolidone, taken twice a day for 7 d. Patients were considered as eradicated when samples taken from the gastric antrum and corpus 12 wk after the end of treatment were negative for *Helicobacter pylori* (*H pylori*) (rapid urease test and histology). Safety was determined by the presence of adverse effects.

RESULTS: Fifty-one patients were enrolled. The eradication rate was 68.8% (31/45). Adverse effects were reported by 31.4% of the patients, and these were usually considered to be slight or moderate in the majority of the cases. Three patients had to withdraw from the treatment due to the presence of severe adverse effects.

CONCLUSION: The association of bismuth, furazolidone, amoxicillin and a proton-pump inhibitor is a valuable alternative for patients who failed to respond to other eradication regimens. It is an effective, cheap and safe option for salvage therapy of positive patients.

Key words: Gastric ulcer; Duodenal ulcer; *Helicobacter pylori*; Retreatment; Furazolidone

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INTRODUCTION

Since the discovery of the etiological role of *Helicobacter pylori* (*H pylori*) in peptic ulcer disease[1], its eradication became the main objective of therapy, and several treatment regimens were developed. Currently, triple therapy with omeprazole, amoxicillin, and clarithromycin remains the best therapeutic option[2]. Despite its efficacy, 10% to 20% of the patients present with treatment failure, demanding alternative therapeutic regimes with variable success rates[3,4]. The reasons for this considerable rate of failure are several, including low patient compliance with treatment[5], and bacterial resistance to antimicrobial agents[6,7]. The development of effective salvage treatments is of paramount importance in this situation.

Furazolidone is a synthetic nitrofuran derivative with bactericidal or bacteriostatic activity against Gram-positive and Gram-negative bacteria, and it is well absorbed in the intestine with no tissue accumulation[8]. It has anti-*H pylori* activity and resistant strains appear to be rare or non-existent in many areas[9], characteristics

that make it a potential option for retreatment of peptic ulcer disease caused by *H pylori*. One of the main limitations for its widespread use is the relatively high incidence of significant adverse effects, reported mainly in European studies[10]. In a country such as Brazil, which has large populations with low socioeconomic levels and a high bacterial resistance to metronidazole[11], furazolidone emerges as an interesting option.

This study aimed to establish the efficacy and safety of a 7-d therapeutic regimen for *H pylori* eradication (omeprazole, bismuth subcitrate, furazolidone, and amoxicillin) in patients with peptic ulcer disease who had been previously treated with other therapeutic regimens without success.

MATERIALS AND METHODS

The study was performed in accordance with the declaration of Helsinki, and was approved by the institutional Ethics Review Board for clinical research, and all patients signed an informed written consent form.

Sample size calculation was determined for a descriptive study of a dichotomous variable, considering the prevalence of peptic ulcer with resistant *H pylori* in 2% of the general population.

Patients were selected from the Outpatient Gastroenterology Clinic of Hospital das Clínicas, Faculty of Medicine, University of Sao Paulo. Fifty-one patients with peptic ulcer who had previously been treated unsuccessfully with one or more eradication regimens for *H pylori* were included in the study. Bacterial persistence after treatment was confirmed by positive rapid urease test and histological examination through a modified Giemsa staining method. Gastric mucosa samples were obtained from the antrum and corpus during upper digestive endoscopy.

Patients younger than 18 years of age were excluded, as were those who presented with severe comorbidity, pregnant patients, infants, patients who had previously undergone gastrectomy, patients with a known history of allergy to the therapeutic regimen drugs, and patients who had used non-steroidal anti-inflammatory drugs, antibiotic therapy, or bismuth salts up to 4 wk before study inclusion.

In an open, cohort study, the patients were invited to use a therapeutic regimen for 7 d that consisted of 20 mg omeprazole, 240 mg colloidal bismuth subcitrate, 1000 mg amoxicillin, and 200 mg furazolidone, taken twice a day. Patients were advised not to ingest alcoholic beverages and to avoid foods related to potential side effects determined for drugs similar to monoamine oxidase (MAO) inhibitors. They were also encouraged to take the full medication regularly and were informed about the importance of adequate use of the medication for successful treatment. No other medication was allowed until the end of the treatment, when patients were evaluated regarding compliance by counting the remaining tablets. Adverse effects were recorded in a questionnaire, and each adverse effect was specifically investigated.

Table 1 Clinical data

Clinical data	*n*
Patients	51
Age (yr)	
Mean	49
Median	48
Interval	23-77
Women	32
Duodenal ulcers	39
Gastric ulcers	7
Duodenal and gastric ulcers	5
Tobacco users	16

Treatment efficacy was determined by bacterial negativity at the rapid urease test and histological examination of gastric antrum and corpus mucosa samples taken during digestive endoscopy performed 12 wk after the end of treatment.

A confidence interval of 95% was calculated for the eradication rate percentiles. The χ^2 method with Pearson coefficient was used for the comparison among the variables, eradication rate for previous treatment, gender, and age, with significance value of $P < 0.05$. Statistical analysis was performed with the statistics software, version 10.0 (SPSS Inc., USA).

RESULTS

Among the 51 patients enrolled in the study, there was no predominance regarding gender, and the median age was 48 years. Duodenal ulcer was most commonly observed (Table 1). Five (10%) patients had already undergone three or more previous treatments (32 and 14 had undergone one and two, respectively). Six patients were excluded from the analysis, three for not undergoing follow-up evaluation, and three for early interruption of the treatment due to adverse effects.

The eradication rate was 68.8% (31/45). Eradication rates were similar regardless of the number of previous treatments (one treatment: 21/32, 65.6%; two treatments: 8/14, 57.1%; three or more treatments: 2/5, 40%). Age and gender did not correlate with eradication rates ($P > 0.05$). Adverse effects were reported by 31.4% of the patients, most of which were considered to be slight or moderate. Three patients had to withdraw from the treatment due to the severe adverse effects (one with nausea, one with diarrhea, and one with dizziness).

DISCUSSION

H pylori infection is highly prevalent in Brazil. Among blood donors without gastrointestinal complaints, positive serology for *H pylori* is found in 68%[12]. In a country with more than 200 million inhabitants, we may estimate that 140 millions individuals are currently infected. If we consider a 10% incidence of peptic ulcer disease or gastric cancer, we will find 14 million people in whom eradication of *H pylori* is mandatory. Despite the good eradication rates achieved with the combination

of omeprazole, amoxicillin, and clarithromycin, 10% to 15% of the patients present with treatment failure[2]. In face of this reality, it is interesting to pursue the development of alternative therapeutic regimens with satisfactory eradication rates, low incidence of adverse effects, and low cost.

The efficacy of different regimens varies according to patient compliance and bacterial resistance to the antibiotics[13]. Clarithromycyn, quinolones and metronidazole should not be used more than once, due to *H pylori* intrinsic or induced resistance[14,15]. For patients with primary treatment failure it would be ideal to test the *H pylori* antimicrobial sensitivity, but the high cost and the lack of laboratories capable of adequately performing sensitivity tests limit this strategy. Retreatment must be based on knowledge of the antimicrobial agents previously used.

In our country, *H pylori* strains show an intrinsic resistance to metronidazole that reaches 50%, which decreases the efficacy of schemes containing this drug or other nitroimidazolics[16-19]. Furazolidone is widely available in public health care facilities in Brazil. Despite that no decrease in susceptibility has been observed[20], and bacterial resistance appears to be rare or non-existent in many areas[18,21,22], even among metronidazole-resistant isolates[23]. The main concern regarding its use is the adverse effects observed, especially among individuals who do not adhere to the dietary restrictions recommended, since this drug belongs to the group of the MAO inhibitors. Asian and European studies have reported a relatively high incidence of adverse effects (31.4%-35%)[10,24,25]. Conversely, two South American[26,27] studies have shown fewer undesirable symptoms when compared to the previously mentioned studies. In the present study, despite a comparable incidence of adverse effects during treatment, only three patients had severe symptoms demanding treatment discontinuation. Lower doses of furazolidone could decrease the incidence of undesirable symptoms, but this strategy can also lead to a lower eradication rate[22,28,29].

The prevalence of smokers was similar to that in the Brazilian population and no significant differences in the results were observed when analyzing the number of previous treatments, gender and age. Even though compliance was good, and the length of treatment was short, this regimen ought to be considered only as an alternative for patients with previous treatment failures. Extending the antibiotic course to 10 d or 14 d could improve eradication rates, despite a greater likelihood of adverse effects[30,31].

Furazolidone appears to be an excellent choice for combination therapy for *H pylori* infection, especially as a substitute for metronidazole in quadruple therapy regimens in areas with high prevalence of metronidazole-resistant strains[32]. In our study, the eradication rate was 68.8%, which was superior to that previously reported[33,34], and, considering the efficacy, safety and potential cost-effectiveness, it seems reasonable to introduce furazolidone-based regimens following the failure of initial eradication attempts[32]. The differences regarding the safety profile between South American and American and European studies may be attributed to the limited clinical experience with this drug in the former regions, where it is unavailable and expensive[33].

In conclusion, our study shows that the association of bismuth, furazolidone, amoxicillin and a proton-pump inhibitor is a valuable alternative for patients who failed to respond to an initial therapeutic regime in a country with high prevalence of *H pylori* metronidazole-resistant strains. This scheme is an effective, cheap and safe option for salvage therapy of *H pylori* positive patients.

COMMENTS

Background

Since the discovery of the etiological role of *Helicobacter pylori* (*H pylori*) in peptic ulcer disease, its eradication became essential to allow for adequate healing and prevention of recurrence. It is well established that triple therapy with omeprazole, amoxicillin and clarithromycyn is the first-line treatment, but it fails in 10% to 20% of the patients, demanding alternative therapeutic regimens.

Research frontiers

Furazolidone has anti-*H pylori* activity, low incidence of resistance, is cheap, and is widely available. Despite these characteristics, not many studies have been performed to evaluate its efficacy for retreatment.

Innovations and breakthroughs

This study provides further evidence of the efficacy and tolerability of a short-term furazolidone-based quadruple regimen in South America.

Applications

Furazolidone-based regimens may be an interesting option for retreatment due to their low cost and low resistance rate, especially in developing countries such as Brazil, where metronidazole-resistant strains are common.

Peer review

Quadruple therapy with the medicine used in this study is not a new regimen. It has been used widely in China with good results. Unfortunately, it is not popularly used outside China. The present study was a small series with not very satisfactory results (efficacy rate 68.8%). However, I think it is still worthwhile to be published in our journal, at least indicating that the regime was also used in a South American country with some success.

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RAPID COMMUNICATION

Identification and characterization of genotype A and D recombinant hepatitis B virus from Indian chronic HBV isolates

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Abstract

AIM: To confirm the presence of recombination, full-length hepatitis B virus (HBV) from chronic patients was sequenced and analyzed.

METHODS: Full-length HBV genomes from 12 patients were amplified and sequenced in an automated sequencer. Phylogenetic analysis was carried out on full-length, Core and preS2/Surface regions using MEGA software. SimPlot Boot Scanning and amino acid sequence analysis were performed for confirmation of recombination.

RESULTS: Eight patients were infected with genotype D strain; one patient with genotype A and three patients had genotype A and D recombination; two of them had cirrhosis and one had hepatocellular carcinoma. Phylogenetic analysis of core and preS2/surface regions separately showed evidence of genotype A and D recombination. The breakpoints of recombination were found to be at the start of preS2 and at the end of surface coding regions.

CONCLUSION: We identified and characterized recombinant A and D genotype HBV in hepatitis B surface antigen (HBsAg)-positive patients.

Key words: Hepatitis B virus; Genotype; Variation; Evolution; Recombination

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INTRODUCTION

Hepatitis B virus (HBV) is an organ-specific virus causing inflammation of the liver, leading to complications such as chronic liver disease (CLD) and hepatocellular carcinoma (HCC). As compared to Europe and North America, the prevalence of HBV infection in Asia is quite high, with 40 million people harboring chronic HBV infection in India[1].

Two features make HBV unique. First, its way of replication, by which it uses the pregenomic RNA as an intermediate step for reverse transcription. Second, the efficient utilization of its compact genome for production of seven different proteins from four open reading frames (ORFs). Major proteins that are encoded from these four ORFs are the envelope, core the X protein and the polymerase.

Nucleotide substitution, deletion, insertion and recombination are the main factors that results in variation of the HBV genome. HBV genotypes are classified into eight genotypes, from A to H, based

on the inter-group divergence of 8% or more in the complete genome nucleotide sequence, or a 4% or greater divergence of the Surface gene[2-4]. Recent studies have reported recombination between the HBV genomes of two genotypes. Two kinds of HBV genotype B have emerged[5-7] i.e. recombinant with genotype C and without recombination with C. Mixed genotype refers to an infection that contains more than one genotype in the same patient and is usually the result of multiple exposures and super-infection, the complete genome of each strain belongs to one genotype. According to Robertson *et al*[8], recombination can be detected when different genes or different regions within the same gene are placed by phylogenetic analysis into different sequence subtypes.

We and others from India have reported the presence of mixed genotype A and D[9-12]. However, despite the presence of mixed genotypes, there are no reports from India about the presence of recombination, especially using the full-length HBV genome sequencing approach.

In the present study, we have identified recombinant genotype A and D in patients with CLD and HCC due to chronic HBV infection.

MATERIALS AND METHODS

Patients and serological markers

Twelve treatment-naive chronic HBV infected patients [five with cirrhosis, five with chronic hepatitis B (CHB), and two with HCC] were enrolled. The serum from these patients was tested for the presence of hepatitis B surface antigen (HBsAg) by ELISA (Abbot Laboratories, North Chicago, USA and Organon Tecknika, Boxtel, Netherlands). In addition, the serum was tested for hepatitis e antigen (HBeAg), antibody to hepatitis e antigen (anti-HBe), hepatitis B core Antigen (IgG anti-HBc) by ELISA (Organon Tecknika, Boxtel, Netherlands). Assessment of the severity of liver disease was made by Child-Pugh score[13]. Approval of the institutional ethical committee was obtained to undertake this study.

HBV DNA quantitation

HBV DNA was quantified by a commercially available hybrid capture assay (Ultra sensitive kit, Digene, USA) with the lower limit of detection being 4700 copies/mL.

Full-length HBV DNA amplification

HBV DNA was extracted by using 0.5 to 1.0 mL of patient's plasma using Sera Lysis Buffer (10 mmol/L Tris, 5 mmol/L EDTA, 50 mmol/L NaCl), SDS (1%) and proteinase K (1 mg/mL), followed by extraction with Tris-saturated phenol (pH 7.9) chloroform and then precipitation with ethanol. The obtained pellet was dried and dissolved in 30 μL of 1 × TE buffer (10 mmol/L Tris 1 mmol/L EDTA), a method described previously[12]. Full-length HBV DNA amplification was done by polymerase chain reaction (PCR), as described by Gunther's method[14]. The Taq polymerase with DNA proof reading activity was used. (Expand high fidelity Taq-Polymerase Roche GmBH Basel, Switzerland). Primers were: P1-CCGGAAAGC TTGAGCTCTTC TTTTTCACCTCTGCCTAATCA (1821-1841), P2-CGGAAAGCTTGAGCTCTTCAAAAAGTTGCA TGGTGCTGG (1823-1806). The reaction conditions for PCR were 94℃ for 5 min, 94℃ for 1 min, 60℃ for 1.5 min; 68℃ for 7 min and extension at 72℃ for 10 min, 35 cycles were performed. Purified full-length HBV DNA from recombinant vector pCF 80 (Tetramer of 3.2 kb HBV cloned in pBR322) was used as a positive control. DNA extracted from serum samples of healthy individuals and commercially available molecular biology grade water served as the negative control. Every set of PCR amplifications included HBV-positive and-negative controls. Primers were designed using the software Primer Express.

Table 1 Primers used for sequencing

Name	Sequence	(nt.)
P1_F	5-TTTTTCACCTCTGCCTAATCA-3	(1821-1841)
SEQ_F1	5-AGGCAACTATTGTGGTTTCA-3	(2194-2212)
SEQ_F2	5-TCTTTAACCCTCATTGGAAA-3	(2516-2535)
SEQ_F3	5-TCACCATATTCTTGGGAACAAGA-3	(2823-2845)
SEQ_F4	5-CTTCCTGCTGGTGGCTCCAGTTC-3	(53-75)
SEQ_F5	5-CTCGTGGTGGACTTCTCTC-3	(253-272)
SEQ_F6	5-ATCCTCAACCACCAGCACG-3	(492-510)
SEQ_F7	5-TATTGGGGGCCAAGTCTGTA-3	(749-768)
SEQ_F8	5-TTTACCCCGTTGCYAGGCA-3	(1144-1162)
SEQ_F9	5-CTCATCTGCCGGACCGTG-3	(1562-1581)
P2_R0	5-AAAAAGTTGCATGGTGCTGG-3	(1825-1841)

Sequencing full-length HBV genomes

PCR-amplified products were purified using the Qiagen Gel purification kit according to their recommended protocol. Internal primers used for sequencing given in Table 1 were used for sequencing in an automated DNA sequencer (ABI Prism 3730 Applied Biosystems, Foster City, USA). The nucleotide sequence data reported in this paper appears in the GenBank/EMBL/DDBJ nucleotide sequence databases with accession numbers EF103275-EF103285 and AY945305. The genome length has been measured according to Galibert *et al*[15].

Data analysis

HBV genotyping was done by phylogenetic analysis using full-length sequences, core and preS2 and surface regions. Briefly, sequences were aligned using the CLUSTALW software[16]. Phylogenetic trees were constructed using the Kimura two-parameter matrix and neighbor-joining (NJ) method by MEGA software version 3.1[17]. To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Recombination was investigated by SimPlot[18] distributed by the author Ray at (http://www.welch.jhu.edu/). Boot scanning was performed for each of the strains using four sequences at a time[19], i.e. putative recombinant sequence, two consensus sequences of the parental genotype and one consensus sequence as an out-group.

Table 2 Baseline characteristics of patients

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
Age (yr)	8	24	20	20	30	29	88	38	45	40	62	50
Gender	M	F	M	M	M	M	M	F	M	M	M	M
Diagnosis	CHB	CHB	CHB	CHB	CHB	Cirr.	Cirr.	Cirr.	Cirr.	Cirr.	HCC	HCC
Bilirubin (mg/dL)	1.1	0.68	1.1	0.6	1.1	1	0.8	0.4	2.1	1.4	0.9	1.2
ALT (IU/L)	46	54	42	59	52	107	78	29	101	98	31	43
Albumin (g/dL)	4	4.1	4.3	4.8	4.3	4	2.2	3.9	3.0	3.7	4.1	4
PT prolongation (s)	2	2	1	1	2	4	4	18	8	5	2	2
Ascites	No	No	No	No	No	No	No	No	Yes	No	No	No
Encephalopathy	No	No	No	No	No	No	No	No	No	No	No	No
CTPscore	5	5	5	5	5	6	8	7	9	6	5	5
HBeAg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Neg
HBV DNA (log copies/mL)	5.4	5.6	6.4	7.2	5.6	6.4	4.9	5.1	5.7	6.1	5.3	6.5
HAI	2	5	6	6	5	-	-	-	-	-	-	-
F Score	1	1	1	3	2	-	-	-	-	-	-	-

Cirr: Cirrhosis; ALT: Alanine aminotransferase; PT: Prothrombin time; CTP: Child-Turcotte-Pugh; HAI; Histological activity index; F: Fibrosis.

