ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of Gastroenterology®

Indexed and Abstracted in:
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Digital Object Identifier. ISI, Thomson Reuters, 2009 Impact Factor: 2.092 (33/65 Gastroenterology and Hepatology).

Volume 16 Number 48
December 28, 2010
World J Gastroenterol
2010 December 28; 16(48): 6035-6162
Online Submissions
www.wjgnet.com/1007-9327office
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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2010-2013

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World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 48 December 28, 2010

World Journal of Gastroenterology
Volume 16 Number 48 December 28, 2010

Contents

AIM AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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NAME OF JOURNAL
World Journal of Gastroenterology

LAUNCH DATE
October 1, 1995

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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PUBLISHING
Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-3115-8812
Telephone: +852-5804-2046
E-mail: baishideng@wjgnet.com
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SUBSCRIPTION
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 245 Yuan for each issue, RMB 11760 Yuan for one year.

ONLINE SUBSCRIPTION
One-Year Price 864.00 USD

PUBLICATION DATE
December 28, 2010

CSSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6035

World J Gastroenterol 2010 December 28; 16(48): 6035-6043
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Oxidative stress and antioxidants in hepatic pathogenesis

Hye-Lin Ha, Hye-Jun Shin, Mark A Feitelson, Dae-Yeul Yu

Hye-Lin Ha, Hye-Jun Shin, Dae-Yeul Yu, Disease Model Research Laboratory, Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea
Hye-Lin Ha, Dae-Yeul Yu, Department of Functional Genomics, University of Science and Technology, Daejeon 305-333, South Korea
Mark A Feitelson, Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA 19122, United States
Author contributions: Ha HL and Yu DY designed the research; Ha HL and Shin HJ performed the research; Ha HL, Feitelson MA and Yu DY wrote the paper.
Supported by The 21st Century Frontier Program in the Functional Human Genome Project, No. HGM0200934; the International Collaboration Program of Science and Technology, No. FGM0600914; the Ministry of Education, Science and Technology, and the KRIBB Research Initiative Program Grant, No. KGM3320911, South Korea
Correspondence to: Dae-Yeul Yu, PhD, Disease Model Research Laboratory, Aging Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Oun-dong, Yuseong-gu, Daejeon 305-806, South Korea. dyyu10@kribb.re.kr
Telephone: +82-42-8604422 Fax: +82-42-8604609
Received: June 2, 2010 Revised: July 12, 2010
Accepted: July 19, 2010
Published online: December 28, 2010

Abstract

Long term hepatitis B virus (HBV) infection is a major risk factor in pathogenesis of chronic liver diseases, including hepatocellular carcinoma (HCC). The HBV encoded proteins, hepatitis B virus X protein and preS, appear to contribute importantly to the pathogenesis of HCC. Both are associated with oxidative stress, which can damage cellular molecules like lipids, proteins, and DNA during chronic infection. Chronic alcohol use is another important factor that contributes to oxidative stress in the liver. Previous studies reported that treatment with antioxidants, such as curcumin, silymarin, green tea, and vitamins C and E, can protect DNA from damage and regulate liver pathogenesis-related cascades by reducing reactive oxygen species. This review summarizes some of the relationships between oxidative stress and liver pathogenesis, focusing upon HBV and alcohol, and suggests antioxidant therapeutic approaches.

Key words: Hepatitis B virus; Hepatitis B virus X protein; Alcohol; Chronic liver disease; Oxidative stress; Antioxidant

Peer reviewer: Thomas Bock, PhD, Professor, Department of Molecular Pathology, Institute of Pathology, University Hospital of Tuebingen, D-72076 Tuebingen, Germany

Ha HL, Shin HJ, Feitelson MA, Yu DY. Oxidative stress and antioxidants in hepatic pathogenesis. *World J Gastroenterol* 2010; 16(48): 6035-6043 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6035.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6035

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent tumor types worldwide. It is the fifth most common cancer and the third leading cause of cancer death[1]. There are multiple etiological agents that are associated with the development of HCC, the most frequent being chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and long-term exposure to the mycotoxin, aflatoxin B1.

HBV is recognized as a major etiological factor in the development of such diseases as fatty liver (steatosis), cirrhosis, hepatocellular adenoma, and HCC[2,3]. The risk of HCC in chronic HBV carriers is more than 100 times greater than in uninfected individuals. In the year 2000, worldwide new cases of HCC had increased to 564 300[4]. More than 80% of these cases occur in developing countries, especially Southeast Asia and sub-Saharan Africa. Some 80%-90% of HCCs develop in cirrhotic liver[5]. After 20-30 years of chronic infection, 20%-30% of patients develop liver cirrhosis. HCC develops at an annual rate of 3%-8% in HBV-infected cirrhotic patients[6].