RESULTS

Patients and virological characteristics

Baseline characteristics of the study population are given in Table 2. The majority of the patients were male (M: 10, F: 2). Of the 12 patients, five had cirrhosis, all diagnosed radiologically; [one decompensated with a Child-Turcotte-Pugh (CTP) score of 8, and four compensated with a CPT score of 5], and five with CHB (all biopsy proven), and two had HCC. Of the 12, eight were HBeAg-positive and the remaining four were anti-HBe positive. The *Eco*RI restriction enzyme site was present in seven of the full-length sequences, whereas it was absent in five. All sequences had a nucleotide (nt.) length of 3182 except genotype A sequence, which had 3221 nt.

Distribution of genotypes

Phylogenetic analysis using complete HBV genomes of genotypes A to H derived from GenBank revealed the presence of genotype A and D in the study population. Genotype D was predominant, accounting for 92% of the study patients (Figure 1). The nature of genotype D was confirmed by the presence of a 33-bp deletion in the preS1 and a 6-bp deletion in the core terminal regions. Whereas in the genotype A sequence, the 33-bp and 6 bp deletions were absent. Phylogenetic analysis of the core revealed the same results as analysis done with complete HBV genomes as shown in Figure 1A and C.

Presence of A and D genotype recombination

Phylogenetic analysis of preS2/surface region of 12 isolates revealed clustering of three more sequences in addition to isolate 60 in the genotype A branch (Figure 1B). Presence of recombination was confirmed by boot scanning SimPlot analysis; all the sequences were subjected to analysis using the consensus sequence of genotype A, D and H as the out-group as shown in Figure 2. Recombination break points, of three recombinant strains were detected in preS2 and surface ORFs. Isolate 113 had break points at nt 595-618; isolate 105 had break points at nt 639-659 and 723-737. Isolate 103 had break points at nt 319-359 and 1170-1184. PreS2 and surface regions showed similarity with genotype A at 18 amino acid positions in the recombinant sequences, whereas it was absent in the surface, core and X ORFs. Four of them were identified in the preS2, whereas 13 in the surface region, as shown in Figure 3A and B.

Major hydrophilic and the "a" determinant regions

As shown in Figure 3C, when analyzed considering only genotype D, the major hydrophilic region (MHR) showed substitutions at 10 amino acid positions. Of the 10 changes, five spanned the "a" determinant region. When similar analysis was done considering genotype A, we could detect a single mutation in isolate 113 at position 144, changing threonine to methionine in the "a" determinant region of the surface region. All the isolates showed the presence of concomitant threonine to proline change at position 131 of the "a" determinant region, which is homologous to the genotype A sequence.

Sequence characteristics of precore/ core and X ORFs

Among the 12 patients, precore stop codon mutation (W28Stop) was found in two patients, and both belonged to the recombinant genotype. We could document the difference in the core nucleotide sequences in the recombinant sequences; however, they were not exactly similar to the typical genotype A pattern. We detected the presence of T1936C nucleotide mutation in the core gene in one of the HCC patients, isolate number 113.

X ORF: In two patients, we detected mutations in X ORF. Both belonged to the recombinant genotype, i.e. isolate 113 mutations were detected at three positions I127T, K130M, V131I, and isolate 105 was harboring a single mutation at position I127L.

DISCUSSION

Phylogenetic analysis based on two different genomic regions, preS2/surface and core, suggested the existence

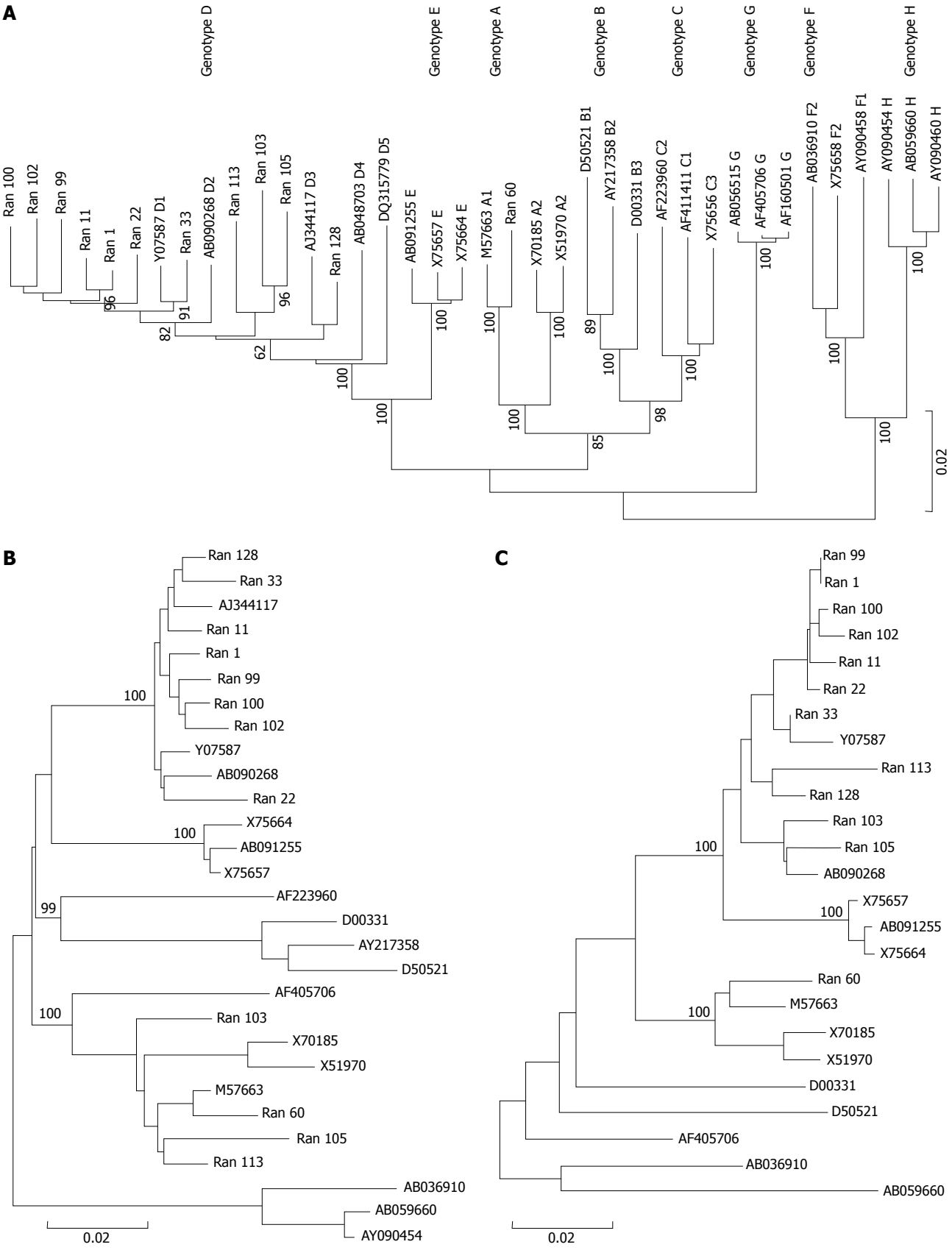

Figure 1 Phylogenetic tree revealed the distribution of genotypes. A: Neighbor joining (NJ) phylogenetic tree was constructed based on the complete genome sequences of 26 HBV reference strains from DDBJ/EMBL/Genbank and 12 Indian HBV isolates in the present study. The bootstrap values obtained from 1000 replicas are shown at the nodes of the main branches. HBV strains from this study are marked as Ran as suffix followed by the isolate number. Reference sequences are denoted by their accession numbers. The capital letter A to H denotes the HBV genotype; B: Phylogenetic analysis done on the PreS2 and surface regions showing three additional (103,105,113) isolates to be grouped in genotype A branch. Phylogenetic trees were constructed by NJ Kimura two-parametric method. Bootstrap values are shown at the nodes of the main branch and at the bottom distance is given; C: Phylogenetic analysis using the Core region. Phylogenetic tree was constructed by NJ Kimura 2-parametric method Bootstrap values are shown at the nodes of the main branch and at the bottom distance given.

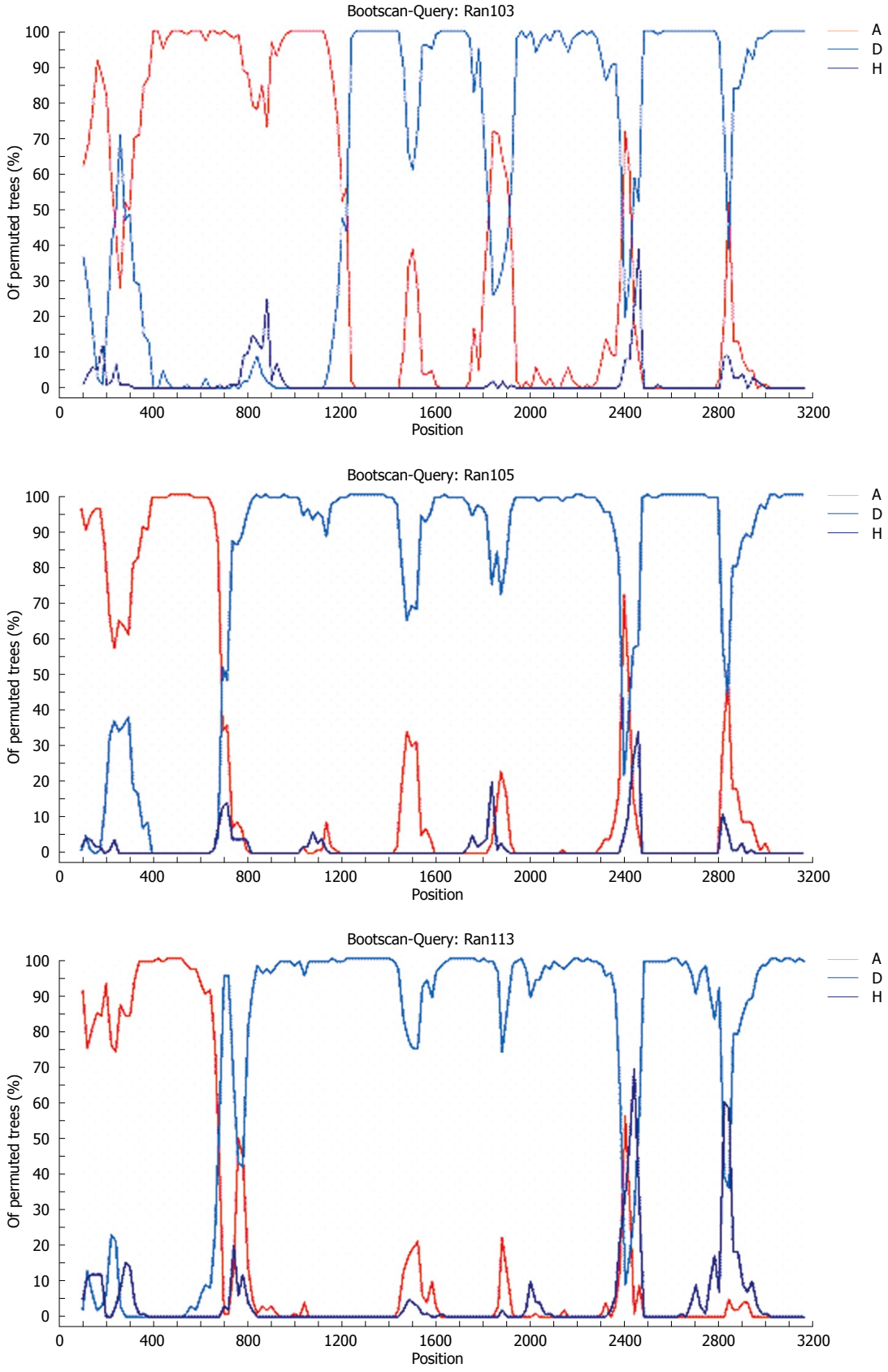

Figure 2 SimPlot analysis demonstrating the recombination in two isolates 103 and105, which were subjected to bootscan analysis over the entire genome using SimPlot program (Lole *et al*[18]).

of recombinant strains in Indian isolates of HBV. On examination of the preS2 and the surface region sequences, a close relation with genotype A sequence was detected in three genotype D sequences respective to core, which was genotype D. Further analysis of corresponding genomes allowed us to map the crossover

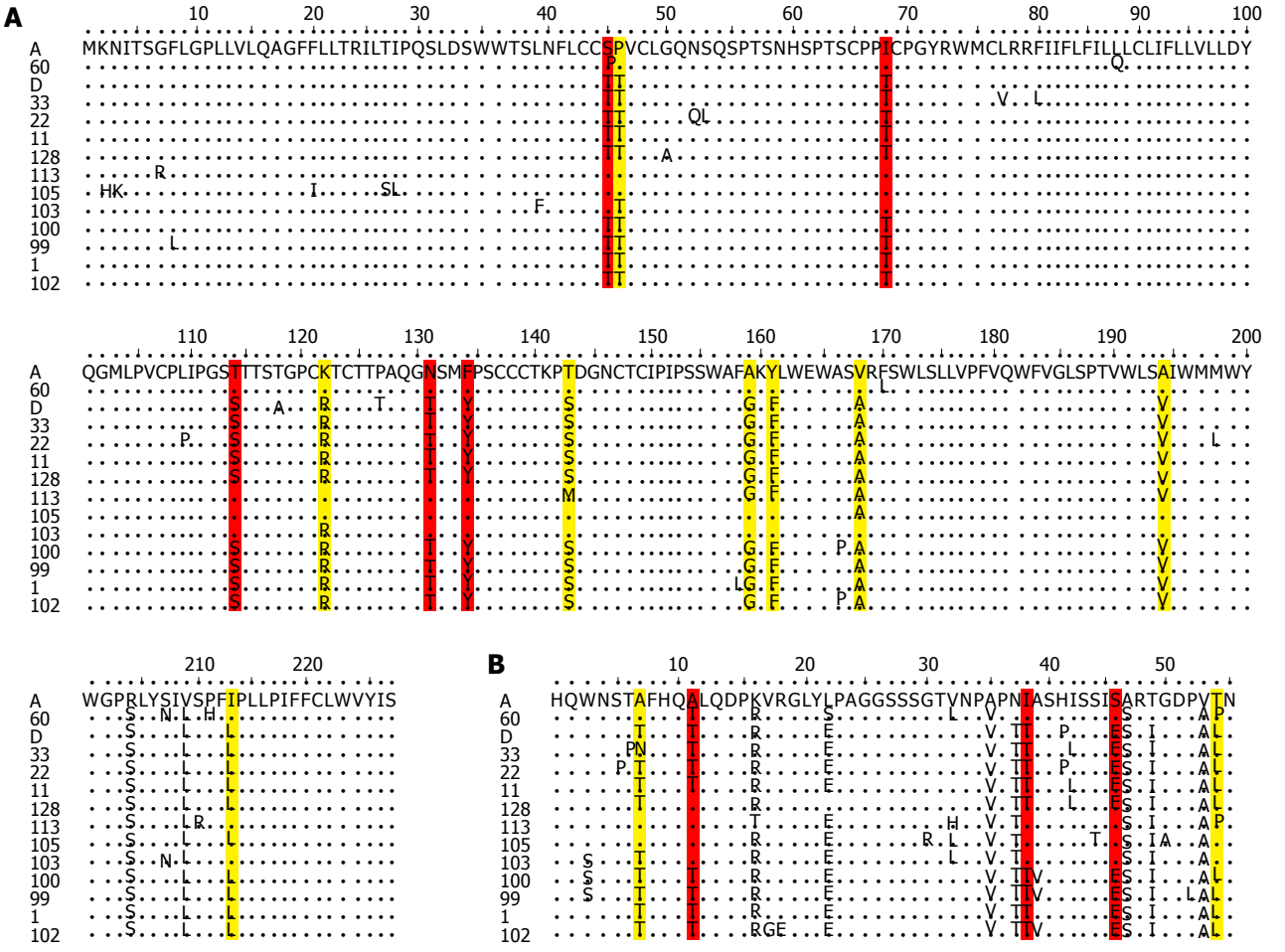

Figure 3 Recombinant sequence similarities with genotype A in PreS2 and Surface region. A: Surface region alignment with genotype A and D amino acids, marked in red shows all three recombinant sequences having similarity with genotype A, whereas yellow marked amino acids shows at least one recombinant sequence similar to genotype A, over all 18 positions; B: Shows same in the preS2 region; C: Shows all the amino acids of MHR of genotype D in black, upward arrow indicates the change of amino acid; D: Shows "a" determinant region and corresponding alignment with 12 isolates.

junctions and to confirm the findings of recombination.

HBV recombination is not a new phenomenon, it is important from an evolutionary as well as epidemiological point of view. As increasing number of full-length HBV genome sequences are reported, and a higher frequency of recombinant hybrid genomes is being recognized. Evidence of HBV recombination from different parts of the world suggests the presence of recombination of HBV genotypes involving A/C, A/D, A/E, B/C, B/D, G/A and G/C strains[20-23]. In Asia, recombination of genotype C/D has been reported from Tibet and China[21,23], whereas recombination of B and C has been detected in Japan[24]. HBV strains from Vietnamese patients also show evidence of

recombination of C and A genotypes[25]. Genotype A and D recombination has only been documented in CHB patients from South Africa[26]. Although the recombination of A and D genotypes has been detected from Italian and Indian HBV strains, such patients were surface-antigen negative[7]. Furthermore, breakpoints of recombination were different from the presently identified recombinant strains[27,28].

A switch in genotype has been documented during change of HBsAg-positive to serologically negative phase[29]. As we detected recombination in the preS2/surface coding region, we focused on the major hydrophilic region, "a" determinant region of surface ORF, speculating that the changes in the MHR and the "a" determinant region could lead to absence of the surface antigen, using standard serological methods. Our analysis revealed changes at 11 amino acid positions when the analysis was done using genotype D (Figure 3C). When the analysis was carried out considering both A and D genotypes, we could detect changes at only a single position substituting (K) lysine with (M) methionine in the "a" determinant region. However, the amino acid substitutions that are supposed to alter the conformation of "a" determinant region were not found.

Compared to genotype D, genotype A is more prevalent in the HBeAg-positive than in the anti-HBe-positive phase[30,31]. It is known that HBV genotype D virus has a selection advantage to form the precore stop codon mutation, as compared to the genotype A virus, the selection being at the pre-genome encapsidation level. However, in the recombinant sequences identified in the present study, we detected the precore stop codon mutation in two of them. The presence of the stop codon in the precore region and co-infection with other genotypes A, C and H are two important features of HBV genotype G[32]. In presently identified recombinant sequences, we detected the presence of Pc G1896A, and this suggests a possible similar situation and a matter for further investigation.

Recent reports suggest the presence of mixed genotypes in Asia. Europe and Africa in CHB patients, including India[9,11,33]. Moreover, higher levels of HBV replication have been shown to be associated with mixed genotype infection[33].

It is quite possible that the PreS, core, X and P proteins are continually expressed but the preS2 region/protein of genotype D is lost for a short interval during recombination with genotype A sequences, mimicking a molecular window period. It is not yet known whether recombination is advantageous for the virus or the host, but it is quite possible that this phenomenon increases the chances of virus survival and doping the host defense system.