In the course of chronic infection, fragments of HBV

DNA integrate randomly into host DNA. Many of these integrated species encode the hepatitis B virus X protein (HBx) and truncated preS polypeptides, which contribute major steps in hepatocarcinogenesis. HBx binds to the DDB1 subunit of a UV-damaged DNA binding protein[7], the latter of which appears to be important for maintaining the integrity of DNA repair[8]. HBx has also been shown to bind to and functionally inactivate p53[9,10].

Therefore, the HBx and HBs proteins represent the two potential candidate proteins involved in HBV-related hepatocarcinogenesis[11-16]. HCC is also a common complication of alcoholic cirrhosis, although ethanol appears to not be directly carcinogenic[17].

OXIDATIVE STRESS

Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. Most cells can tolerate a mild degree of oxidative stress, because they have sufficient antioxidant defense capacity and repair systems, which recognize and remove molecules damaged by oxidation. The imbalance can result from a lack of antioxidant capacity caused by disturbances in production and distribution, or by an overabundance of reactive oxygen species (ROS) from other factors. ROS are potential carcinogens because of their roles in mutagenesis, tumor promotion, and progression[18]. If not regulated properly, the excess ROS can damage lipids, protein or DNA, inhibiting normal function[19]. ROS alterations in different signaling pathways may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death. These events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation[20]. ROS may also activate cellular signal pathways, such as those mediated by mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K), p53, β-catenin/Wnt and associated with angiogenesis[21-23]. Importantly, HBx stimulates the activities of MAPK, NF-κB, PI3K, and β-catenin (as well as other pathways) that are thought to contribute importantly to the development of HCC. Perhaps this is why carriers with chronic liver disease (CLD) develop a high incidence of HCC, while asymptomatic carriers do not.

OXIDATIVE STRESS EFFECT ON CHRONIC LIVER DISEASE AND LIVER FIBROSIS

Several *in vitro* and *in vivo* observations suggest that oxidative stress and associated damage could represent a common link between different forms of chronic liver injury and hepatic fibrosis. For example, oxidative stress contributing to lipid peroxidation is one of the critical factors involved in the genesis and the progression of nonalcoholic steatohepatitis and liver cancer[24,25]. Viral infection or alcohol abuse greatly increased the highly variable miscoding etheno-modified DNA like epsilondA [1,N(6)-etheno-2'-deoxyadenosine] levels by triggering lipid peroxidation. Patients with chronic hepatitis, liver cirrhosis, and HCC due to HBV infection had more than 20 times higher urinary epsilondA levels[25] compared to uninfected individuals with no liver disease.

Among the mechanisms involved in mediating the process of liver fibrosis, an important role is played by ROS[26]. During the progression of liver injury, hepatic stellate cells (HSCs) become activated, which produce extracellular matrix such as collagen I [27]. Collagen I gene regulation has revealed a complex process involving ROS as a key mediator[28-30]. ROS-sensitive cytokines contribute to HSC activation during inflammation through paracrine signals released from immune cells[31]. The activated HSCs become responsive to platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. PDGF facilitates the progression of hepatic fibrosis in human CLD. It increased the accumulation of hydrogen peroxide in HSCs. Specifically PDGF-induced increases in collagen deposition and liver fibrosis is markedly reduced by treatment with the anti-oxidant drug Mn-TBAP[32,33]. TGF-β increases ROS production and decreases the concentration of glutathione (GSH)[34]. In this context, it is important to note that HBx trans-activation activity is stimulated by ROS. Given that HBx is also associated with the development of HCC in both human carriers and in transgenic mice, and that HCC is associated with chronic inflammation, this underscores the importance of inflammation in the context of chronic HBV infection to hepatocarcinogenesis.

HBV INFECTION AND OXIDATIVE STRESS

Many groups have shown that HBV can induce oxidative stress using HBV transgenic mice or HBV DNA transfection of cells *in vitro*, while oxidative stress is also common among HBV infected patients with CLD[35-41]. Oxidative stress also precedes the development of HCC in transgenic mice that overproduce and accumulate intracellular HBsAg. Several studies have found that the total peroxide level, a parameter of oxidative stress, is significantly higher in patients with chronic hepatitis compared to asymptomatic carriers, and positively correlated with alanine aminotransferase (ALT) levels, suggesting that oxidative stress plays a critical role in hepatic injury. Oxidative stress is also associated with the severity of the disease. Lipid peroxidation and oxidative DNA damage are enhanced in patients with HBV infection.