One of the reasons for enhanced HCC development in young African adults could be high HBsAg expression in genotype-A-harboring patients[34] HBV genotype A directs the high level of synthesis of HBsAg in proportion to viral DNA, core protein and HBeAg[35].

The frequency of detection of spliced viral genomes is higher in CHB cases compared to acute and resolved HBV infections. The generation of recombinant HBV could be intracellular, as the ratio of full-length and spliced genomes isolated from the intracellular compartment was significantly higher than from extracellular space. This indicates that, compared to those containing spliced genomes, nucleocapsids containing full-length genomes are preferentially enveloped and released from the cell, and could be one of the reasons for severe liver disease[36]. It would be worth while to study the co-infection of two genotypes, and to establish whether the changes accumulate in one cell or together in the newly infected cells. Genetic exchanges between different viral strains within the infected hepatocytes could be one of the possible reasons for recombination.

HBV infection is the predominant factor for the development of HCC in India[37]. Several reports suggest integration of the preS/S region in cancerous liver tissue[38-40]. Binding of the PreS region with fibronectin and transactivation of TGF α could lead to development of cirrhosis and HCC[41-43]. It is quite unique that HBV uses its strongest promoter preS2/S for expression of the host cellular genes, which are advantageous for the virus itself.

HBV recombinant sequences were analyzed with the orangutan and gibbon monkey hepatitis virus. However, we could not find any association of recombinant sequences with them, and the reason for such transmission was clarified (data not shown). Secondly, phylogenetic analysis was done using the Italian and Indian recombinant sequences reported previously[27,28]. However, they were not clustering in the same region as detected in the present recombinant sequences (data not shown).

There are three theories proposed for evolution of HBV: The new world origin theory[5], co-evolution theory[3,44] and co-speciated theory[45,46]. We postulate a competitive selection theory in which the virus and the host cellular machinery compete, and involvement of various unidentified ways by the virus to combat the host defense mechanisms. A few of these could be the splicing, integration, recombination and down-regulation of MHC I . On the other hand, host APOBEC response to edit the viral genome, CTL proteosome complex and various host genetic factors, taking into consideration ethnicity, may play a part as well. Recombinant detection of mixed genotypes, however, may be the tip of the iceberg as a template switch over, splicing and extensive editing by all APOBEC 3 proteins, which have not been well studied.

It can be argued that, in our study, the HBV sequences were analyzed only at one stage of the disease, the process of sequential changes and the time points were not tracked. This was a preliminary study and such studies are quite cumbersome and expensive.

In summary, we identified new A/D recombinants from Indian CLD and HCC patients. To the best of our knowledge, this is the first report which describes recombinant A and D genotype from HBsAg-positive

patients from Asia, and indicates the association of recombinant HBV genotype with HCC. The results of the present study warrants further larger studies to identify populations of recombinant viruses in different clinical categories of HBV patients.

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COMMENTS

Background

Recombination is common in retroviruses, especially human immunodeficiency virus (HIV). As the hepatitis B virus (HBV) uses the reverse transcription step using the pre-genomic RNA, the rate of mutation accumulation is at a much higher rate compared to other DNA viruses. India, being highly populated, harbors the second largest pool of HBV carriers. Recombination is also one of the mechanisms of sequence variability and could account for the non-response to antiviral therapy as well as vaccine. Though recombination from the Indian subcontinent has been detected, authors for the first time report recombination of A and D genotype in HBsAg-positive chronic HBV patients.

Research frontiers

Non-response to antiviral drugs and vaccine is one of the hot research related to the article.

Innovations and breakthroughs

This is believed to be the first report describing the recombinant genotype on the Indian subcontinent.

Applications

Large-scale studies are warranted to determine the prevalence and profile of the recombinant genotype on the Indian subcontinent. The affect of antiviral therapy on the recombinant virus is also warranted.

Terminology

Mixed genotype refers to an infection that contains more than one genotype in the same patient, and is usually the result of multiple exposures and super-infection, the complete genome of each strain belongs to one genotype. Recombinant genotype can be detected when different genes or different regions within the same gene are placed by phylogenetic analysis into different sequence subtypes.

Peer review

This is an interesting paper, which confirms the presence of recombination, and full-length HBV from chronic patients were sequenced and analyzed. Authors identified and characterized recombinant A and D genotype HBV in HBsAg-positive patients.

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RAPID COMMUNICATION

Effect of a fermented milk containing *Bifidobacterium lactis* DN-173010 on Chinese constipated women

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Abstract

AIM: To investigate the effect of a fermented milk containing *Bifidobacterium lactis* DN-173010 and yogurt strains (BIO®) on adult women with constipation in Beijing.

METHODS: A total of 135 adult females with constipation were randomly allocated to consume for 2 wk either 100 g of the test fermented milk or 100 g of an acidified milk containing non-living bacteria (control). Stool frequency, defecation condition scores, stool consistency and food intake were recorded at baseline and after 1 and 2 wk in an intention-to-treat population of 126 subjects. In parallel, safety evaluation parameters were performed.

RESULTS: At baseline, no differences were found between groups. Following consumption of test product, stool frequency was significantly increased after 1 wk (3.5 ± 1.5 *vs* 2.4 ± 0.6, $P < 0.01$) and 2 wk (4.1 ± 1.7 *vs* 2.4 ± 0.6, $P < 0.01$), *vs* baseline. Similarly, after 1 and 2 wk, of test product consumption, defecation condition (1.1 ± 0.9 *vs* 1.9 ± 1.2, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.9 ± 1.2, $P < 0.01$, respectively) and stool consistency (1.0 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$ and 0.6 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$, respectively) were significantly improved. Compared with the control group, stool frequency was also significantly increased (3.5 ± 1.5 *vs* 2.5 ± 0.9, $P < 0.01$ and 4.1 ± 1.7 *vs* 2.6 ± 1.0, $P < 0.01$, respectively), and defecation condition (1.1 ± 0.9 *vs* 1.6 ± 1.1, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.01$, respectively) and stool consistency (1.0 ± 0.8 *vs* 1.4 ± 1.0, $P < 0.05$ and 0.6 ± 0.8 *vs* 1.3 ± 1.0, $P < 0.01$, respectively) significantly decreased after 1 and 2 wk of product consumption. During the same period, food intake did not change between the two groups, and safety parameters of the subjects were within normal ranges.

CONCLUSION: This study suggests a beneficial effect of a fermented milk containing *B. lactis* DN-173010 on stool frequency, defecation condition and stool consistency in adult women with constipation constipated women after 1 and 2 wk of consumption.

Key words: Probiotic; *Bifidobacterium lactis* DN-173010; Fermented milk; Constipation; Stool frequency; Stool consistency

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INTRODUCTION

In the Chinese National Product Standard of GB2746-1999, fermented milk is a product prepared with cow's milk or milk powder as raw material by degreasing, partly degreasing or non-degreasing and fermentation. Bacteria used to ferment milk, typically lactic acid bacteria (LAB), are bacteria that can produce lactic acid during the metabolism process. At present, common LAB used as fermentation agents include: *Streptococcus thermophilus*, *Lactobacillus delbruekii sp bulgaricus*,

Lactobacillus acidophilus, and *Lactobacillus casei*. The health effects of yogurt validated by research include: regulation of intestinal and colon flora, prevention or treatment of diarrhea, regulation of immune function, decreasing symptoms of inflammatory bowel diseases, improvement of lactose intolerance, lowering blood cholesterol level, and prevention of certain types of cancers[1,2]. These effects are a result of the yogurt characteristics due to the fermentation by yogurt symbiosis[3].

Constipation is a common problem, and generally refers to less than 2-3 stools per week, accompanied by small, dry, and/or hard defecation and discharge difficulty. This trouble is commonly reported in many regions of the world including Asia[4] North America[5], and Europe[6], Functional constipation is caused by non-organic or drug factors. Constipation can result in some discomforts such as abdominal distension, abdominal pain, headache, dizziness and poor appetite[7]. The symptoms of constipation can interfere with quality of life. An epidemiological study conducted in Beijing concluded that 6.1% of the adult population was suffering from the symptoms of functional constipation[8]. The disorder is more common in women and elderly people[9]. For example, in Beijing, the incidence of constipation in males and females is 1/4.59. High risk factors include anxiety, work fatigue, bad mood and working at a sedentary job[10].

Probiotics are defined as live micro organisms that, when administered in adequate amounts confer a health benefit on the host[11]. *Bifidobacterium lactis* DN-173010 survives complete transit through the digestive tract and is recovered live in stools in large quantities relative to the quantity initially ingested[12-14]. Three separate clinical studies have demonstrated that daily consumption of fermented milk containing *B. lactis* DN-173010 in association with the yogurt starters *L. bulgaricus* and *S. thermophilus* for 15 d improves gastrointestinal transit. This effect is enhanced with increased daily intake (effect of three pots/d > two pots/d > 1 pot/day) in elderly subjects free of any gastrointestinal pathology[15,16], and in a group of healthy women aged 18-45 years[17]. An exploratory study was designed to examine the effect of this product, compared to heat-treated yogurt, on quality of life and symptoms in irritable bowel syndrome (IBS)[18]. This large scale (267 IBS constipation predominant, Rome II criteria), double-blind, randomized, controlled, parallel group study over 6 wk demonstrated that daily consumption of this product alleviates bloating and improves digestive comfort as assessed by the Functional Digestive Disorders Quality of Life questionnaire. An increase in stool frequency was also observed in subjects with the lowest stool frequency (< 3 or 4 bowel movements/wk) without any change of stool consistency. A fifth study performed on fermented milk containing *B. lactis* DN-173010 alone also demonstrated a reduced transit time in healthy men and women[19]. The results of these five studies support a strong link between improved stool frequency and the strain *B. lactis* DN-173010, and indicate that further research should be carried out to investigate the potential use of this fermented milk product for improving stool parameters in subjects with constipation.

The aim of this study was to investigate the effect of a fermented milk (Bio®) combining *B. lactis* DN-173010 and yogurt strains on functional constipation parameters of adult women in Beijing.

MATERIALS AND METHODS

Study population

A total of 135 women, age 25-65 years old, were recruited in Beijing Hospital. They had a diagnosis of constipation according to the following criteria: less than three stools per week; increased stool hardness; non-organic constipation and habitual constipation.

The following were excluded from the study: Those unable to orally ingest or unable to administer according to instruction; those unable to express complaint clearly; those with constipation symptoms caused by surgical operation within 30 d; those with recent defecation difficulty due to severe organic diseases (colon carcinoma, severe enteritis, intestinal obstruction, inflammatory bowel disease); those with defecation difficulty accompanied by pain; those with acute gastrointestinal tract disease developing within 30 d; those who were pregnant or menstruating; with severe whole body diseases such as cardiovascular, liver, kidney and hematopoietic system; those taking short-term products which may have influenced the results.

Study protocol

One week before product consumption (baseline period), general conditions, safety evaluation parameters, dietary intake and defecation functional parameters (stool frequency, defecation condition scores, stool consistency) were recorded. Thereafter, eligible constipated women were randomly allocated to consume, daily for 2 wk, one pot of either the test product (67 cases) or the control product (68 cases). Subjects were stratified by age, daily eating habits, and constipation causes to the extent possible to ensure inter-group comparability, which could possibly have influenced the results. During the study period, subjects maintained their usual lifestyle and eating habits. The study was approved by the Ethical Review Committee of the Chinese Academy of Preventive Medicine, Institute of Nutrition and Food Hygiene. All subjects provided written informed consent before inclusion in the study.

Study products

The test product was a fermented milk (BIO®, Danone (Shanghai) Consulting Co., Ltd, Shanghai, China), containing *B. lactis* DN-173010 [1.25×10^{10} colony forming unit (cfu) per pot], together with the two classical yogurt ferments, *S. thermophilus* and *L. bulgaricus* (1.2×10^9 cfu/pot).

The control product was an acidified milk without any ferments or probiotics. Both the test and control products were without flavor, and had similar appearance,

color, texture, taste and lactose concentration level. Each serving, corresponding to one pot, contained 100 g. Both products were specially prepared for the study and provided by Danone (Shanghai) Consultation Corp., Ltd.

Assessments and study criteria

The general conditions, such as the mental status, sleep, eating habit, and blood pressure, were evaluated 1 wk before product consumption.

The safety evaluation parameters blood, urine, and stool routine examinations [red blood cell (RBC) count, white blood cells (WBC) count, hemoglobin (Hb) in blood; RBC, WBC, protein in urine; stool properties, RBC and worm ova in feces], liver and kidney function examinations [glutamate pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), urea nitrogen, creatine, and blood sugar)] were examined once 1 wk before and 1 and 2 wk after product consumption.

Chest X-ray, electrocardiography and abdominal B-ultrasound examinations were done 1 wk before product consumption.

Dietary intake was assessed after 1-2 d, 7-8 d, and 13-14 d of product consumption by food record method for 48 h, to monitor eating habits.

Defecation functional parameters: stool frequency, defecation condition scores and stools were recorded at baseline and 1 and 2 wk after product consumption.

According to extent of defecation difficulty, the defecation condition scores were categorized into four grades[20]: Grade Ⅰ (0 points): Normal defecation; grade Ⅱ (1 point): Only bearing down and discomfortable sensation. grade Ⅲ (2 points): Obvious bearing down and discomfortable sensation, or frequent defecation with difficult and little defecation, seldom abdominal pain or anal burning sensation; grade Ⅳ (3 points): Often abdominal pain or anal burning sensation to influence defecation. According to classification method of Bristol, stool consistency was classified into three grades[20]: Grade Ⅰ (0 points): Like sausage or snake, smooth and soft; like sausage, with fissure on the surface; grade Ⅱ (1 point): Sausage-shaped, with lumps; noncohesive lumps, with coarse edges; grade Ⅲ (2 points): Separating hard lumps, like fruit kernel (difficult discharge).

Statistical analysis

All analysis were conducted on the intention-to-treat (ITT) population, corresponding to subjects having consumed at least one pot of product.

Descriptive statistics were reported as mean ± SD or frequency for all variables, unless otherwise stated. Statistical comparative analysis between two groups was performed by *t* test, χ^2 test and the sum of rank, by SPSS statistical software.

RESULTS

General information of the subjects

Female volunteers (n = 135) were recruited for the study; four cases in test group and five cases in control group were withdrawn from the study. The general conditions of the other 126 subjects in two groups are described in Table 1. A *t* test was used for age and weight, χ^2 test for eating pattern and amount, and non-parametric rank sum tests for the three defecation parameters, to compare difference between the two groups. There were no significant differences ($P > 0.05$).

Table 1 General basic information of subjects before product consumption

Parameters		Control group (n = 63)	Test group (n = 63)	P value
Eating pattern	Regular	59	56	0.344
	Irregular	4	7	
Appetite	Good	17	21	0.437
	Common	46	42	
	Poor	0	0	
Eating amount	Large	3	4	0.927
	Common	52	51	
	Small	8	8	
Age (yr)		46.4 ± 6.7	46.4 ± 9.8	0.992
Body weight (kg)		62.5 ± 10.4	61.2 ± 9.6	0.478
Stool frequency (n/wk)		2.4 ± 0.6	2.4 ± 0.6	0.746
Defecation condition score		1.9 ± 1.2	1.9 ± 1.2	0.914
Stool consistency		1.6 ± 1.1	1.5 ± 1.1	0.408

Table 2 Stool frequency (n/wk, mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	2.4 ± 0.6	2.5 ± 0.9	2.6 ± 1.0[a]
Test group	63	2.4 ± 0.6	3.5 ± 1.5[b,c]	4.1 ± 1.7[b,c]

Note: Self comparison between before and after product consumption, [b]$P < 0.01$, [a]$P < 0.05$; compared with control group, [c]$P < 0.01$.

Results of blood, urine and stool routine assays, and liver and kidney function examinations were all in normal range before and after product consumption, and there were no clinical changes between the two groups. The chest X-ray, electrocardiogram and abdominal B-ultrasound examinations indicated that the subjects were healthy.

Stool frequency of the subjects

The stool frequency at baseline and after 1 and 2 wk of product consumption is shown in Table 2. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, stool frequency was significantly increased after 2 wk of control product consumption (2.6 ± 1.0 *vs* 2.4 ± 0.6, $P < 0.05$) but no differences were found after 1 wk. In the test product group, stool frequency was significantly improved after 1 (3.5 ± 1.5 *vs* 2.4 ± 0.6, $P < 0.01$) and 2 wk (4.1 ± 1.7 *vs* 2.4 ± 0.6, $P < 0.01$), respectively. Compared with control group, stool frequency was also significantly increased after 1 and 2 wk of product consumption (3.5 ± 1.5 *vs*

Table 3 Defecation condition score (mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	1.9 ± 1.2	1.6 ± 1.1[a]	1.6 ± 1.1
Test group	63	1.9 ± 1.2	1.1 ± 0.9[b,d]	0.8 ± 1.0[b,d]

Note: Self comparison before and after test, [a]$P < 0.05$, [b]$P < 0.01$; compared with control group, [d]$P < 0.01$.

Table 4 Stool consistency score (mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	1.6 ± 1.1	1.4 ± 1.0	1.3 ± 1.0[a]
Test group	63	1.5 ± 1.1	1.0 ± 0.8[b,c]	0.6 ± 0.8[b,d]

Note: Self comparison before and after test, [a]$P < 0.05$, [b]$P < 0.01$; compared with control group, [c]$P < 0.05$, [d]$P < 0.01$.

Table 5 Food intake amount of the subjects (g, mean ± SD)

	day 1-2		day 6-7		day 13-14	
	Control group	Test group	Control group	Test group	Control group	Test group
Staple food	341 ± 110	324 ± 117	350 ± 109	334 ±115	351 ± 117	353 ± 123
Fruits and vegetables	287 ± 227	341 ± 235	296 ± 212	322 ± 223	305 ± 244	318 ± 201
Meat and egg	118 ± 120	119 ± 100	114 ± 104	123 ± 103	121 ± 95	111 ± 105
Total daily intake	745 ± 343	784 ± 322	761 ± 294	779 ± 316	776 ± 344	783 ± 315

2.5 ± 0.9, $P < 0.01$ and 4.1 ± 1.7 *vs* 2.6 ± 1.0, $P < 0.01$, respectively) as shown in Table 2.

Defecation condition scores of the subjects

Defecation condition scores at baseline and after 1 and 2 wk of product consumption are shown in Table 3. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, defecation condition score was significantly improved after 1 wk of control product consumption (1.6 ± 1.1 *vs* 1.9 ± 1.2, $P < 0.05$), but no differences were found after 2 wk. In the test product group, defecation condition score was significantly improved after 1 (1.1 ± 0.9 *vs* 1.9 ± 1.2, $P < 0.01$) and 2 wk (0.8 ± 1.0 *vs* 1.9 ± 1.2, $P < 0.01$), respectively. Compared with control group, defecation condition scores were also significantly improved (1.1 ± 0.9 *vs* 1.6 ± 1.1, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.01$), respectively, after 1 and 2 wk of product consumption.