Mitochondria are a major source of ROS. ROS can form through electron leakage from the mitochondrial respiratory chain[42]. HBx itself targets mitochondria and directly interacts with voltage-dependent anion channel 3. It alters the mitochondrial membrane potential and increases the endogenous ROS level[43-46]. HBx expression also induces oxidative stress through calcium signaling and activates cellular kinases, leading to the activation of transcription factors NF-κB, signal transducer and activator of transcription 3, and others *via* phosphorylation[47,48]. It is observed that HBV-induced oxidative stress also stimu-

lates the translocation of mitogen-activated protein kinase Raf-1 to mitochondria. This activation involves both the Src- and the PAK-mediated phosphorylation of the Raf-1 activation domain[49]. HBx also induces lipid peroxidation *via* down-regulation of SeP expression, resulting in increased expression of tumor necrosis factor-α in the human hepatoblastoma cell line, HepG2[50].

Activity of the anti-oxidant enzymes CuZn-SOD and GSH-Px was found to be the lowest in chronically infected patients compared with other groups[51,52]. Detection of an increase in MDA levels, which is a product of lipid peroxidation in HBV infected groups, indicates that oxidative stress is increased in HBV infection[52,53]. After treatment with interferon-α and lamivudine, however, there was a decrease in the products of lipid peroxidation and an increase in the antioxidant enzymes, such as CuZn-SOD and GSH-Px, compared with pretreatment[53].

The marker 8-hydroxydeoxyguanosine (8-OHdG) is useful in estimating DNA damage induced by oxidative stress. Importantly, hepatic 8-OHdG accumulation was detected in patients with chronic hepatitis B[39,54]. Further, HBV replication causes oxidative stress in HepAD38 liver cells, with more than 3 fold increases in the GSSG/GSHtot ratio[37].

HuH-7 cells carrying the pre-S mutant (a truncated form of preS/S polypeptide) exhibited enhanced levels of ROS and oxidative DNA damage through endoplasmic reticulum (ER) stress pathways. Oxidative DNA damage has also been observed in livers of transgenic mice carrying the pre-S mutant[36]. HepG2-HBx cells and the livers of HBx mice also showed increased ROS levels (Figure 1), mtDNA deletion, and declines in the mitochondrial membrane potential compared to controls (data not shown). Through DNA chip analysis, several ROS-related molecules, such as members of the CYP450 families, were altered in HBx transgenic mice. The cytochrome p450s are a superfamily of hemeproteins that serve as terminal oxidases[55]. A major function of these p450s is to convert compounds into more polar metabolites[56]. Detoxification by cytochrome p450 can also produce ROS[57,58]. CYP2E1, a member of the p450 family that oxidizes ethanol, generates oxidative stress in the mitochondrial compartment of hepatocytes. This has been suggested to play a role in hepatotoxicity, as observed in ALD-related patients[59-61]. In a mouse model of nonalcoholic steatosis, CYP2E1 also plays key roles in ROS production and contributes to the pathogenesis of liver damage[62,63]. Thus, the involvement of mitochondria in the production of free radicals resulting from ethanol metabolism, and the fact that elevated free radical formation stimulates HBx activities, combined with the ability of mitochondria to oxidize ethanol may help to explain the apparent synergistic effects of chronic ethanol intake and HBx expression on the pathogenesis of CLD and HCC.

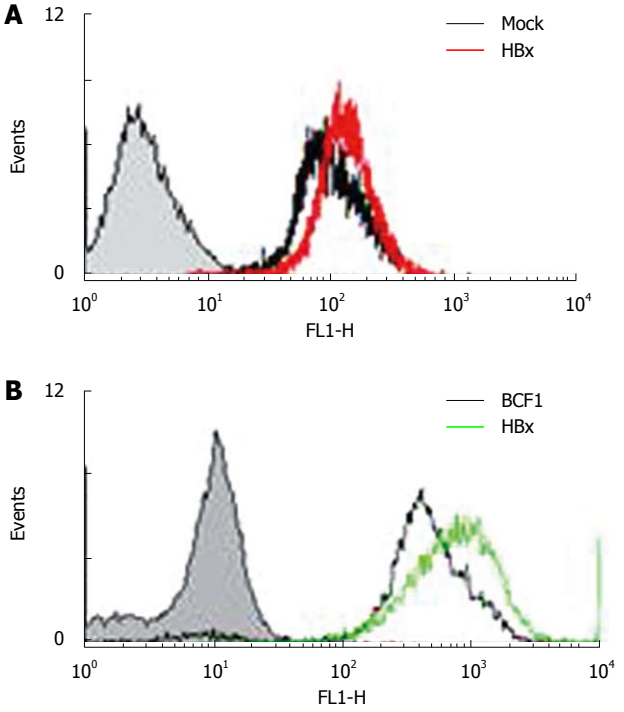

Figure 1 Increased reactive oxygen species in hepatitis B virus X protein transfected HepG2 stable cell line and hepatitis B virus X protein transgenic mouse hepatocytes. Reactive oxygen species (ROS) was detected by FACS caliber using dichlorofluorescein diacetate (DCFA-DA). A: HepG2 cell line stably transfected with hepatitis B virus X protein (HBx) showed a higher level of ROS compared to control cells; B: ROS production was checked after 4 wk of male HBx and control mouse hepatocyte growth. HBx mice hepatocytes generate more ROS than control mice.

LIVER PATHOGENESIS BY ALCOHOL-INDUCED OXIDATIVE STRESS

Chronic alcohol consumption has long been associated with progressive liver disease[64,65]. The liver is the major site of ethanol metabolism and thus sustains the most injury from chronic alcohol consumption. In alcohol-related liver disease, free radicals play a part in the pathogenesis of liver damage. Acute and chronic ethanol treatment increases ROS production, lowers cellular antioxidant levels, and enhances oxidative stress in many tissues, especially the liver[66,67]. It induces an accumulation of cysteine, a glutathione precursor/metabolite in the liver, probably due to gamma-glutamyltransferase induction[68]. Acetaldehyde produced by the oxidation of alcohol is able to inhibit the repair of alkylated nucleoproteins, to decrease the activity of several enzymes, and to damage mitochondria. Acetaldehyde also promotes cell death by depleting the concentration of reduced glutathione, by inducing lipid peroxidation, and by increasing the toxic effects of free radicals. Finally, acetaldehyde has been shown to directly stimulate proliferation of HSC and to increase collagen synthesis[69-71].

Chronic ethanol treatment has long been known to depress mitochondrial function[72-74]. The occurrence of DNA fragmentation in peripheral blood lymphocytes reflects a direct genotoxic effect of alcohol, HBV, and/or HCV, and suggests that the same genotoxic effect may operate in the liver and contribute to hepatocarcinogenesis[75].

Alcohol is also metabolized by mitochondrial CYP2E1. Ethanol exposure to VL-17A cells increased CYP2E1, decreased the activity of antigen-trimming enzymes

Table 1 Serum glutamate oxalate-transferase and glutamate-pyruvate-transferase values of wild and Hepatitis B virus X protein mice

Groups	Age (mo)	No. of animals	Treatment	Duration (wk)	GOT (U/L)	GPT (U/L)
HBx-tg	8	8	25% alcohol	12	193 ± 83.5	87.3 ± 35.5
	8	4	Normal water	12	60 ± 13.8	82 ± 19
C57BL/6J	8	8	25% alcohol	12	119 ± 31.9	61.7 ± 11.5
	8	9	Normal water	12	42 ± 11	68 ± 6

GOT: Glutamate oxalate-transferase; GPT: Glutamate-pyruvate-transferase; HBx: Hepatitis B virus X protein.

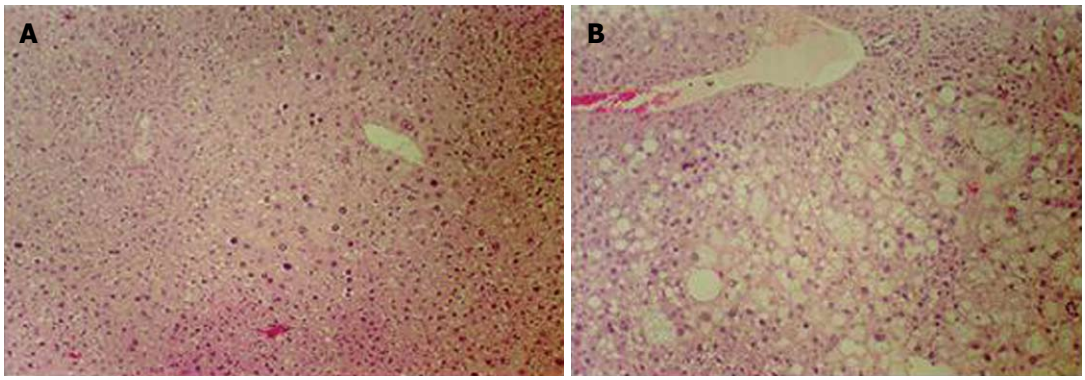

Figure 2 Chronic ethanol consumption caused liver damage in hepatitis B virus X protein transgenic mice. Ethanol fed hepatitis B virus X protein (HBx) tg mouse liver (B) showed severe liver damage, hepatocyte enlargement and fatty changes compared with water fed HBx (A). Original magnifications 100 ×.

like proteasome peptidase and leucine aminopeptidase (LAP). This defect may potentially result in decreased MHC class I -restricted antigen presentation on virally infected liver cells[68].