Stool consistency scores of the subjects

Stool consistency at baseline and after 1 and 2 wk of product consumption is showed in Table 4. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, stool consistency score was significantly decreased after 2 wk of control product consumption (1.3 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.05$), but no differences were found after 1 wk. In the test product group, stool consistency score was significantly improved after 1 (1.0 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$) and 2 wk (0.6 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$), respectively. Compared with control group, stool consistency score was also significantly decreased after 1 and 2 wk of product consumption, (1.0 ± 0.8 *vs* 1.4 ± 1.0, $P < 0.05$ and 0.6 ± 0.8 *vs* 1.3± 1.0, $P < 0.01$), respectively.

Food intake of the subjects during product consumption

Food intakes of the subjects was surveyed by 48 h dietary recall at initial stage (1st-2nd day), intermediate stage (6th-7th day) and end stage (13th-14th day) of product consumption. The mean daily intakes of staple food, fruits and vegetables, and meat and eggs of the three times were calculated and statistically analyzed by *t* test between the two groups (Table 5). Food intake throughout the study did not differ between groups.

DISCUSSION

Consumption of the fermented product tested in this study was well tolerated by all the participants, and no adverse effects were reported. An acidified milk was used as the control material in this study. Lactose in milk can cause intolerance characterized by rugitus, abdominal distension, abdominal pain, even diarrhea as a severe symptom. The incidence rate of lactose intolerance reaches 90% in Chinese adults. For example, Yang *et al*[21] have shown that lactose intolerance occurred in 87% of the 7-8 and 11-13 years old Chinese children. In order to prevent diarrhea, due to milk intake in the control group, extrinsic lactase was added to control milk samples during the manufacturing process. This resulted in an equal lactose content between the test and control products.

Several reviews[22-25] have described that some probiotics could improve lactose digestion and eliminate the symptoms of intolerance. A recent study has shown that a yogurt enriched with *B. lactis* DN-173010 and *B. longum* in capsules modifies the composition and metabolic activities of the colonic microbiota and alleviates symptoms in Chinese lactose-intolerant subjects[26].

BIO® is a fermented milk product which contains a mixture of live bacterial cultures; *B. lactis* DN-173010 (1.25×10^{10} cfu/pot) and yogurt starters *L. bulgaricus* and *S. thermophilus* (1.2×10^{9} cfu/pot). It has been shown to increase slow transit and, therefore, was tested in women with constipation to determine whether it would modulate bowel habits. Food intake and blood parameters remained constant throughout the study.

The results of this study indicated that stool frequency was significantly increased by 40% and 58% after 1 and 2 wk of product consumption, respectively ($P < 0.01$). In addition, after 1 wk of consumption, defecation condition scores (31% and 50%, $P < 0.01$) and stool consistency (29%, $P < 0.05$ and 25%, $P < 0.01$) were also significantly improved from baseline values in women consuming test product.

Three separate clinical studies have already demonstrated that daily consumption of a fermented milk containing *B. lactis* DN-173010 improves gastrointestinal transit time in elderly subjects[15,16], and in a group of healthy women with slow transit time[17]. Stool frequency was also significantly improved after product consumption in IBS subjects compared to controls with respect to subjects with a stool frequency < 4 stools per week at baseline[18]. A fifth study performed on fermented milk containing *B. lactis* DN-173010 alone also demonstrated a reduced transit time in healthy men and women with slow transit time[19]. Our finding that probiotics may normalize bowel movements is in line with those in some previous studies[27-30]. Some studies have shown that milk or yogurt fermented with different types of probiotics may increase the daily stool number in constipated subjects. In a double-blind, placebo-controlled study performed in 70 subjects with chronic constipation, a probiotic beverage containing *Lactobacillus casei Shirota* administered for a 4-wk period was significantly better than placebo in improving severity of constipation, stool frequency and consistency[27]. Likewise, a preparation containing *Escherichia coli* Nissle 1917 (a probiotic strain) was compared to placebo in a double-blind clinical trial in 70 subjects with chronic constipation, showing that the *E. coli* preparation was significantly better than placebo in increasing stool frequency[28]. Moreover, intake of a fermented milk product containing *L. casei* strain Shirota for 2 wk in a placebo-controlled double-blind cross-over design improved the state of bowel movements and stool quality in healthy subjects with a stronger tendency to constipation[29]. Finally, in an open trial in elderly subjects, a commercial mixture of *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii* improved defecation frequency by 24%, but no reduction in laxative use was observed[30]. To date, no clinical studies have been performed to measure defecation conditions/straining on constipated subjects. Few studies have been focused on the effects of probiotics on the intestinal function in healthy people, and the observed effects depended on the strain used. Three randomized, double-blind, placebo-controlled human clinical trials[31-33] have been performed to investigate the effect of a fermented product containing probiotic strains. In these clinical studies, product consumption exerted a beneficial effect on the bowel functions, but with no significant effect compared to the placebo. Findings in previous studies are inconsistent possibly due to lesser statistical power, the use of different probiotic strains and different subject population. Thus, probiotics may be effective in subjects with mild to moderate constipation and controlled and well-designed studies in this type of subjects are warranted[34]. In any case, our study is the first showing the significant efficacy of fermented milk consumption on stool frequency and consistency, as well as defecation conditions in constipated subjects.

The patients' ability to achieve normal bowel habits without being in pain, and to control bowel movements, are important elements of physical well-being. This was shown by studies investigating the relationships between quality of life and gastrointestinal symptoms in persons with constipation[35,36]. The surveys revealed an impaired quality of life in constipated individuals in comparison with healthy persons, depending on the severity of the constipation. In addition, Guyonnet *et al*[18] have shown that daily consumption of a fermented milk containing *B. lactis* DN-173010 improves quality of life and symptoms in IBS compared to heat-treated yogurt. These results support the hypothesis of a relationship between improved stool frequency, transit time, quality of life and a fermented milk containing *B. lactis* DN-173010, and indicate that further research should be carried out to investigate the potential use of this fermented milk product in improving quality of life in subjects with constipation.

In conclusion, the present large-scale study showed a beneficial effect of a fermented milk containing *B. lactis* DN-173010 on stool frequency and consistency, as well as on defecation conditions of women with constipation. Further studies are required to elucidate mechanisms of such effects to provide additional scientific evidence to support the use of such probiotic food to relieve constipation.

COMMENTS

Background

In recent years, probiotics have been studied for their efficacy on gastrointestinal disorders. *Bifidobacterium lactis* DN-173010, a probiotic strain, has already demonstrated health benefit on the gastrointestinal transit. Positive results have been obtained with consumption of a fermented milk containing *B. lactis* DN-173010 and yogurt strains on gut transit time in healthy people with normal to slow transit time. Equally, encouraging positive results have been obtained on stool frequency in Irritable Bowel Syndrome subjects with predominant constipation. These results indicate that research should be carried out to investigate the potential use of this fermented milk product in improving stool parameters in subjects with constipation.

Research frontiers

Constipation is reported in many regions in the world. An epidemiological study conducted in Beijing concluded that 6.1% of the adult population was suffering from the symptoms of functional constipation but no clinical study has been carried out in this population. This is why we decided to investigate the effect of a fermented milk (BIO®) combining *B. lactis* DN-173010 and yogurt strains on constipation functional parameters of adult women in Beijing.

Innovations and breakthroughs

This research demonstrates the first positive results on the ability of the *B. lactis* DN-173010 to improve stool frequency, stool consistency and defecation conditions in adult constipated women. It is believed to be the first study to investigate the effect of *B. lactis* DN-173010 leaded in another ethnic population with a different diet than European populations. Finally, this study shows the first evidence of the positive effect of *B. lactis* DN-173010 on constipated subjects.

Peer review

The contribution by Yue-Xin Yang *et al* studied the effect of a fermented milk containing *B. lactis* DN-173010 and yogurt strains (BIO®) in adult constipated women in Bejing China. The authors determined that the fermented milk containing *B. lactis* DN-173010 had a beneficial effect on stool frequency, stool consistency, and defecation of women with constipation. This is a well-written report.

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RAPID COMMUNICATION

Immunogenicity and immunoprotection of recombinant PEB1 in *Campylobacter-jejuni*-infected mice

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Abstract

AIM: To construct a prokaryotic expression vector carrying *Campylobacter jejuni peb1A* gene and express it in *Escherichia coli*. Immunoreactivity and antigenicity of rPEB1 were evaluated. The ability of rPEB1 to induce antibody responses and protective efficacy was identified.

METHODS: *peb1A* gene was amplified by PCR, target gene and prokaryotic expression plasmid pET28a (+) was digested with *Bam*HI and *Xho*I, respectively. DNA was ligated with T4 DNA ligase to construct recombinant plasmid pET28a(+)-*peb1A*. The rPEB1 was expressed in *E. coli* BL21 (DE3) and identified by SDS-PAGE. BALB/c mice were immunized with rPEB1. ELISA was used to detect the specific antibody titer and MTT method was used to measure the stimulation index of spleen lymphocyte transformation.

RESULTS: The recombinant plasmid pET28a (+)-*peb1A* was correctly constructed. The expression output of PEB1 protein in pET28a (+)-*peb1A* system was approximately 33% of total proteins in *E. coli*. The specific IgG antibody was detected in serum of BALB/c mice immunized with rPEB1 protein. Effective immunological protection with a lower sickness incidence and mortality was seen in the mice suffering from massive *C. jejuni* infection.

CONCLUSION: rPEB1 protein is a valuable candidate for *C. jejuni* subunit vaccine.

Key words: *Campylobacter jejuni*; Prokaryotic expression; Immunogenicity

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INTRODUCTION

Campylobacter jejuni is one of the leading causes of bacterial diarrhea in travelers, children, and military personnel in regions where water and food sources are commonly contaminated[1]. Moreover, *C. jejuni* is an infectious agent most often associated with Guillain-Barre syndrome (GBS), a post-infectious polyneuropathy[2-5]. Campylobacter has been reported in many geographic regions and its incidence varies with the season. Campylobacter outbreak and sporadic cases occur in developed countries, but the risk of developing campylobacteriosis is greater in travelers, children, and military personnel in regions where water and food sources are commonly contaminated. Currently, no commercial vaccines are available for the prevention of campylobacter-induced diseases in humans or for the reduction/elimination of colonization in poultry.

The development of vaccines has been hampered because the pathogenesis of campylobacter infections is poorly understood. The live-attenuated or killed whole-cell campylobacter vaccine candidates have raised questions about its safety. The protein PEB1, encoded by *peb1A* genes, is considered a common antigen and a major cell adherence molecule of *C. jejuni*[6]. The *peb1A* gene contains 780 bases encoding a 259-residue polypeptide. The peptide sequence starting at residue 27 matches that determined from amino-terminal sequencing of mature PEB1 from *C. jejuni*. The molecular mass of mature PEBl (amino acids s 27-259) is 25.5 kDa. In this study, we constructed a prokaryotic expression vector carrying *C. jejuni peb1A* gene minus its signal sequence and expressed it in *E. coli*. These

vaccine candidates were evaluated in mice for their ability to induce antibody responses specific to rPEB1 immunization and to protect the candidates against oral challenge with *C. jejuni*.

MATERIALS AND METHODS

Animals

BALB/c mice, at the age of 6-8 wk, were purchased from Center of Experiment Animal of Sun Yat-sen University and housed in cages for 7 d before use.

Bacterial strains and culture conditions

C. jejuni was grown in brucella agar plates at 37℃ in a microaerobic environment. *E. coli* JM109 used for amplification of the recombinant plasmid pET28a (+) was grown in a LB medium supplemented with kanamycin (50 μg/mL) at 37℃.

Construction of pET28a (+)-peb1A

Primers were designed according to the sequence of the *C. jejuni peb1A* gene (Genbank, ATCC700819) minus its signal sequence. The sequence of up primer is 5'-GC GGATCCGCAGAAGGTAAACTTGAGTCTAT-3' and the sequence of down primer is 5'-CCGCTCGAGTTA TAAACCCCATTTTTTCGCT-3'. The restriction sites of *Bam*HI and *Xho*I (underline) were introduced into the sequences of up and down primers, respectively, for gene cloning.

As a first step in amplification of the *C. jejuni peb1A* gene, template DNA was extracted from the *C. jejuni* genome. In a 50-μL Eppendoff tube, 30.5 μL of ddH_2O, 5 μL of 2 mmol/L dNTP, 5 μL 10 × PCR buffer, 0.5 μL of Taq polymerase, 1 μL of template DNA were added. The PCR product was subjected to electrophoresis on 1.5% agarose, purified using a DNA purification kit and then subjected to digestion with *Bam*HI and *Xho*I. The digested PCR product was purified and inserted into pET28a (+) digested with the same restriction enzyme to construct pET28a (+)-*peb1A*. pET28a (+)-*peb1A* was transfected into *E. coli* JM109. After propagation, pET28a (+)-*peb1A* was identified with restriction enzyme by direct sequencing.

Protein expression and purification

The *peb1A* gene from *C. jejuni* was expressed in *E. coli* as hexahistidine tagged proteins in pET-28a (+). *E. coli* BL21 (DE3) containing *peb1A* clone was grown in LB broth containing 30 μg/mL kanamycin. Cells were incubated at 37℃ with shaking at 250 r/min for 3-4 h until the culture reached an OD of 0.3-0.4. Then, IPTG was added to the LB broth at a final concentration of 1 mmol/L to induce expression of the target protein PEB1. Culture was continued for 6 h and BL21(DE3) cells were harvested at 1, 2, 3, 4 and 6 h, respectively, by centrifugation. The pellet of BL21(DE3) cells was resuspended in 1 × LEW buffer containing 50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, pH 8, and subjected to ultrasound in ice water. To evaluate the solubility and inclusion body formation, the resulting supernatant and sediments were separated by centrifugation at 12000 r/min for 10 min at 4℃, and subjected to SDS-PAGE for expression of recombinant PEB1 (rPEB1), which was purified by nickel chromatography under native conditions.

Vaccination

BALB/c mice were injected with 100 μL of PBS or with PBS containing 25, 50 or 100 μg of rPEB1 protein emulsified with an equal volume of CFA or IFA. Mice in each vaccination group (n =10 mice) were immunized four times at 1-wk intervals by intramuscular and subcutaneous injection. Following vaccination, the mice were monitored for adverse effects. Blood was collected from mice at various time points before and after immunization, and allowed to clot. The tubes were spun at 3000 r/min for 10 min, and the serum was collected into a clean microcentrifuge tube. Serum samples were logged in and stored at -20℃.

ELISA was used to evaluate the level of antibody response to anti-PEB1. Briefly, rPEB1 was used as the solid phase. After blocking with PBST supplemented with 10% fetal calf serum, the serum from mice was added. After extensive washing, bound antibodies were detected with goat anti-mouse IgG labeled with horseradish peroxidase. Antibody titers were determined by the serial end-point dilution method. The titer of serum was expressed as group geometric mean ± SD of the mean of individual animal values, which represented the average of duplicate assays.

T-cell proliferation assays

BALB/c mice immunized with rPEB1 or PBS (control) were sacrificed on day 60 after the first immunization. Splenocytes were harvested from the mice, co-cultured with rPEB1 (2 μg/mL) or with PHA in RPMI1640 for 54 h before addition of MTT (10 μL per well), and incubated at 37℃ for 3 h. The supernatant was transferred into a new Eppendorf tube. Absorbance of the converted dye was measured at a wavelength of 570 nm with a spectrophotometer.

Protective efficacy of oral challenge with C. jejuni

BALB/c mice at the age of 7-9 wk without specific pathogen were used in the study. The vaccinated mice were challenged with *C. jejuni* strain 81-176 in the oral model. We compared the protective efficacy of rPEB1 in immunized and non-immunized mice. Deaths occurred in challenged and control mice were recorded for more than 7 d. Illness index was scored as follows: 2 = dead, 1 = lethargic with ruffled fur and lower activity, and 0 = healthy.

RESULTS

Construction of pET28a (+)-peb1A

A single band at the 720-bp site was well shown in *C. jejuni* genome amplified by PCR. Recombinant plasmid pET28a (+)-*peb1A* analyzed by restriction enzyme digestion and DNA sequence was correctly constructed.

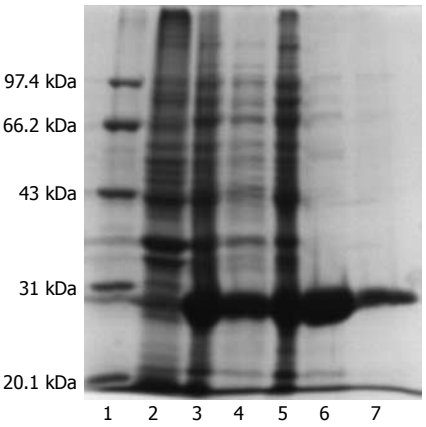

Figure 1 Analysis of expression pattern of recombinant protein by SDS-PAGE. Lane 1: protein marker; lanes 2-3: *E. coli* BL (DE3) transformed with pET28a(+) and pET28a(+)-*peb1A* respectively after induced with IPTG for 4 h; lanes 4-5: supernatant and precipitate of sonicated broken *E. coli* BL (DE3) transformed with pET28a(+)-*peb1A*; lanes 6-7: Purified recombinant protein.

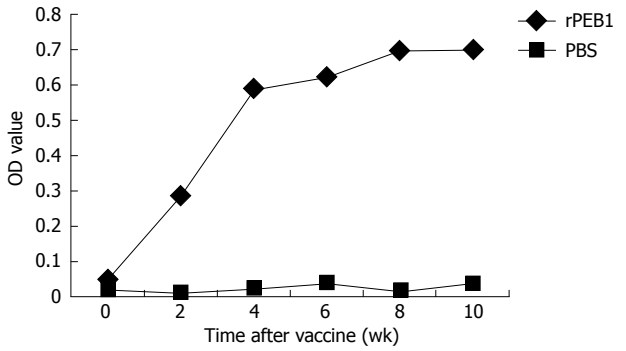

Figure 2 OD values of specific serum IgG antibody levels after immunization with rPEB1.

Expression and purification of recombinant protein

A rPEB1 protein with an expected molecular weight of 29kD was efficiently expressed in *E. coli* BL (DE3). The rPEB1 was mainly observed in supernatant of the *E. coli* BL (DE3) lysate and purified to approximately 96% purity by Ni-NTA resin after ultrasonication. The expression output of PEB1 protein in pET28a (+)-*peb1A* system was approximately 33% of total proteins of *E. coli* (Figure 1).

Strong immune response of BALB/c mice immunized with rPEB1

In subcutaneous and intramuscular injection groups, no apparent side effects were noted in mice and delivery of rPEB1 with CFA caused a ruffled fur appearance in all mice that lasted < 24 h, suggesting that injection of rPEB1 was safe. The mice in subcutaneous and intramuscular injection groups were immunized with rPEB1 interfused in CFA or IFA. PBS was substituted for rPEB1 in the control group. Anti- rPEB1 serum was detected 2 wk after the first immunization in both subcutaneous and intramuscular injection groups (Figure 2). Compared to the PBS group, significantly higher levels of serum IgG were detected in ⩾ 90% of the animals when 50 μg or higher rPEB1 was delivered with the adjuvant. Vaccination with 100 μg rPEB1 with the adjuvant induced antigen specific serum IgG, which was indistinguishable from that in 200 μg recipients (Table 1). A clear vaccine dose-dependent response was seen for the response magnitude and a strong immune response was observed in mice after immunization with rPEB1. The highest end point dilution titer of anti-rPEB1 serum was 1:5600.