Alcohol-induced inflammatory and innate immune responses in Kupffer cells, due to elevated gut-derived plasma endotoxin levels, increase ROS-induced damage, and profibrogenic factors such as acetaldehyde or lipid peroxidation products, contribute to activation of HSCs[76]. Following a fibrogenic stimulus such as alcohol, HSCs transform into activated collagen-producing cells. There is much current interest in the likely synergistic interactions between hepatitis viruses and alcohol, especially with respect to generating oxidative stress.

Alcohol exacerbates pathological changes in HBx transgenic mice

C57BL/6J (control) and HBx transgenic mice 8 mo of age were fed with water or 25% ethanol liquid diets for 12 wk (Table 1). Glutamate oxalate-transferase (GOT) and glutamate-pyruvate-transferase (GPT) levels, both indicators of liver damage, were elevated in control and HBx ethanol-fed groups, but not in the water-fed groups. However, HBx mice showed higher levels of GPT (87.3 ± 35.5 U/L) and GOT (193 ± 83.5 U/L) than wildtype mice (GPT: 61.7 ± 11.5 U/L, GOT: 119 ± 31.9 U/L). This result indicated that HBx transgenic mice developed more severe liver damage from ethanol than control mice. This was confirmed by histological evaluation of the liver, which showed the development of more severe liver injury only in the HBx transgenic mice. Hyperplastic nodules, found in both the water- and ethanol-fed groups of HBx transgenic mice, were more frequent among the ethanol-treated group (Figure 2). Control mice fed ethanol showed mild steatosis (data not shown), but the alcohol-treated HBx transgenic liver had severe steatosis and hepatomegaly compared to the untreated controls (Figure 2). Thus, even moderate ethanol consumption promoted oxidative stress and liver injury in HBx transgenic mice, implying that compromised antioxidant defense promotes alcohol liver injury.

ANTIOXIDANT ENZYMES AND THE REDUCTION OF OXIDATIVE STRESS

Given that ROS production is a natural process, and that persistent, high levels of ROS could be damaging, the human body has developed antioxidant systems aimed at their neutralization. A variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS. These include superoxide dismutase (SOD), which detoxifies the superoxide ion, catalase and the GSH peroxidase system, peroxiredoxins, which inactivate hydrogen peroxide (H_2O_2), and glutathione peroxidase, whose function is to detoxify cellular peroxides. Further, ceruloplasmin and ferritin help remove metals, such as iron, that promote oxidative reactions. There are also nonenzymatic, low-molecular-weight antioxidants, such as GSH, vitamin E, ascorbate (vitamin C), vitamin A, ubiquinone, uric acid, and bilirubin[77,78].

A CuZn-SOD is present in the cytosol and in the space between the inner and outer mitochondrial membranes, while a manganese-containing SOD is present in the mitochondrial matrix. Both of these enzymes are critical for prevention of ROS-induced toxicity[79].

Catalase is found primarily in peroxisomes; it catalyzes a reaction between two H_2O_2 molecules, resulting in the

formation of water and O_2. In addition, catalase can promote the interaction of H_2O_2 with hydrogen donors so that the H_2O_2 can be converted to one molecule of water, and the reduced donor becomes oxidized (peroxidatic activity of catalase).

The Prx family has the capacity to decompose H_2O_2 *in vivo* and *in vitro*. All Prx enzymes contain a conserved Cys residue that undergoes a cycle of peroxide-dependent oxidation and thiol-dependent reduction during catalysis. Mammalian cells express six isoforms of Prx (Prx I to VI), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) based on the number and position of Cys residues that participate in catalysis. Prx I to Prx IV are members of the 2-Cys Prx subgroup. Prx I and Prx II exist in the cytosol. Prx III, which is synthesized with a mitochondrial targeting sequence, is imported into and matures within mitochondria. Prx IV is a secreted protein[80-83]. Prx V is expressed ubiquitously; it localizes to mitochondria and peroxisomes[84] and possesses antioxidant activity equivalent to that of catalase[85]. All peroxiredoxins have two cysteine residues, but Prx VI has only one at position 47. Prx VI is the only peroxiredoxin whose target is glutathione rather than thioredoxin. It is mostly cytosolic.

ANTIOXIDANT THERAPY FOR CHRONIC LIVER DISEASE

As discussed above, oxidative stress plays a central role in HBV- and alcohol-induced liver damage. There are several possible strategies for preventing this stress[34]. Among them is the addition of antioxidant agents to antiviral drugs for patients with chronic hepatitis B.

Curcuminoids

For example, curcuminoids, the main yellow pigments in Curcuma longa (turmeric), have been used widely and for a long time in the treatment of sprains and inflammation[86]. Curcumin is the main component of turmeric, and two minor components are also present as curcuminoids. Curcuminoids possess antioxidant activity[87]. They protect DNA against oxidative attack, thereby lowering the risk for mutations and other genetic damage[88,89]. They also activate detoxification enzymes such as glutathione S-transferase[90]. Curcumins can down-regulate NF-κB, a nuclear transcription factor and critical upstream regulator of genes that control acute and chronic inflammation cascades[91,92]. Curcumin exerts beneficial effects in animal models of liver injury and cirrhosis[93,94]. Curcumin prevents alcohol-induced liver disease in rats by blocking activation of NF-κB[95] and by induction of HO-1[96]. Curcumin inhibits the fibrogenic progression of murine steatohepatitis[97]. It inhibits extracellular matrix formation by enhancing HSC matrix metalloproteinase expression *via* $PPAR_\gamma$ and suppresses connective tissue growth factor expression[98]. CLL extract also represses HBV replication by enhancing the level of p53 protein[99].

Silymarin

Silymarin is a purified extract from milk thistle [*Silybum marianum* (L.) Gaertn], composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin, and silychristin. This extract has been used as a remedy for almost 2000 years[100] and continues to be used as a medicine for many types of acute and chronic liver diseases. Silybin is an effective antioxidant, conserving GSH in liver cells while stabilizing the liver cell membranes against oxidative attack[100,101].

Inhibition of liver fibrogenesis in clinical trials, and promotion of liver regeneration[102,103] have been inconsistent with these treatments. In clinical trials among patients with viral hepatitis[104], alcoholic liver damage[105], and/or other liver diseases, silymarin and silybin lowered liver enzymes and (at times) improved antioxidant status, but did not consistently improve symptoms[104,105]. It is routinely used in the clinic as a hepatoprotectant. Silymarin exerts beneficial effects on the early stages of chronic liver disease, preventing and delaying the onset of HBV-related liver carcinogenesis[106-110].

Mechanistically, the anti-inflammatory and anticancer effects of silybin and the other flavonolignans are related to the potent inhibition of NF-κB. Silybin is a potent inhibitor of NF-κB activation, as induced by a variety of anti-inflammatory agents[111].

Green tea

Green tea, a product of the plant Camellia sinensis (family Theaceae), contains polyphenols, specifically catechins of the flavan-3-ol class and their gallate derivatives. They are potent antioxidant and anti-inflammatory agents[112]. The flavan-3-ol structure makes them efficient scavengers of superoxide, singlet oxygen, nitric oxide, and peroxynitrite[113]. They up-regulate antioxidant and other detoxifying enzymes and protect DNA from oxidative damage[114-116]. Like other flavonoids, the green tea catechins can down-regulate NF-κB and AP-1, both of which may promote chronic inflammation and carcinogenesis when abnormally activated[117].

When treated with natural green tea extract, cells supporting HBV replication had reduced virus gene expression and reduced cell growth[118].

Vitamins C and E

Vitamin C is essential to a healthy diet as well as a highly effective antioxidant. It is a substrate for ascorbate peroxidase. Vitamin E is a fat-soluble antioxidant that is the major antioxidant found in lipid-phase membranes. It blocks the production of ROS formed when fat undergoes oxidation[119]. Several studies have clearly shown that serum levels of vitamin E are significantly reduced in patients with alcoholic liver disease[120,121]. Vitamin E levels also negatively correlate with production of oxidative stress products and directly correlate with the extent of liver damage[122]. Therefore, maintenance of normal concentrations of vitamin E seems to be essential to prevent lipid peroxidation induced by alcohol consumption. Works from several laboratories have indicated that mitochondrial damage may present a common early event in cell injury[123]. Mitochondrial damage was prevented by vitamin E[124]. Vitamin E or C alone or in combination can facilitate scavenging free