Table 1 OD values of specific serum IgG antibody levels in BALB/c mice after immunization with recombinant PEB1 protein vaccine

Groups	Immune route	Serum IgG (mean ± SD)
Control	Subcutaneous	0.157 ± 0.010
P50 μg	Subcutaneous	0.365 ± 0.019[1]
P100 μg	Subcutaneous	0.521 ± 0.024[1]
P200 μg	Subcutaneous	0.619 ± 0.028[1]
Control	Muscular	0.157 ± 0.010
J50 μg	Muscular	0.350 ± 0.016[1]
J100 μg	Muscular	0.641 ± 0.019[1]
J200 μg	Muscular	0.638 ± 0.023[1]

[1]$P < 0.01$ *vs* control group.

Table 2 Illness index of BALB/c mice after oral challenge with wild-type *C. jejuni*

Groups	Cases	Healthy	Sickness	Dead	Illness index	Protective rate (%)
Control	6	0	2	4	9.14 ± 0.90	0
J50 μg	6	2	1	3	5.71 ± 0.49	33.3
J100 μg	6	4	1	1	3.00 ± 0.82	75
J200 μg	6	3	2	1	3.14 ± 0.90	66.7

T-cell proliferation assay of splenocytes in BALB/c mice

The proliferating response of splenocytes was generated in immunized mice when they were stimulated by PHA or rPEB1 protein. The stimulation index (SI) value for the immunized group was significantly higher than that for the control group, suggesting that proliferation of T cells from immunized mice could be stimulated by rPEB1. No difference in SI was observed in different groups immunized with different doses of rPEB1 compared with the PHA control group (Figure 3).

Protective efficacy of oral challenge with wild-type C. jejuni

Fourteen days following vaccination, animals immunized with different doses of rPEB1 were challenged with wild-type *C. jejuni*. The results are summarized in Table 2. Fifty micrograms rPEB1 failed to protect mice against *C. jejuni* infection and no significant difference was observed in illness pattern of PBS recipients. The efficacy of rPEB1 vaccine was significantly higher in animals challenged with *C. jejuni* than in those of the control group, indicating that rPEB1 vaccine could eradicate *C. jejuni* infection (Figure 4).

DISCUSSION

PEB1, a surface-exposed conserved antigen in *C. jejuni*,

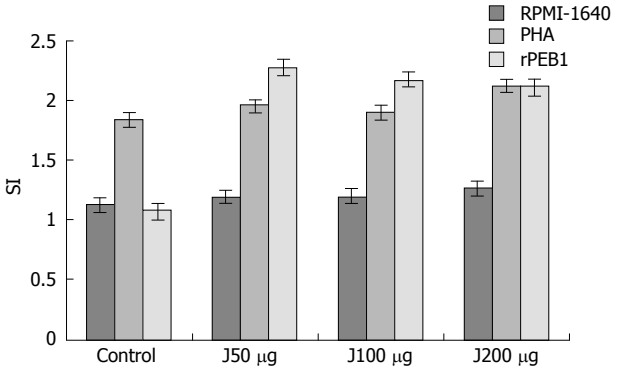

Figure 3 Stimulation index of immunized BALB/c mice spleen lymphocytes stimulated by intramuscular injection of rRPEB1.

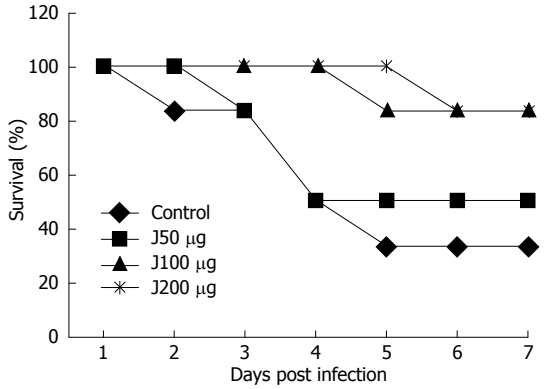

Figure 4 Survival of BALB/c mice immunized with rPEB1 after oral challenge with wild-type *C. jejuni* in control and vaccination groups.

is commonly recognized in convalescent sera from infected patients and involves binding of *C. jejuni* to eukaryotic cells[7]. Pei *et al*[8] have reported that PEB1 is a homolog of cluster 3 binding proteins of bacterial ABC transporters and a *C. jejuni* adhesion cell-binding factor 1. They determined the role of PEB1 in *C. jejuni* adherence and noted that the rate and duration of intestinal colonization by its mutants are significantly lower and shorter than those of the wild-type strain in mouse challenge test[8]. The adherence to epithelial cells is essential for the establishment of colonization in the gastrointestinal tract. It has also been shown that the PEB1 can adhere to Hela cells[9]. Inactivation of the *peb1A* locus significantly reduces *C. jejuni* adherence to Hela cells[10,11]. Moreover, the particulate PEB1 is the only antigen, known to elicit a prominent immune response[12,13]. We believe that PEB1 may be used as a vaccine for *C. jejuni* infection, which was confirmed by the fact that we successfully constructed a fusion gene containing *C. jejuni peb1A* gene and expressed rPEB1 protein in *E. coil* BL21 (DE3). rPEB1 with adjuvant CFA/IFA was used to immunize BALB/c mice, in which strong specific humoral immune responses were induced. High specific anti-rPEB1 and significantly higher specific T-cell proliferation were detected in BALB/c mice 3 wk after their first immunization. Furthermore, rPEB1 vaccination was found to have an effect on reducing the illness index of BALB/c mice after oral challenge with wild-type *C. jejuni*.

Sizemore *et al*[14] have reported that live and attenuated *Salmonella Typhimurium* strains expressing PEB1 can induce antibody responses specific to PEB1 following oral immunization, and have the ability to protect mice against infection with *S. Typhimurium* strains by reducing or eliminating systemic dissemination and intestinal colonization of wild-type *C. jejuni* strain 81-176. However, they noted that attenuated salmonella can stimulate production of serum IgG in mice and cannot protect mice against challenge with wild-type *C. jejuni*[14]. They believe that a small amount of antigen, available at the time of vaccination, may play a role in the absence of serum IgG[14]. Our results indicated that immunization with a low dose (50 μg) of rPEB1 could stimulate specific humoral immune responses and could not protect mice against challenge with wild-type *C. jejuni*.

The results of the animal protection test using 100 μg rPEB1 showed that most immunized mice remained healthy after *C. jejuni* challenge. Eighty percent of the control mice were lethargic with ruffled fur and lower activity and died 2 d post-challenge. The protective rate of rPEB1 immunization was 75% in the 100 μg rPEB1 group.

In conclusion, rPEB1, as a candidate vaccine, offers several advantages. It can lead to strong immune response, and provide a protective efficacy. Further study is needed to address its mechanism.

COMMENTS

Background

Despite the growing importance and widespread recognition of campylobacter enteritis as a major international public health problem, no commercial vaccines are available for the control of campylobacter-associated enteric disease in humans, or for the reduction/elimination of colonization in poultry.

Research frontiers

This study looked for the best induction of protective immune responses when the immunogenic campylobacter protein PEB1 was expressed.

Innovations and breakthroughs

The results of this study showed that rPEB1 was successfully expressed in *Escherichia coli* and immunization of mice through systemic routes could induce strong and specific serum IgG responses and splenocyte proliferation.

Applications

Based on the results of our study, further investigation should be focused on mucosal immune responses, which may be more important to *Campylobacter jejuni* subunit vaccines.

Peer review

The results of this study are interesting. The authors evaluated immunoreactivity and antigenicity of rPEB1, identified the ability of rPEB1 to induce systemic immune responses and its protective efficacy.

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RAPID COMMUNICATION

Midkine accumulated in nucleolus of HepG2 cells involved in rRNA transcription

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Abstract

AIM: To investigate the ultrastructural location of midkine (MK) in nucleolus and function corresponding to its location.

METHODS: To investigate the ultrastructural location of MK in nucleolus with immunoelectronic microscopy. To study the role that MK plays in ribosomal biogenesis by real-time PCR. The effect of MK on anti-apoptotic activity of HepG2 cells was studied with FITC-conjugated annexin V and propidium iodide PI double staining through FACS assay.

RESULTS: MK mainly localized in the granular component (GC), dense fibrillar component (DFC) and the border between the DFC and fibrillar center (FC). The production of 45S precursor rRNA level was decreased significantly in the presence of MK antisense oligonucleotide in the HepG2 cells. Furthermore, it was found that exogenous MK could protect HepG2 from apoptosis significantly.

CONCLUSION: MK was constitutively translocated to the nucleolus of HepG2 cells, where it accumulated and mostly distributed at DFC, GC components and at the region between FC and DFC, MK played an important role in rRNA transcription, ribosome biogenesis, and cell proliferation in HepG2 cells. MK might serve as a molecular target for therapeutic intervention of human carcinomas.

Key words: 45S rRNA; Anti-apoptosis; Proliferation; Midkine

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INTRODUCTION

Midkine (MK) is a cysteine-rich basic protein with a molecular weight of 13 Ku, which is strongly expressed during mid-gestation embryogenesis[1]. MK can be detected in most carcinoma specimens at a high level in a tissue type-independent manner, including those of esophageal, gastric, gall bladder, pancreas, colorectal, breast, and lung carcinomas, and Wilms' tumors[2-7]. Furthermore, MK exhibits several cancer-related activities, which include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic activity[8-10]. Recently, Shibata *et al*[11] have shown that exogenous MK is endocytosed by cultured mouse L cells and then transported to the nucleus. However, more detailed information on the location and biological mechanism of MK within cells still remains to be elucidated. In a previous study, using green fluorescent protein (GFP) as a tracking molecule we have found that MK is exclusively localized to the nucleus and nucleolus in HepG2 cells[12]. We have also found that MK both with and without signal peptide is exclusively localized to the nucleus and accumulats in the nucleolus of DU145 and MCF7 cell lines[13].

In the present study, we demonstrated the ultrastructural location of MK in the nucleolus with immunoelectron microscopy. As is known, the nucleolus is a region of the nucleus that is known to be the locus for ribosomal biogenesis. This prompted us to hypothesize that MK may be involved in RNA

transcription and processing. We studied the effect of MK on rRNA synthesis and found 45S rRNA production level decreased in response to down-regulation of MK expression. Since cell proliferation and cancer survival require continuous protein synthesis that depends on a constant supply of ribosomes[14], 45S rRNA production may be affected by endogenous MK level, suggesting that cell proliferation is directly related to MK level. Investigating this possibility, we demonstrated that cell proliferation was inhibited by down-regulation of MK. Moreover, we reported that exogenous human MK is involved in anti-apoptotic activity of HepG2 cells.

MATERIALS AND METHODS

Immunoelectron microscopy

HepG2 cells were fixed with 3% paraformaldehyde and 1% glutaraldehyde at 4 degree for 2 h, and then sequentially dehydrated with 30%, 50% and 100% ethanol and embedded in Lowicryl K4M. Sections of 50 nm were cut and mounted on nickel grids. Non-specific binding was blocked with 1% BL (50 mmol/L PBS, pH 7.0, 1% BSA, 0.02% PEG20000, 100 mmol/L NaCl, 1% NaN_3) for 30 min at room temperature. Then, sections were incubated for 1 h at room temperature with anti-MK primary antibody (rabbit polyclonal to human MK, Abcam, UK) at a dilution of 1:100. After another treatment with BL, sections were incubated with 15 nm colloidal gold-labeled second antibody (goat polyclonal antibody to rabbit IgG, 15nm gold; Abcam, UK). Finally, sections were stained sequentially with uranyl acetate for 15 min and lead nitrate for 10 min. The ultrastructural distribution of MK was examined and photographed with a Hitachi H-800 transmission electron microscopy.

Antisense treatment

The sequence of MK morpholino antisense oligomer was as follows: MK-As (5'-AGGAAGCCTCGGTGCTGCA TCTCGC-3'). The sequence for MK-Sen was as follows: (5'-CGCTCTACGTCGTGGCTCCG AAGGA-3'). MK-Sen is a control oligonucleotide that has the same base composition as MK-As, but in the reverse sequence, and thus does not hybridize with MK mRNA. MK-As and MK-Sen were transfected into HepG2 cells in the presence of Lipofectamine-Plus (Life Technologies, Inc) in accordance with the manufacturer's instructions.

Real time PCR assay

The real-time PCR was performed with an RT-PCR kit (Takara, Japan) according to the manufacturer's instructions using GAPDH primers (5'-AACGACCCCTT CATTGAC-3' and 5'-TCCACGACA TACTCAGCAC-3'), MK primers (5'-AAACCGAACTCCAGGACCAGAGA C-3' and 5'-AACACTCGCTGCCCTTCTTCAC-3') and 45S primers (5'-CGCCGCTAGAGGTGAAATTC-3' and 5'-CATTCTTGGCAAATGCTTTCG-3'). Samples were amplified in a 7500 Real Time PCR system for 40 cycles using the following PCR parameters: 95℃ for 30 s, 58℃ for 1 min, and 72℃ for 1 min.

Cell proliferation assay

A total of 1.0×10^4 HepG2 cells were added to each well of a 96-well microtiter plate and allowed to attach overnight. Oligonucleotides at concentrations of 0.2, 0.4 and 0.6 μmol/L were transfected into HepG2 cells with Lipofectamine Plus (Life Technologies, Inc) following the manufacturer's instructions. The effects of antisense oligodeoxynucleotide on cellular viability were measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

Analysis of apoptosis through FACS

1.0×10^5 HepG2 cells were seeded in six-well plates. Three wells were pre-treated with 500 ng/mL MK and three wells were treated with PBS as control for 3 h. Cells then were treated with 10^{-6} mol/L adriamycin, harvested 20 h later through trypsinization, and washed twice with cold PBS. The cells were centrifuged at 3000 r/min for 5 min, then the supernatant was discarded and the pellet was resuspended and incubated with FITC-conjugated annexin V and propidium iodide (Pharmingen) for 15 min at room temperature in the dark, and then analyzed by FACS.

RESULTS

The ultrastructural location of MK in HepG2 cells nucleolus

In a previous study, we demonstrated that MK exclusively localizes to the nucleus and nucleolus in HepG2 cells, using GFP as a tracking molecule[12]. At the ultrastructural level, the nucleolus includes three components: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC)[15]. Although we have found that MK accumulats in the nucleolus, the exact actions that MK performed on the nucleolus are still unclear. Therefore, we employed immunogold-labeling electron microscopy to investigate the ultrastructural location of MK, and found MK mainly localized in the GC, DFC and the border between the DFC and FC (Figure 1).

MK involved in rRNA transcription

In order to make clear that the function of MK corresponds to its ultrastructural location in the nucleolus, we investigated the role that MK plays in ribosomal biogenesis by real-time PCR. We found the production of 45S precursor rRNA in the HepG2 cells was decreased significantly in the presence of MK antisense oligonucleotide (Figure 2). This suggests that endogenous MK plays an important role in cancer cell proliferation.

MK promotes cell proliferation

Due to the central importance of rRNA transcription in cell growth, decreased rRNA transcription will slow cell proliferation. In this study, we showed that MK-As reduced cell proliferation rates by 41%, 48%, 58% after transfection of 0.2, 0.4 and 0.6 μmol/L MK antisense

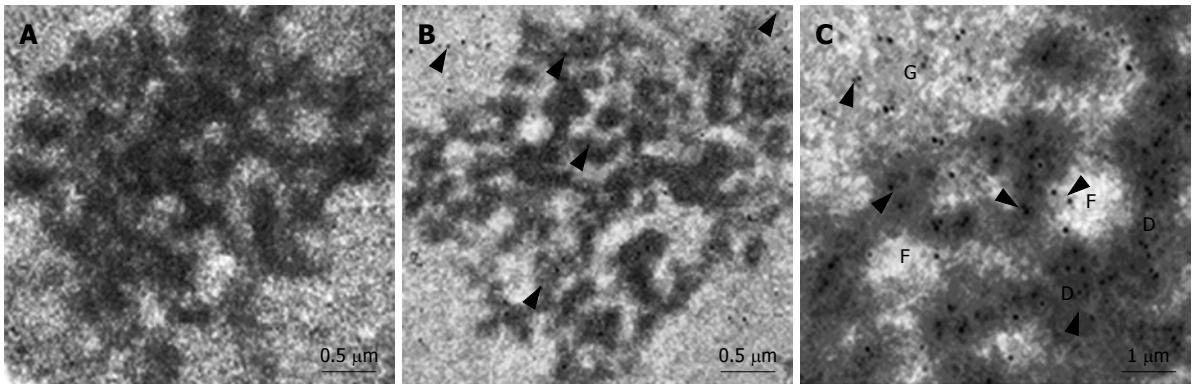

Figure 1 The location of MK in HepG2 cells nucleolus using immunogold labeling electron microscopy. HepG2 cells labelled without MK antibody were performed as the control. No immunogold particles of MK were seen (A). The MK protein (arrow) mostly localized to the DFC, GC and the region between FC and DFC (B, C) [scale bar represents 0.5 μm (A, B) and 1 μm (C)].

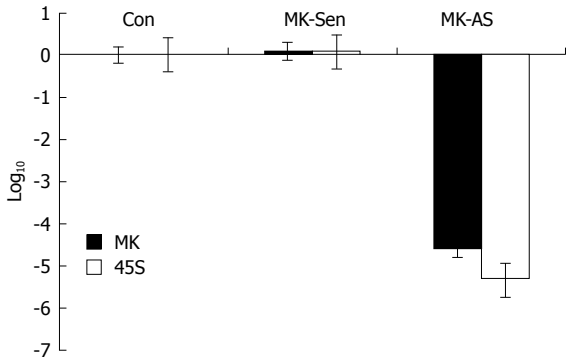

Figure 2 45S rRNA transcription could be regulated by endogenous MK level. It was shown that 45S rRNA transcription was decreased significantly in response to downregulation of MK expression, through real-time PCR analysis ($P < 0.05$).

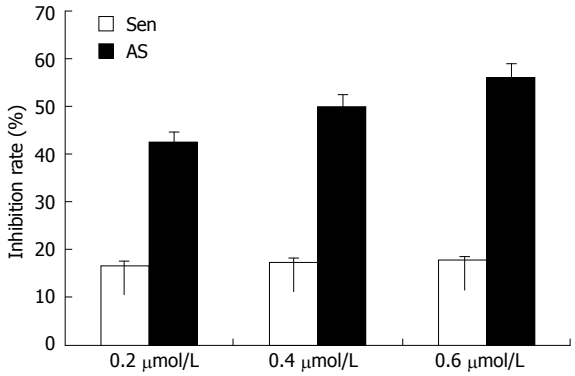

Figure 3 Effect of MK on proliferation of HepG2 cells. HepG2 cells were transfected with 0.2, 0.4 and 0.6 μmol/L MK-As or MK-Sen for 24 h, and were analyzed by MTT assay. Data show that HepG2 cell proliferation and growth were inhibited by downregulating the MK expression with antisense MK transfection ($P < 0.05$).

oligonucleotide into 10^4 HepG2 cells. The control MK-Sen did not inhibit cell proliferation significantly (Figure 3).

MK mediates anti-apoptotic activity

In order to investigate whether MK plays a role in the apoptosis of cancer cells, we added exogenous MK to HepG2 cells in the presence of adriamycin, inducing apoptosis. The results showed that exogenous MK could protect HepG2 from apoptosis significantly (Figure 4). This led us to suggest that MK may be a drug target for curing cancer.