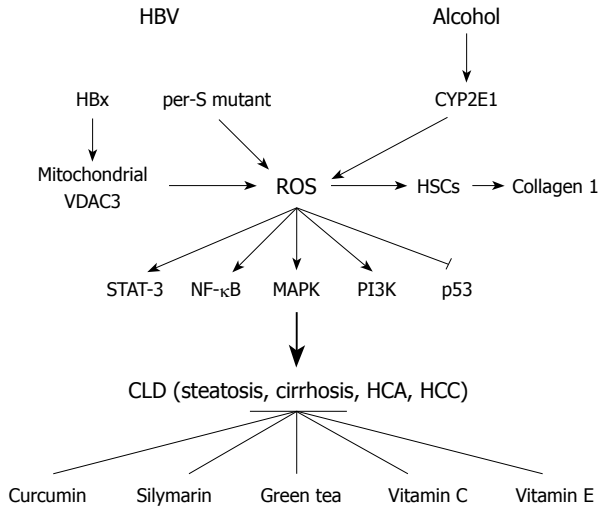

Figure 3 Summary of hepatitis B virus and alcohol induced reactive oxygen species effects on chronic liver disease and antioxidant's protective effects. HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein; VDAC3: Voltage-dependent anion channel 3; ROS: Reactive oxygen species; HSCs: Hepatic stellate cells; STAT-3: Transducer and activator of transcription 3; NF-κB: Nuclear factor κB; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; CLD: Chronic liver disease; HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma.

radicals generated in liver tissue[125]. Pretreatment with vitamin C against imidacloprid-induced oxidative liver stress in mice is better than post-treatment administration[126]. Pretreatment with vitamin E reduced the degree of oxidative stress[90], although this vitamin produced only slight changes in hepatic injury[127]. In the mouse model, vitamin E supplementation restored alcohol-induced redox status, reduced apoptosis, and prevented oxidative stress[128]. In addition, vitamin E in doses of 600 mg daily was effective in suppressing HBV replication and normalizing ALT in a significant proportion of chronically infected patients with CLD[129]. In this context, it will be important to determine whether anti-oxidants reduce HBxAg expression and/or function in cultured cells, or promote the resolution of CLD in human carriers and/or among human carriers with CLD who are also chronic alcoholics. If so, then anti-oxidant treatments may reduce the risk for progressive CLD lesions ultimately resulting in HCC, and/or eliminate the synergy between HBV and chronic alcoholism in the pathogenesis of alcoholic liver disease.

CONCLUSION

In summary (Figure 3), HBV and alcohol-induced liver injury are multi-step processes involving several mechanisms. The ability of HBV and alcohol to induce oxidative stress and the role of ROS in HBV- or alcohol-triggered liver damage is an important area of research, particularly because that information could be of major therapeutic value in protecting the liver. As basic information continues to emerge regarding the role of oxidative stress in disease development and the mechanisms underlying ROS-related cellular toxicity, these findings will lead to more rational antioxidant therapeutic approaches.

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S- Editor Wang JL **L- Editor** O'Neill M **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6044

World J Gastroenterol 2010 December 28; 16(48): 6044-6045
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Hepatic organ protection: From basic science to clinical practice

Rene Schmidt

Rene Schmidt, Department of Anesthesiology and Critical Care Medicine, Freiburg University Medical Center, Hugstetter Strasse 55, D-79106 Freiburg, Germany
Author contributions: Schmidt R wrote this editorial.
Supported by The International Anesthesia Research Society
Correspondence to: Rene Schmidt, MD, DESA, Department of Anesthesiology and Critical Care Medicine, Freiburg University Medical Center, Hugstetter Strasse 55, D-79106 Freiburg, Germany. rene.schmidt@uniklinik-freiburg.de
Telephone: +49-761-2702306 Fax: +49-761-2702396
Received: June 28, 2010 Revised: July 28, 2010
Accepted: August 4, 2010
Published online: December 28, 2010

Abstract

Hepatic ischemia and reperfusion (I/R) injury during liver surgery is still the main cause of postoperative liver failure and the subsequent rise of mortality in these patients. During the last few years, a multitude of underlying mechanisms have been extensively characterized and many different protective approaches have been evaluated under experimental conditions. Some of them have already found their way into small sized clinical trials. In this Topic Highlight series of articles, we present recent insights into promising protective concepts including the regulation and optimization of hepatic blood flow, molecular mechanisms of preconditioning and pharmacological approaches with the aim of limiting hepatic I/R injury. Leading international experts present the latest experimental evidence in their fields stressing clinically relevant ideas, which are now on the edge of entering clinical practice.