DISCUSSION

In this study, we showed that MK was constitutively translocated to the nucleus of HepG2 cells, where it accumulated (Figure 1) and mostly distributed at the region between the FC and DFC, and GC components of the nucleolus (Figure 2). In a previous study, it has been demonstrated that each component of the nucleolus corresponds to a special biological function: the nascent transcripts appear in the junction region between the FC and DFC and accumulate in the DFC, and continue during the intranucleolar migration of the rRNA towards the GC. This implies that the role of the MK in the nucleolus is possibly related to control of rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly, which could be the downstream elements of controlling cell proliferation. The finding that MK is involved in rRNA transcription in HepG2 cells in our study is significant for understanding cancer biology. One of the hallmarks of cancer is sustained cell growth and this can only be achieved by increased protein synthesis. To accommodate this need, there must be an increase in ribosome biogenesis. The role MK plays in rRNA transcription in cancer cells suggests that up-regulation of MK expression in various cancer cells not only induces tumor growth, but also directly contributes to cell proliferation. Thus, inhibitors targeting MK will be more effective than those that inhibit cancer cell proliferation alone. rRNA transcription regulates ribosome production and consequently, the translation potential of a cell, and increasingly expressed ribosomal proteins and rRNA transcription is an important factor in cancer transformation[16]. It is conceivable that deregulation of rRNA transcription may be an important determinant in transformation of cancer cells. Continuous nuclear translocation of MK in cancer cells can be possible one of the contributing factors in determinant of transformation. Indeed, inhibiting

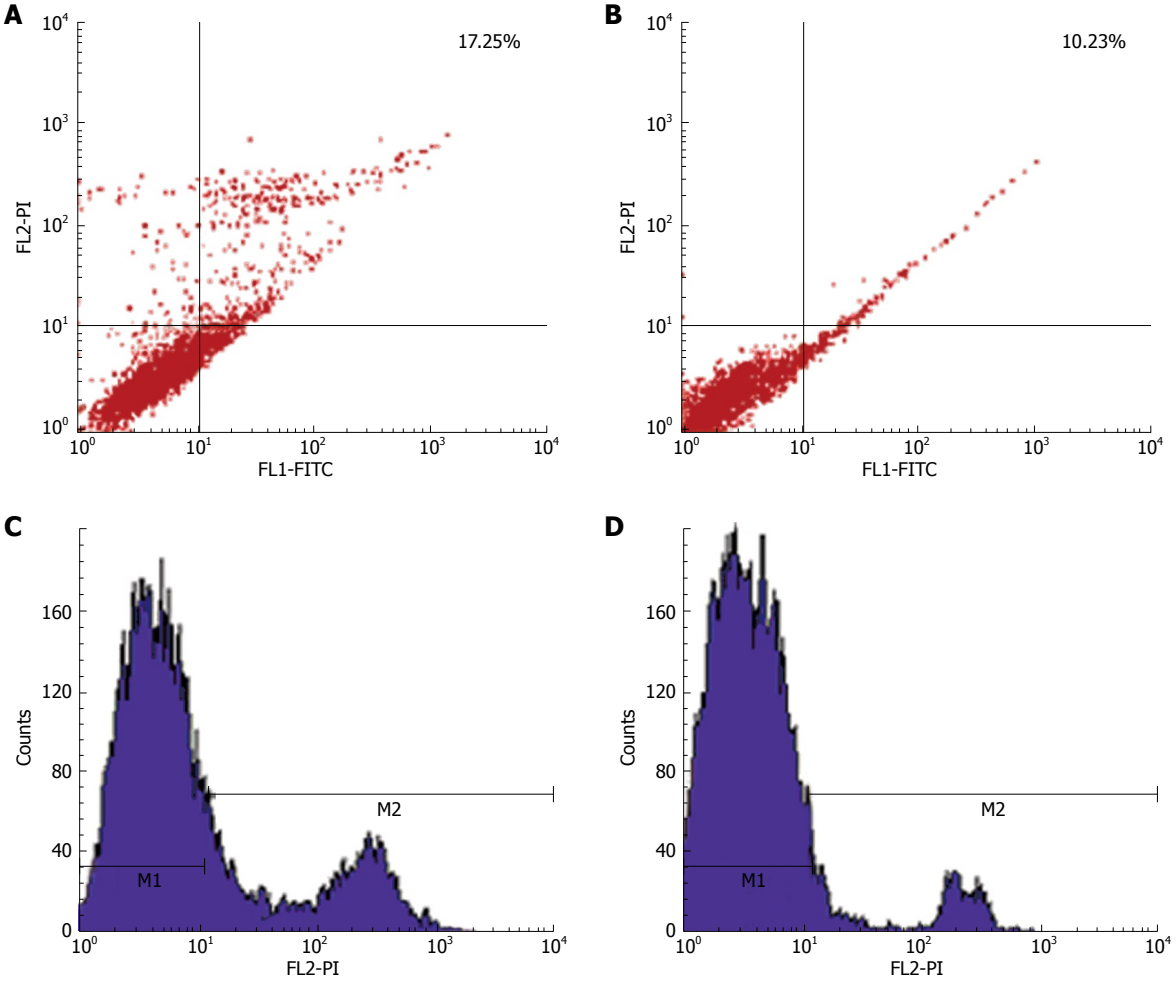

Figure 4 Exogenous MK mediates its anti-apoptotic activity. HepG2 cells are induced to apoptosis by 10^{-6} mol/L adriamycin for 20 h. It shows that about 17.25% of cells enter apoptosis (A, C), while 500 ng/mL exogenous MK showed its anti-apoptotic of activity (B, D).

MK expression reduced tumorigenicity and reversed the malignant phenotype of cancer cells, by antisense oligodeoxynucleotide targeted to MK. It is therefore possible that other effects such as repression of rRNA transcription of cancer cells also contributed to the marked anticancer activity. In conclusion, the results showed that MK played an important role in rRNA transcription, ribosome biogenesis, and cell proliferation in HepG2 cells. However, the mechanism by which MK stimulates rRNA transcription is still unclear at present. More extensive work is needed to understand how MK is translocated to the nucleus, whether it interacts with the RNA polymerase I machinery or binds to DNA.

COMMENTS

Background

Midkine (MK) has been found to play important roles in carcinogenesis, including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic, and angiogenic cancer-related activities. In a previous study, it has been demonstrated that each component of the nucleolus corresponds to a special biological function: the nascent transcripts appear in the junction region between the fibrillar center (FC) and dense fibrillar component (DFC) and accumulate, in the DFC, and continues during the intranucleolar migration of the rRNA towards the granular component (GC). This implies that the role of the MK in the nucleolus is possibly related to control of rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly, which could be the downstream elements of controlling cell proliferation. In a previous study, we found that MK exclusively localized to the nucleus and nucleolus in HepG2 cells. However, it is unclear what is the function of MK in the nucleus and nucleolus.

Research frontiers

MK expression is increased in many human carcinomas, such as esophageal, stomach, colon, pancreatic, thyroid, lung, breast, urinary bladder, uterine, ovarian, prostate and hepatocellular carcinomas, osteosarcoma, neuroblastoma and glioblastoma. This phenomenon is observed in about 80% of cases in many types of carcinomas. Furthermore, MK exhibits several cancer-related activities, which include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic activity.

Innovations and breakthroughs

In our study, we demonstrated that MK may be involved in 45S rRNA transcription. Since rRNA transcription is essential for tumor growth and proliferation, inhibition of MK-stimulated transcription of rRNA may be developed into a novel method to inhibit the growth of tumors.

Applications

The results may provide valuable evidence for the further study on the functions of MK in the nuclus and its mechanisms, in which rRNA transcription and ribosome assembly are involved. MK might serve as a molecular target for therapeutic intervention in human carcinomas.

Terminology

FC, DFC and GC are three components of the nucleolus. The nucleolus can be observed with the light microscope and its structure has more recently been clarified using the electron microscope.

Peer review

In this manuscript, authors report that the oncogenic function of MK could be associated with its role in 45S rRNA transcription. This finding is interesting.

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RAPID COMMUNICATION

Inhibition of pancreatic carcinoma cell growth *in vitro* by DPC4 gene transfection

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Abstract

AIM: To detect the expression of DPC4 in malignant and non-malignant specimens of human pancreas, and observe the inhibition of retroviral pLXSN containing DPC4 on pancreatic carcinoma cells *in vitro*.

METHODS: The expression of DPC4 was determined in 40 pancreatic adenocarcinoma and 36 non-malignant pancreatic specimens by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohisto-chemistry. Furthermore, we constructed retroviral vectors containing DPC4, which then infected the pancreatic carcinoma cell line BxPC-3. Cell growth *in vitro* after being infected was observed, and the vascular endothelial growth factor (VEGF) mRNA level in the daughter cells was determined by semi-quantitative PCR assay.

RESULTS: The RT-PCR assay showed a positive rate of DPC4 mRNA in 100% (36/36) of normal specimens, compared to 40% (16/40) in adenocarcinoma specimens. The regional and intense positive cases of DPC4 expression in adenocarcinoma detected by immunohistochemistry were 10 and four, whereas it was all positive expression in normal tissues. There was a significant difference of DPC4 expression between them. The stable expression of DPC4 in the pancreatic carcinoma cells BxPC-3 could be resumed by retroviral vector pLXSN transfection, and could inhibit cell growth *in vitro*. Rather, DPC4 could decrease VEGF mRNA transcription levels.

CONCLUSION: The deletion of DPC4 expression in pancreatic carcinoma suggests that loss of DPC4 may be involved in the development of pancreatic carcinoma. The retroviral vector pLXSN containing DPC4 can inhibit the proliferation of pancreatic carcinoma cells, and down-regulate the level of VEGF.

Key words: Gene therapy; Pancreatic carcinoma; Retroviral vector

Peer reviewer: MA Hernandez Bartolome, General Surgery, Hospital Universitario Getafe, Getafe (Madrid) 28904, Spain

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INTRODUCTION

Chromosome 18q is lost in a high proportion of colorectal and pancreatic cancers. Three candidate tumor suppressor genes, DCC (deleted in colorectal carcinoma), DPC4 (deleted in pancreatic carcinoma, locus 4) and Smad2 have been identified in this chromosome region. The tumor suppressor DPC4, which was identified by Scott Kern in 1996[1], is frequently lost in many tumor cells, especially in pancreatic cells. DPC4, also named as Smad4, belongs to the evolutionarily conserved family of Smad proteins that are crucial intracellular mediators of signals from transforming growth factor-β (TGF-β)[2]. TGF-β regulates a wide variety of biological activities. Smad proteins can transduce the TGF-β signal at the cell surface into gene regulation in the nucleus.

Here, we detected the expression of DPC4 in 40 pancreatic adenocarcinoma and 36 non-malignant pancreatic specimens by RT-PCR and immunohistochemistry; then, we reintroduced the DPC4 gene in the pancreatic carcinoma cell line BxPC-3 (null for DPC4[3]), by transferring the retroviral vector pLXSN containing the DPC4 gene, in order to study inhibition of DPC4 gene expression in the pancreatic carcinoma cells *in vitro*.

MATERIALS AND METHODS

Patients and methods

Forty malignant pancreatic carcinoma and 36 corresponding non-cancerous tissues were obtained from the First Affiliated Hospital of Suzhou University and the Wuxi's People Hospital from 2003 to 2006. The clinical and pathological data from this patient population were readily available from pathology reports and a regularly updated clinical database. There were 28 males and 12 females with pancreatic carcinomas, and the average age of the patients was 55.18 ± 11.29 years old (mean ± SD). Tumor fragments were obtained in sterile conditions from different areas of the specimen and immediately placed in supplemented RPMI-1640 medium.

Reverse-transcriptase polymerase chain reaction (RT-PCR) for DPC4 mRNA

Total RNA was extracted from tissues with a single-step method. Randomly primed cDNAs were reverse-transcribed from 4 μg total RNA, which was extracted from about 1 g fresh specimen, using a cDNA synthesis kit in a 20-μL mixture. The 2-μL mixture was increased to 100 μL by adding 10 mmol/L Tris-HCI (pH 8.3), 50 mmol/L KCl_2, 1.5 nmol/L $MgCl_2$, 200 μL of each deoxynucleotide triphosphate, 6 U Taq polymerase, and 50 pmol of each of the specific oligonucleotide primers for DPC4. PCR amplification was performed in a DNA thermal cycler and consisted of 30 or 40 cycles of denaturation for 30 s at 94℃, annealing for 30 s at 58℃, and extension for 90 s 68℃. Final extension proceeded for 1 min at 68℃, Internal control for RNA quality was obtained with β-actin, which was amplified at 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 2 min for 30 cycles. PCR amplification was performed by using primers 5'-CGGAATTCATGGACAATATGTCTATTACG-3' and 5'-GCGGATCCTCAGTCTAAAGGTTGTGG-3' for the DPC4 cDNA fragment. The product was about 1.6 kb. The PCR primers for β-actin were 5'-ACACTGTGCCCATCTACGAGG-3' and 5'-AGGGGCCGGACTCGTCATACT-3'. The product was 621 bp. All the products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemical analysis for DPC4

Tissues were routinely fixed in neutral formalin and embedded in paraffin. After being deparaffinized, the slides were placed in a solution of 3% hydrogen peroxide for 5-10 min to block the activity of endogenous peroxidase. After being washed with distilled water and then dipped in PBS for 5 min, the slides were closed in normal sheep blood serum and then heated to room temperature for 10 min. Non-specific binding was blocked with a protein solution for 10 min, and then each slide was labeled with a 1:100 dilution of monoclonal antibody to DPC4 (murine anti-human; LAB Vision). Anti-DPC4 antibody was detected by adding secondary antibodies (rabbit anti-murine; Maxim Biotech, Fuzhou). After being incubated at 37℃ for 1 h, the slides were washed by PBS. The sections were counterstained with hematoxylin. Positive cells were stained dark brown in the nuclei and/or cytoplasm, and the staining was graded into three categories: no staining, weak staining, or heavy staining. Positive staining was considered as expression of DPC4. Normal pancreatic ducts, islets of Langerhans, acini, lymphocytes, and stromal fibroblasts showing moderate to strong expression of DPC4 served as positive internal controls for each section.

Construction of the retroviral vectors, cell culture and transfection

The human DPC4 cDNA was amplified from Smad4/DPC4-pBluescript plasmid (as a gift from Scott Kern) by PCR. The identity of the amplified fragments was confirmed by cycle sequencing using the manufacturer's directions (Shanghai Sangong Biological Engineering Technology and Servical Co., Ltd), then subcloned to the retroviral vector pLXSN to obtain pLXSN/DPC4+ recombinant with direct insertion, and packaged with GP+E86 and PA317 amphotropic packaging cells. AntiG418 clones were acquired and named as PA317/pLXSN DPC4+ cells. As a control, the empty vector pLXSN also was packaged with GP+E86 and PA317 cells and the antiG418 clones were named as PA317/pLXSN. The virus titer was elevated through cross infection from GP+E86 to PA317 cells and reached 6.0×10^5 pfu/L. DPC4 gene integration in PA317/pLXSN DPC4+ or PA317/pLXSN cells was confirmed by PCR assay.

Retroviral supernatant was obtained from the producer cell lines and maintained at 32℃ in 5% CO_2 atmosphere for 24-48 h. The BxPC-3 lines (purchased from Shanghai Institute for Biological Science, Chinese Academy Science) were transduced using the following protocol. One milliliter of the filtered supernatant was added to 4×10^5 target cells in the presence of 8 μg/mL polybrene. Cells and retroviral supernatant were incubated at 37℃ for 4 h. Medium from producer cells was then replaced by RPMI-1640 medium supplemented with 20% fetal calf serum and then in the presence of 2 μg/mL polybrene at 24-h intervals. After the last infection, the daughter cells were subjected to an initial period of selection in 0.2 mg/mL G418 for 4 d and then in 0.5 mg/mL for 1 wk. The positive cells were named as BxPC-3/DPC4. As a positive control, daughter cells transduced by empty vector were named as BxPC-3/pLXSN. As a negative control, mother cells were named as BxPC-3/-.

Western blot analysis

10^7 cells were lysed at 4℃ in a lysis buffer containing 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA), and 25 mmol/L Tris (pH 7.5). The lysates were cleared by centrifugation and boiled for 5 min at 100℃ in Laemmli's SDS-PAGE sample buffer containing 100 mmol/L DTT. Proteins were resolved at 100 V on 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% non-fat dry milk, incubated with a Smad4/DPC4

monoclonal antibody (Neo Markers), and then incubated with the secondary clonal antibody. An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA) was used for detection.

MTT colorimetric growth assay for cells proliferation in vitro

Cell growth was determined by the MTT colorimetric growth assay as described previously. Cells were plated in three duplicate wells of a 96-well microtiter plate at 5×10^3 cells/well in 100 μL. After incubation at 37℃ in 5% CO_2, the cells were visually determined on each of the microtitration plates and 25 mL of RPMI-1640 containing 5 mg/mL of MTT was added to each well. Incubation was continued at 37℃ for 3 h. The content of each well was removed, and 200 mL of isopropanol containing 5% 1 mol/L HCl was added to extract the dye. After 30 min of incubation at room temperature and gentle agitation, the Absorbance (*A*) was measured with a microtitration plate spectrophotometer at 550 nm. The *A* of the blank, which consisted of an uninoculated plate incubated together with the inoculated plates, was subtracted from the *A* of the inoculated plates.

Semi-quantitative PCR assay

A semi-quantitative RT-PCR assay was performed to confirm the expression of vascular endothelial growth factor (VEGF) mRNA. Total RNA was extracted from the three cells, and 1 mg total RNA was reverse-transcribed into first strand cDNA in a reaction primed by oligo (dT) 12-18 primer using Superscript Ⅱ reverse transcriptase (Invitrogen). Two microliters of the first strand cDNA were used as template for the PCR reactions using Taq polymerase (Life Technologies, Inc.). The PCR reaction started at 94℃ for 2 min, followed by 35 cycles (94℃ for 30 s, 56℃ for 45 s, and 72℃ for 45 s), and ended with a 7-min incubation at 72℃. The primers of VEGF were 5'-GGGCCTCCGAAACCATGAACTT-3' and 5'-CGCATCAGGGGCACACAG-3'. The product size was 259 bp. Expression of β-actin was monitored as an internal control; the primers for β-actin were 5'-ACACTGTGCCCATCTACGAGG-3', 5'-AGGGGCCGGACTCGTCATACT-3', and the products was 621 bp. All RT-PCR products were separated by electrophoresis in 1.2% agarose gels and autoradiographed. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using the Fisher's exact probability test and χ^2 analysis using the SAS statistical software. A two-tailed Student's *t* test was used for statistical analysis of comparative data. Values of $P < 0.05$ were considered significant.

RESULTS

RT-PCR for the DPC4 expression in the malignant pancreatic tissue

The RT-PCR assay showed a positive rate of DPC4 mRNA in 100% (36/36) in all normal specimens, compared to 40% (16/40) in adenocarcinoma specimens ($n = 76$, $\chi^2 = 31.5692$, $P < 0.0001$; Figure 1).

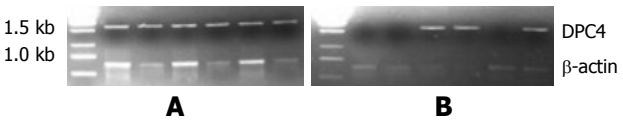

Figure 1 The mRNA of DPC4 by RT-PCR. A: Non-malignant pancreatic species; B: Malignant pancreatic species.

Immunohistochemical analysis for DPC4 expression in the malignant tissue

The regional and intense positive expression of DPC4 protein revealed by immunohistochemistry was 10 and four respectively, and all positive rate accounted for 35% (14/40), whereas there was all positive expression in normal tissues ($n = 76$, $\chi^2 = 35.568$, $P < 0.0001$; Figure 2).

PCR amplification of the DPC4 gene from Smad4/DPC4-pBluescript plasmid

The PCR product was about 1.6 kb in the electrophoresis gel as expected (Figure 3). pucm-T/DPC4 gene sequence was identified by Shanghai Sangong Biological Engineering Technology and Servical Co., Ltd., and agreed with our expected result.

Stable expression of DPC4 in the pancreatic carcinoma cell BxPC-3 after transfection demonstrated by RT-PCR and Western bloting

As an internal control, β-actin segments of about 838 bp were obtained in all three cell lines, which indicated that DNA was distilled effectually. DPC4 was expressed only in BxPC-3/DPC4 cells, but not the other control cells, BxPC-3/- and BxPC-3/pLXSN (Figure 4). An approximately 60-kDa protein blot, DPC4 protein, was obtained in BxPC-3/DPC4, but not in the BxPC-3/pLXSN or BxPC-3/- cells (Figure 5).

MTT for the cells grown in vitro

The BxPC-3/DPC4 cells grew much more slowly than the BxPC-3/pLXSN and BxPC-3/- cells. At day 7, the ratio of proliferation inhibition was about 50% ($F = 9.65$, $P = 0.0209$, BxPC-3/DPC4 *vs* BxPC-3/pLXSN; $F = 11.03$, $P = 0.0160$, BxPC-3/DPC4 *vs* BxPC-3/-; Figure 6).

Semi-quantitative PCR for VEGF mRNA

All three cell types had positive expression of VEGF and β-actin. However, semi-quantitative PCR assay showed the level of VEGF mRNA was much lower in BxPC-3/DPC4 than BxPC-3/pLXSN or BxPC-3/- (Table 1).

DISCUSSION

The majority of patients who present with pancreatic carcinoma have little chance of undergoing operative

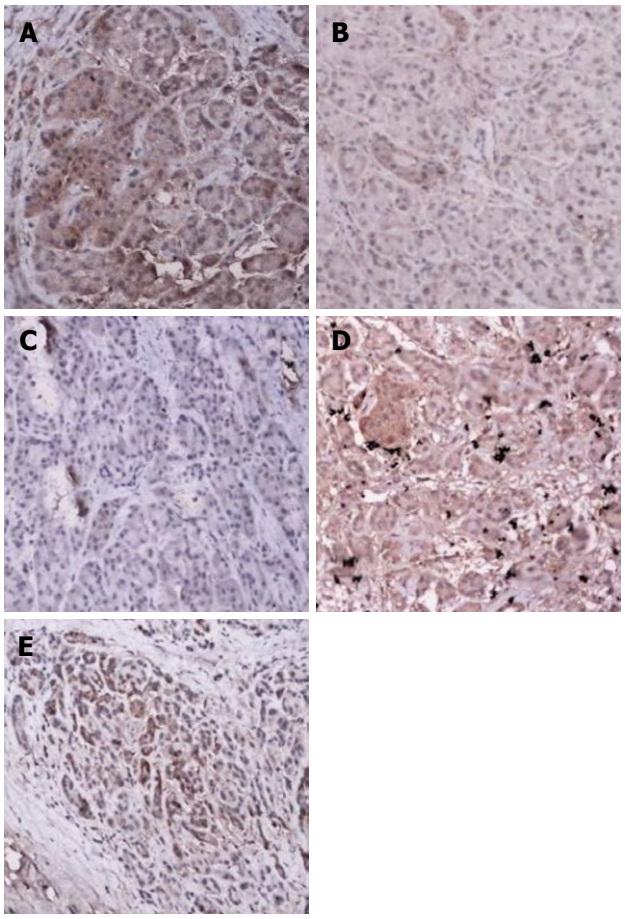

Figure 2 Immunohistochemical staining (x 100). A: Intense positive expression of DPC4 in the nuclei and/or cytoplasm in the normal pancreatic tissue; B: Weak expression of DPC4 in normal pancreatic tissue; C: Pancreatic carcinoma showed loss of DPC4 expression; D: DPC4 expression in an adenocarcinoma with a wild-type DPC4 gene; E: Intense positive expression of DPC4 in the malignant pancreatic tissue.

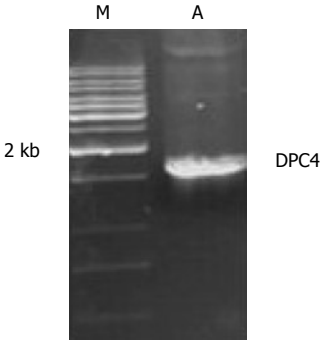

Figure 3 PCR amplification for the DPC4 gene. A: PCR product; M: Marker.

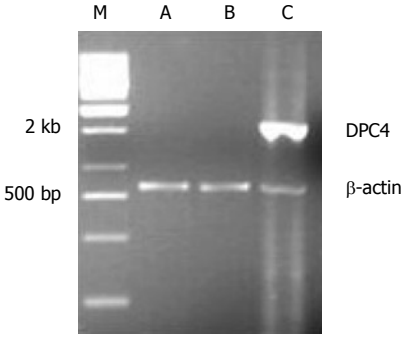

Figure 4 The expression of DPC4 mRNA in BxPC-3. M: Marker; A: BxPC-3/-; B: BxPC-3/pLXSN; C: BxPC-3/DPC4.

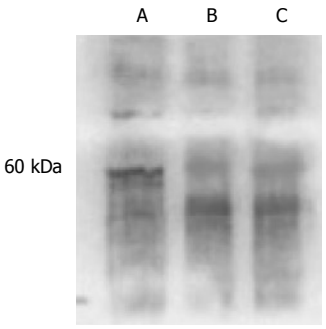

Figure 5 Westen blot for DPC4 in BxPC-3 cells. A: BxPC-3/DPC4; B: BxPC-3/pLXSN; C: BxPC-3/-.

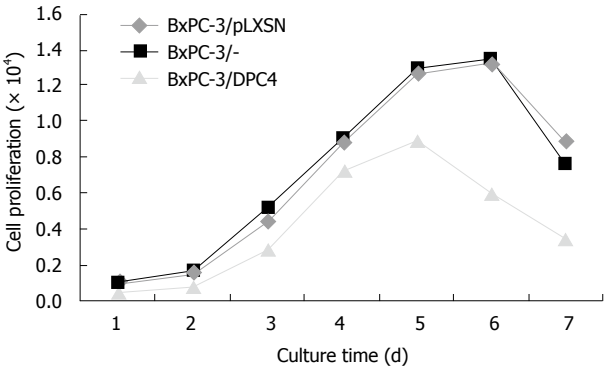

Figure 6 Cell growth curve. *P* = 0.0209, BxPC-3/DPC4 *vs* BxPC-3/pLXSN; *P* = 0.0160, BxPC-3/DPC4 *vs* BxPC-3/-.

Table 1 Semi-quantitative PCR for VEGF mRNA

	BxPC-3/DPC4	Bxpc-3/pLXSN	Bxpc-3/-	Ratio
VEGF	0.1887 ± 1.2399	0.3875 ± 2.0478	0.3910 ± 1.0714	2.0535
[1]*P*		0.035	0.021	

[1]*vs* BxPC-3/DPC4.

treatment, and non-operative treatments can offer little survival advantage. It is necessary to look for some other effective treatment for pancreatic carcinoma. TGF-β inhibits cell growth and/or induces apoptosis. In epithelium, disruption of the TGF-β signaling cascade is considered an important mechanism by which tumor cells can escape growth suppression. In a number of cancers, resistance to TGF-β growth inhibition is associated with mutations either in receptor Ⅱ or in the signal transducers. DPC4/Smad4 belongs to the evolutionarily conserved family of Smad proteins that are linked to the TGF-β superfamily of cytokines, forming a complex with R-Smads in response to ligand stimulation. Disruption of DPC4 can result in a TGF-β signal blackout and is involved in the regulation of cell differentiation, as well as the inhibition of cell proliferation. So, DPC4 is an essential signaling intermediate in the TGF-β receptor-mediated pathway. Abrogation of DPC4 function might cause a breakdown in this signaling pathway and loss of transcription of genes critical to cell-cycle control. Cells might therefore become TGF-β resistant and escape from TGF-β-mediated growth control and thereby contribute to tumorigenesis. C-terminal truncation of DPC4 protein

prevents DPC4 homomeric complex formation and heteromeric complex formation with activated Smad2. Furthermore, the mutant protein is unable to be recruited to DNA by transcription factors and hence cannot form transcriptionally active DNA-binding complexes[4]. Otherwise, oligo-ubiquitination positively regulates DPC4 function, whereas poly-ubiquitination primarily occurs in unstable cancer mutants and leads to protein degradation[5].

In the present study the relative contributions of three genes located at the 18q21 region (DCC, Smad2 and DPC4/Smad4) to progression and dissemination of human colorectal and pancreatic tumors were determined. DPC4 inactivation, always accompanied by alteration of all of the other three genes (K-ras, p53, p16)[6], has also been detected in colon, biliary tract, esophageal, gastric, ovarian, head and neck, lung and prostate cancer, especially in pancreatic carcinoma including one mutation and seven homozygous deletions[7-12]. Supporting evidence for the above observation was provided by Takaku and colleagues[13], who constructed knock-out mice with the DPC4 gene. Although DPC4-null mice were embryonically lethal, the heterozygotes of DPC4 were fertile and appeared normal up to the age of 1 year. However, gastric polyps developed in three of 15 heterozygous mice at the age of 50 wk, and in all heterozygous mice at the age of 100 wk. In addition, duodenal polyps were found in mice older than 50 wk. Morphologically, these polyps resembled those of human juvenile polypsis. These results suggest that inactivation of DPC4 is one of the early events in polyp formation in the DPC4 mice, which is analogous to human familial juvenile polyposis. Bartsch *et al*[14] have also revealed that the expression of DPC4 protein is associated with histopathological grades of pancreatic cancer. Meanwhile, DPC4 inactivation is associated with a poor prognosis[15-19]. Consistent with these reports, DPC4 was shown to be inactivated about in half of pancreatic cancer tissue in our study, whether mRNA or protein. Together with its deletion in pancreas carcinomas, these results suggest that DPC4 has the properties of a tumor suppressor gene, which indicates that it is involved in the carcinogenesis and development of pancreatic carcinoma and is a late event in pancreatic carcinogenesis. The present study can clarify the role of DPC4 in the development of pancreatic carcinoma. In a few substantial studies, significant prognostic markers for pancreatic carcinoma have been reported; markers such as tumor size, lymph node involvement, status of resection margins, DNA ploidy, degree of differentiation, and perineural invasion are inconclusive. In addition, preoperative estimation of tumor size and lymph node involvement is difficult. Deletion of DPC4 in pancreatic carcinoma and loss of DPC4 expression in those patients with poorly differentiated adenocarcinomas was significantly higher than that in those with well and moderately differentiated adenocarcinomas. Therefore, DPC4 gene might preserve phenotypic characteristics under normal conditions and control the malignant progression of pancreatic carcinoma. DPC4 may be proposed as a predictor of prognosis. Recently it was reported that expression of DPC4 can enhance the tumor response to drug treatment[20]. Hoever, it is regretable that we can not draw a consistent conclusion because of deficiency of the detailed clinical and survival data about these pancreatic carcinoma patients in our present study.

Since DPC4 plays a pivotal role in regulating all TGF-β superfamily signal pathways, it is reasonable to postulate that resumption of expression of DPC4 in pancreatic carcinoma cells can inhibit cell proliferation. In fact, DPC4 can induce growth inhibition in breast and colon tumor cells[21,22]**.** In order to develop an effective therapeutic intervention for patients with pancreatic cancer, we developed a new gene therapy that targets the genetic character of pancreatic cancer, using retroviruses that are selectively replication-competent in tumor cells. The DPC4 transcripts were cloned and subjected to sequence analysis. We performed reconstitution experiments of DPC4 in human pancreatic adenocarcinoma cell line BxPC-3. The wild-type DPC4 DNA was amplified from Smad4/DPC4-pBluescript plasmid by PCR and was enclosed successfully in the retroviral vector pLXSN. The pancreatic carcinoma cells BxPC-3 stably expressing DPC4 were obtained by retroviral transfection of DPC4 expression vectors and by selecting stable clones with G418. Stable transfection of BxPC-3 cells null for DPC4, accompanied by control vectors with DPC4 expression and an empty vector control, yielded similar numbers of G418-resistant clones. RT-PCR and Western blot analysis revealed restored expression of DPC4 in daughter clones derived from expression vector transfection. It was a feasible way to transfer the wild-type DPC4 gene to the DPC4-null cancer cells by pLXSN transfection. Some have reported that the DPC4 expression can inhibit growth of many tumor cells. In breast and colon carcinoma, DPC4 inhibited cell proliferation and induced anoikis[23,24]. Dai *et al*[25] have explored an inducible system in which DPC4 protein is activated by translocation to the nucleus, when cell lines that stably express wild-type or mutant DPC4 proteins fused to a murine estrogen receptor domain, are treated with 4-hydroxytamoxifen. This induced DPC4-mediated transcriptional activation and a decrease in growth rate, attributable to cell cycle arrest at the G1 phase and induction of apoptosis. In our study, MTT showed that the restored expression of DPC4 in the pancreatic cells can inhibit proliferation by approximately 50% *in vitro*. These data show that restored expression of functional DPC4 can be efficiently obtained via retrovirus-mediated gene transfer. Supporting our hypothesis, we found that restoration of DPC4 significantly delayed tumor growth *in vitro*.

The present study indicates that DPC4-inducible apoptosis has the greater consequence in growth control. Indeed, the period of the greatest growth suppression temporally was better correlated with apoptotic responses than with cell cycle arrest. This induced DPC4-mediated transcriptional activation and a decrease

in growth rate, attributable to cell cycle arrest at the G1 phase and induction of apoptosis in approximately 55% of pancreatic adenocarcinomas[24,25]. To date, two major apoptotic pathways, the death receptor and the mitochondrial pathway, have been well documented in mammalian cells. However, the involvement of these two apoptotic pathways, particularly the death receptor pathway, in TGF-β1-induced apoptosis is not well understood. Kim *et al*[21] have reported that apoptosis of human gastric SNU-620 carcinoma cells induced by TGF-β1 is caused by the Fas death pathway, in a Fas-ligand-independent manner, and that the Fas death pathway activated by TGF-β1 is linked to the mitochondrial apoptotic pathway.

Most solid tumor growth is dependent on angiogenesis, and the tumor growth and invasion can be inhibited through anti-angiogenesis. Serum levels of VEGF can decrease significantly after radical resection of the tumor. Elevated preoperative serum VEGF level is a significant prognostic factor, although not independent of stage, for patient survival[26,27]. A decrease in the levels of VEGF could be observed upon restoration of DPC4 expression in cell lines. This effect of DPC4 was found in the present study, implicating DPC4 for the first time as an inhibitor of pancreatic tumor angiogenesis. We found the level of VEGF mRNA level was decreased in the BxPC-3 cells after DPC4 resumption, as demonstrated by the semiquantitative RT-PCR. The retrovirus transfer of DPC4 in DPC4-null cells restored its expression and function, and may be correlated with the suppression of angiogenesis and invasion. However, it is not clear how DPC4 controls VEGF. Other experimental evidence indicates that DPC4 regulates an angiogenic switch by decreasing the expression of VEGF and increasing the levels of angiogenesis inhibitor thrombospondin-1 (TSP-1). It has been reported that DPC4 downregulates VEGF transcription and the secreted matrix metalloproteinase-2 (MMP-2) and MMP-9 expression levels consistently in pancreatic adenocarcinoma cell lines. There was a significant reduction in the tissue immunoreactivity of MMP-2 (a protease activated in angiogenic vasculature), and MMP-9 in samples from mice bearing DPC4-transfected tumors, compared to those from control groups. MMPs have been implicated in primary and metastatic tumor growth and angiogenesis, as well as in tumor invasion and progression. VEGF is a strong inducer and activator of MMP-2, while MMP-9 has been shown to increase the availability of VEGF to its receptors and identifying TSP-1 and VEGF as relevant tumor targets[21].

In summary, our study describes the deletion of tumor suppressor DPC4 in pancreatic carcinoma, and restoration of expression of DPC4 in human cancer cell lines growing *in vitro* showed the expected results. DPC4 decreased the expression of VEGF. We demonstrated that DPC4 mediates growth inhibition in pancreatic tumour cells even without TGF-β present, and suggest that DPC4 has the potency of a tumor suppressor gene.

ACKNOWLEDGMENTS

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COMMENTS

Background

Pancreatic carcinoma patients have poor survival, even those who have undergone surgery. The tumor suppressor DPC4 belongs to the evolutionarily conserved family of Smad proteins that are crucial intracellular mediators of signals from TGF-β and is frequently lost in many tumor cells, especially in pancreatic cells. The deletion of DPC4 is involved in the carcinogenesis and development of pancreatic carcinoma. How to further study the role of DPC4 and resumption of the DPC4 gene expression in the PC cell line by the transferring of the vectors containing DPC4 gene in order to inhibit PC growth is becoming a hot topic.

Research frontiers

It is necessary to develop a new modality of treatment for pancreatic cancer. Gene therapy strategies may provide therapeutic benefits with a more favorable risk-benefit ratio than the current conventional treatments. With the advances in understanding the pathogenesis, progression, and metastasis of pancreatic carcinoma that have been achieved, studies on gene therapy for pancreatic carcinoma have been attempted in different ways, such as inhibiting oncogenes, and activating tumor suppressor genes. New specific target genes and further development of gene technology may bring a break-through in this field.

Innovations and breakthroughs

We demonstrated that DPC4 can mediate growth inhibition in pancreatic tumor cells even without TGF-β present and reestablish one of the key regulatory controls of cell proliferation. Although numerous attempts have been made and different approaches have been used to identify the target genes, only limited success has been achieved. Our data showed that VEGF may be one of the DPC4-regulated downstream target genes, which will extend our understanding of the mechanism for DPC4 as an inhibitor of pancreatic tumor angiogenesis.

Applications

DPC4 is an important tumor suppressor. Further study on the biological nature of DPC4 may contribute to the study of the etiology of pancreatic cancer, and offer a theoretical basis for gene therapy of pancreatic cancer.

Peer review

In this study the authors demonstrated that deletion of DPC4 in pancreatic carcinoma, and restoring DPC4 expression in pancreatic carcinoma cells could effectively inhibit cancer cell growth *in vitro,* even without the presence of TGF-β. Re-expression of DPC4 in pancreatic carcinoma cells can downregulate VEGF mRNA expression and anti-angiogenesis therapy may represent a promising therapeutic option.

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CASE REPORT

Solitary fibrous tumor of the liver expressing CD34 and vimentin: A case report

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Abstract

A case of a successfully treated solitary fibrous tumor (SFT) of the liver is reported. An 82-year-old female presented with left upper abdominal discomfort, a firm mass on palpation, and imaging studies revealed a large tumor, 15 cm in diameter, arising from the left lobe of the liver. A formal left hepatectomy was performed. Microscopic evaluation showed spindle and fibroblast-like cells within the collagenous stroma. Immunohistochemistry disclosed diffuse CD34 and positive vimentin, supporting the diagnosis of a benign SFT. The patient remained well 21 months after surgery. SFT of the liver is a very rare neoplasm of mesenchymal origin. In most cases it is a benign lesion, although some may have malignant histological features and recur locally or metastasize. With less than 30 reported cases in the literature, little can be said regarding its natural history or the benefits of adjuvant radiochemotherapy. Complete surgical resection remains the cornerstone of its treatment.

Key words: Liver neoplasm; Solitary fibrous tumor; CD34; Vimentin

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INTRODUCTION

Solitary fibrous tumor (SFT) is a rare neoplasm of mesenchymal origin that occurs preferentially in the pleura, meninges, orbit, upper respiratory tract, thyroid and peritoneum. In extremely unusual cases, SFT may arise from the liver parenchyma of adult patients[1]. In the English literature, less than 30 cases of SFTs of the liver have been reported[2-5].

Clinical or radiological findings are not specific and cannot exclude malignancy. Preoperative cytology may be inconclusive or misleading. Immunohistologically, CD34, vimentin and desmin should be used as markers to precisely diagnose an SFT of the liver[3]. In most cases, there is low cellularity with minimal atypia or necrosis, making this a benign entity. Occasionally, a large size, high mitotic rate, cellular pleomorphism, atypia and central necrosis are interpreted as features suggestive of malignant behavior. As a result of its rarity, overall experience is insignificant. The outcome of an SFT of the liver is mostly related to resectability,

although correlated with neither pathological grade nor tumor size[4]. Thus, complete surgical removal of the neoplasm is most commonly proposed. We describe a new case of SFT of the liver and review the literature.

CASE REPORT

An 82-year-old female patient was referred to our institution on account of persistent abdominal discomfort and a palpable firm mass in the left upper quadrant. She had a past medical history of severe sleep-apnea syndrome and was recovering from a recent episode of mild gallstone pancreatitis. Laboratory tests, including liver biochemical profile and tumor markers were within normal range. Ultrasonography revealed an ovoid mass in the left lobe of the liver. Dynamic computed tomography (CT) and magnetic resonance imaging demonstrated a large, space-occupying lesion arising from the left lobe and compressing the stomach, pancreas and hepatoduodenal ligament. The lesion showed an early arterial enhancement and delayed venous washing out. The tumor developed in a polycystic liver parenchyma. It was well defined and measured about 15 cm in diameter. No direct invasion of the great vessels or adjacent intraperitoneal structures was seen (Figure 1). Preoperative fine needle aspiration biopsy under CT guidance, performed in another institution, was suggestive of a primary hepatocellular carcinoma. The patient underwent formal left hepatectomy and cholecystectomy. On gross examination, the resected specimen measured 18 cm × 15 cm × 8 cm and weighed 1275 g (Figure 2A). On the cut section, the tumor was firm, lobulated, well-demarcated, greyish-white, with a whorled and fasciculated surface and focal myxoid degeneration (Figure 2B). No cirrhosis or fibrosis was observed in the peripheral liver parenchyma. Resection margins were free of tumor. Pathological examination showed a highly cellular neoplasm composed of monomorphic spindle and fibroblast-like cells arranged in a storiform pattern. They were intermingled with dense bundles of collagen. In some areas, tumor cells were arranged around ectatic vessels in a hemangiopericytoma-like pattern (Figure 3A). No mitotic figures or nuclear polymorphism were noted. Immunohistochemical studies revealed a strong cytoplasmic positivity for CD34 (Figure 3B), vimentin (Figure 3C), desmin and Bcl-2. An absence of staining for HHF-35, S-100, CD117, muscle-specific actin and cytokeratin was observed. The percentage of ki-67 positive tumor cells was less than 5%. On the basis of the aforementioned results, pathological evaluation ascertained the presence of a benign SFT of the liver.

The patient recovered uneventfully after the procedure. She was discharged home on the 6th postoperative day and followed-up on an outpatient basis. Twenty-one months after surgery, the patient was stll doing well with no evidence of recurrence.

DISCUSSION

SFTs of the liver are unusual neoplasms with fewer than 30 previous cases reported in the world literature. There is a 2:1 female-to-male predominance with the ages of affected individuals ranging from 16 to 83 years. The mean age at diagnosis is 55 years and the average follow-up period reaches 27 months[1]. Most SFTs of the liver are usually found as giant lesions growing in either the right or the left lobe of a non-cirrhotic liver, causing non-specific symptoms of fullness and pressure, gastrointestinal obstruction, weight loss or hypoglycemia[3,5]. In addition, the development of a primary SFT on a background of polycystic liver disease, as in the present case, has never been reported before.

As a result of their extreme rarity, overall experience of SFTs is limited. In addition, the difficulty of

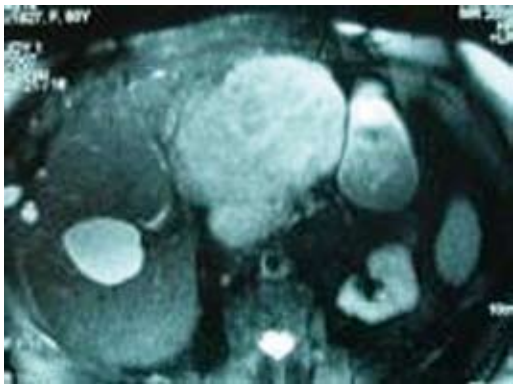

Figure 1 Magnetic resonance imaging demonstrating a large, 15 cm in size, well-circumscribed lesion in the left hepatic lobe, compressing the stomach, pancreas and hepatoduodenal ligament.

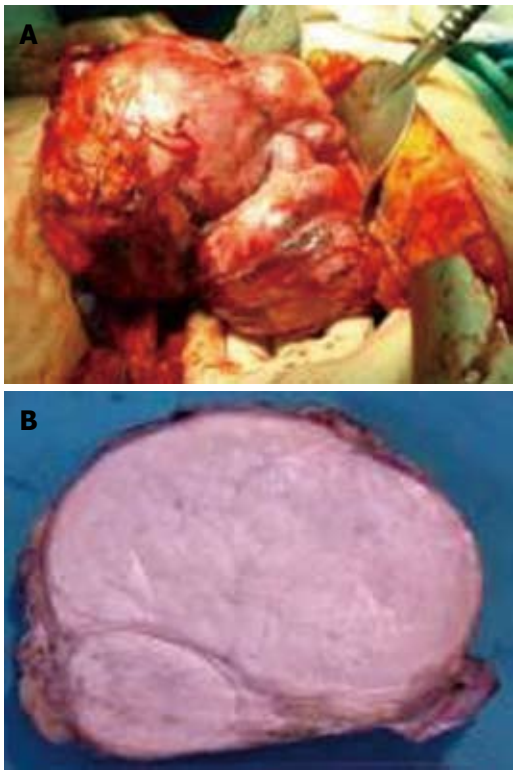

Figure 2 Gross appearance of a firm, well-demarcated and lobulated SFT of the liver (A) and greyish-white SFT of the liver with whorled and fasciculated surface and focal myxoid degeneration on cut section (B).

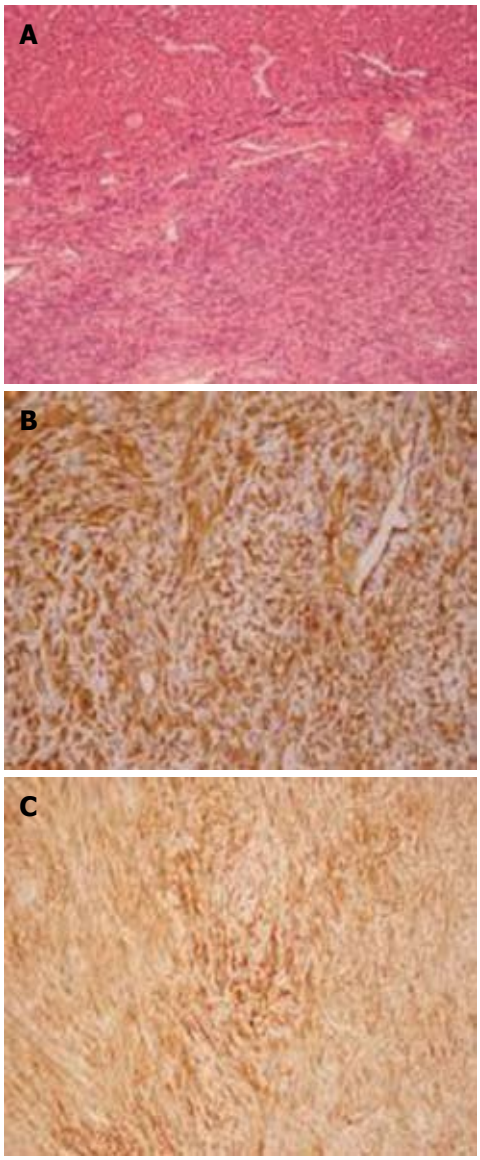

Figure 3 Tumor cells. A: Microscopy showing a tumor composed of uniform collagen-forming spindle cells arranged in interlacing fascicles and well-encapsulated and differentiated from the adjacent non-cirrhotic liver parenchyma (HE, x 100); B: CD34 immunohistochemical staining demonstrating diffusely strong reactivity (x 200); C: Tumor cells showing diffuse immunohistochemical positivity for vimentin (x 200).

interpretation of histological pictures and the huge volume at presentation still raise problems in terms of correct preoperative diagnosis and proper clinical management[6]. In the present case, the diagnosis of SFT of the liver was based on the association of characteristic histological and immunohistochemical features, i.e. high cellular proliferation of spindle cells arranged in a storiform pattern, together with the immunohistochemical staining profile of CD34 (+), vimentin (+), Bcl-2 (+) and cytokeratin (-), which is highly suggestive for SFTs. These characteristics differentiate it from other liver tumors, such as primary hepatocellular carcinoma (CD34-negative), leiomyoma (smooth-muscle actin-positive and CD34-negative), inflammatory pseudotumor (forms fibrous tissue made by collagen fibers with fibro and myofibroblast and plasma cells), fibrosarcoma (forms a "herring bone" pattern), and epithelioid hemangioendothelioma (factor Ⅷ positivity)[7]. The pathological features described for the presented case (low mitotic rate, no nuclear atypia and cellular pleomorphism) are in keeping with those of a benign SFT of the liver. This was confirmed by the favorable course of the patient, who was alive and disease-free 21 mo after surgery.

Radical surgical removal of the tumor with clear margins of resection is the mainstay of treatment. Since most SFTs of the liver are usually found to represent large, well-circumscribed lesions, complete removal necessitates the performance of major hepatectomies (≥ 3 segments). Owing to the size of most SFTs of the liver and their tendency to displace surrounding structures, they are virtually altering the intra-abdominal anatomy in an unpredictable manner. Although an SFT is not a primarily malignant disease, it seems to be of utmost importance that tumor-free resection margins be achieved in order that locoregional recurrence is prevented[8,9]. In light of this, surgical treatment of a hepatic SFT poses a tremendous challenge to the liver surgeon.

Little can be written about the possible benefits of adjuvant radio- and/or chemotherapy in these patients, as reported data are scarce. As SFT of the liver is often a benign neoplasm, postoperative chemotherapy or radiotherapy should not be necessary and is reserved for when resection is incomplete or pathological examination reveals features of malignancy[3,10].

In conclusion, SFTs of the liver are extremely rare neoplasms in adult patients and their incidence is unknown. Precise diagnosis is solely based on the correct interpretation of unique pathological and immunohistochemical features. Aggressive surgical management remains the treatment of choice. The outcome of SFTs is mostly related to resectability, rather than on pathologic grade or tumor size. Since the current number of reported cases is very limited, all efforts should be made to ensure careful follow-up of all identified patients. Only then we can reliably comment on the behavior and definite prognosis of SFTs of the liver.

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CASE REPORT

Acute ulcerative jejunal diverticulitis: Case report of an uncommon entity

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Abstract

Jejunal diverticulosis is a rare entity with variable clinical and anatomical presentations. Its reported incidence varies from 0.05% to 6%. Although there is no consensus on the management of asymptomatic jejunal diverticular disease, some complications are potentially life threatening and require early surgical treatment. We report a case of an 88-year-old man investigated for acute abdominal pain with a high biological inflammatory syndrome. Inflammation of multiple giant jejunal diverticulum was discovered at abdominal computed tomography (CT). As a result of the clinical and biological signs of early peritonitis, an emergency surgical exploration was performed. The first jejunal loop showed clear signs of jejunal diverticulitis. Primary segmental jejunum resection with end-to-end anastomosis was performed. Histopathology report confirmed an ulcerative jejunal diverticulitis with imminent perforation and acute local peritonitis. The patient made an excellent rapid postoperative recovery. Jejunal diverticulum is rare but may cause serious complications. It should be considered a possible etiology of acute abdomen, especially in elderly patients with unusual symptomatology. Abdominal CT is the diagnostic tool of choice. The best treatment is emergency surgical management.

Key words: Jejunal diverticulum; Diverticulitis; Surgery; Tomography

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INTRODUCTION

The majority of jejunal diverticulosis cases are discovered incidentally during radiological investigations. Symptomatic presentations are rare and generally complicated. Jejunal and jejuno-ileal localization is nearly three times less frequent than duodenal, but about four times likely to develop complications[1]. Asymptomatic cases require neither medical nor surgical treatment. Rarity of mild or chronic presentations explains the absence of clear consensus on therapeutic strategy and conservative management[2]. Complicated presentations remain a diagnostic challenge because of non-specific and ambiguous symptomatology. Surgical exploration is the treatment of choice for almost all acute complicated cases[3].

CASE REPORT

An 88-year-old male patient with a 6-year history of hypertensive terminal renal failure managed by hemodialysis was admitted for acute abdominal pain. Physical examination revealed right lower quadrant tenderness. C-reactive protein was 260 mg/L and white blood cell count was 19 g/L. There were no clinical or biological signs of bleeding. Abdominal computed tomography (CT) scan showed multiple small bowel giant diverticula, initially attributed to the ileum because of their localization in right iliac fossa. The diameters of these diverticula were between 3.5 cm and 6 cm and some showed clearly inflammatory infiltration

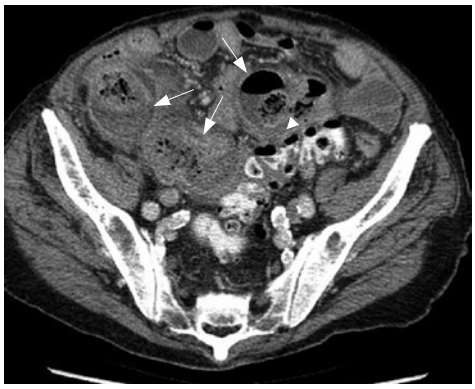

Figure 1 Axial CT image illustrating multiple diverticula (white arrows) developed on the mesenteric border of the jejunum, surrounded by inflammatory mesenteric fat. The arrowhead shows the communication between the diverticula and jejunal lumen.

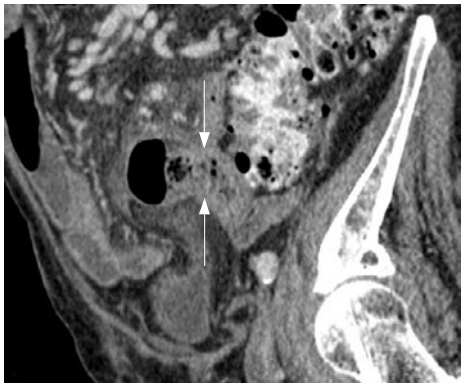

Figure 2 Multiplanar reconstructions on a sagittal view demonstrating a communication between the diverticula and jejunum (white arrow).

(Figures 1 and 2). There was no pneumoperitoneum or intraperitoneal fluid. Emergency laparotomy confirmed the presence of inflamed diverticula on the proximal jejunal portion (Figure 3). Treatment was the resection of a 35-cm jeujunal segment, removing all visible diverticula. End-to-end anastomosis was performed. Histopathology report confirmed an ulcerative jejunal diverticulitis with some areas of perforation and acute local peritonitis. The patient made an excellent postoperative recovery with normalization of inflammatory parameters and rapid intestinal transit recovery. He was discharged on the 12th postoperative day.

DISCUSSION

The present case shows an exemplary preoperative diagnosis and the surgical management of jejunal diverticula in its typical presentation.

The difficulty is that diverticular small bowel disease is an uncommon entity with an incidence between 0.05% and 6%. Jejuno-ileal diverticulosis remains asymptomatic in about 80% of cases, but chronic clinical manifestations may be under-or misdiagnosed with dyspepsia and irritable bowel syndrome. The mean age

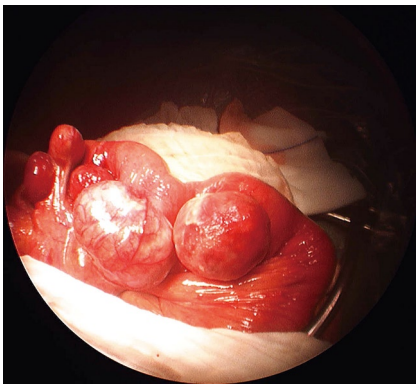

Figure 3 Inflamed giant jejunal diverticula on macroscopic operator view.

of symptomatic patients is up to 60 years in all reported studies. Only less than 10% of the affected individuals develop acute complications such as inflammation, perforation, hemorrhage and obstruction[4].

Complicated jejuno-ileal diverticulosis is generally present in elderly males with non-specific unexplained central abdominal pain, associated with clinical and biological septic syndrome. Some cases have a history of chronic symptoms such as vague abdominal discomfort, fullness, recurrent central and upper abdominal cramping pain caused by pseudo-obstruction or bacterial overgrowth. Yet the diagnostic accuracy of complicated acute jejunal diverticulosis based on simple clinical evaluation is still extremely poor[5,6].

Abdominal CT with double-oral and intravenous contrast may allow the diagnosis based on the following findings: focal area of out-pouching of the mesenteric side of the bowel filled or not filled with feces-like materials, focal asymmetric wall thickening, or inflammatory process adjacent to a loop of jejunum[7,8]. In some cases, use of coronal or sagittal reformatted images is helpful in identifying the bowel segment with diverticula (Figure 2). Today, multi-slice CT is very helpful in diagnosing jejunal diverticulosis and appears clearly superior to conventional enteroclysis for small intestine diseases[9].

Radiology-proven complicated presentations of jejuno-ileal diverticulosis require emergency surgical management. Emergency resection of perforated, inflamed, bleeding or obstructed bowel segment with primary anastomosis is safe and gives the best outcome.

Jejunal diverticulum is rare and generally discovered in complicated presentations only. Interpretation of abdominal CT findings is the key to its correct diagnosis. The treatment of choice is surgical excision of the affected jejunum segment with primary end-to-end anastomosis.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
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June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
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November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
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International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **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

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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