Key words: Heme oxygenase-1; Hepatectomy; Hepatic organ protection; Ischemia/reperfusion injury; Liver blood flow; Liver transplantation; Preconditioning

Peer reviewer: Andrej Khandoga, MD, Institute for Surgical Research Ludwig-Maximilians-University of Munich, Marchioninistr. 27, 81377 Munich, Germany

Schmidt R. Hepatic organ protection: From basic science to clinical practice. *World J Gastroenterol* 2010; 16(48): 6044-6045 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6044.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6044

The sequence of hepatic ischemia and reperfusion (I/R) is frequently associated with the destruction of liver cells thus contributing to postoperative liver failure and increased mortality. In liver transplantation, up to 30% of delayed graft function are caused by the consequences of hepatic I/R injury[1]. Intermittent clamping of hepatic blood inflow (e.g. Pringle's maneuver), an established technique to reduce blood loss during major liver resection, could have similar adverse effects by I/R mediated mechanisms. Furthermore, van der Bilt and colleagues demonstrated that I/R induced by vascular clamping is a strong stimulus that promotes the outgrowth of micrometastasis in the liver[2]. Therefore, the development of therapeutic concepts to prevent hepatic I/R injury has become the focus of intensive research efforts during the last few years. In this Topic Highlight series, we have put together a group of international experts providing an update on the latest achievements in their fields stressing clinically relevant ideas with the aim of protecting the liver against I/R injury. The maintenance of macro- and microvascular perfusion after hepatic ischemia plays a crucial role in the prevention of liver cell injury. In the first article of the present review series, Eipel *et al*[3] discuss recent insights into the regulation of hepatic blood flow and in particular the relevance of the "hepatic arterial buffer response", an important intrinsic mechanism of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow. The authors present detailed experimental and clinical information stressing the crucial importance of the hepatic arterial buffer response

as a regulatory mechanism to maintain adequate liver function and metabolic homeostasis. The second contribution focuses on preconditioning, an important phenomenon mediating cytoprotection in many different organs including the liver. Alchera *et al*[4] provide an interesting overview on the molecular mechanisms of liver preconditioning with special emphasis on the development of pharmacological approaches aimed at activating intrinsic protective systems in patients undergoing liver surgery. The next review concentrates on one of the most powerful inducible enzymes known today: heme oxygenase-1 (HO-1). HO-1 metabolizes heme into iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin. Upregulation of HO-1 and administration of each of its reaction products has been shown to play a pivotal role in the maintenance of cellular function after sublethal stress in nearly all organ systems including the liver. However, the development of therapeutic strategies that utilize the protective effect of HO-1 induction is hampered by the fact that most pharmacological inducers of this enzyme perturb organ function by themselves and that gene therapy for upregulation of HO-1 has potential negative side effects, which currently preclude its clinical application under these conditions. Hence, most substances used for upregulation of HO-1 under experimental conditions are not available for use in patients because of their toxicity and undesirable or unknown side effects[5]. During the last years, a few non-toxic HO-1 inducing compounds have been identified in animal experiments including the β_1-agonist dobutamine, the phosphodiesterase-Ⅲ-inhibitor olprinone, and the volatile anesthetics isoflurane and sevoflurane. Isoflurane has been shown to profoundly protect the liver against I/R injury by upregulation of HO-1 gene expression under experimental conditions[6,7]. As a consequence, volatile anesthetics are currently being evaluated for their potential to induce HO-1 and protect the liver against I/R in humans. In the present series of reviews, Richards *et al*[8] summarize HO-1 mediated protective effects within the liver and point to its therapeutic potential in detail. The protective role of nitric oxide (NO) in the context of hepatic I/R injury is then nicely presented by Siriussawakul and coworkers in their contribution[9]. The authors discuss the influence of endogenous NO on hepatic I/R injury and the potential therapeutic role of inhaled NO, nitrite and other NO donors in ameliorating hepatic I/R injury. Next, Mathes systematically describes the current knowledge on the antioxidant and other protective actions of melatonin in the liver. Melatonin, the "hormone of darkness", has recently been shown to exert abundant hepatoprotective effects in a multitude of experimental studies. Mathes illustrates this topic in depth and highlights possible approaches for its beneficial use in patients[10]. Finally, Gurusamy *et al*[11] present the currently available clinical data concerning protective strategies in liver surgery and review the significance of these studies in an evidence-based approach.

The present Topic Highlight series "Hepatic organ protection: From basic science to clinical practice" is far from being a complete reference of all experimental evidence concerning liver protection. It presents fascinating clinically relevant experimental concepts aimed at the identification of surgical techniques and pharmacological compounds, which now have to be validated in large randomized clinical trials.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6046

World J Gastroenterol 2010 December 28; 16(48): 6046-6057
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Regulation of hepatic blood flow: The hepatic arterial buffer response revisited

Christian Eipel, Kerstin Abshagen, Brigitte Vollmar

Christian Eipel, Kerstin Abshagen, Brigitte Vollmar, Institute for Experimental Surgery, Medical Faculty, University of Rostock, 18055 Rostock, Germany
Author contributions: Eipel C and Abshagen K performed the research; Vollmar B wrote the paper.
Correspondence to: Brigitte Vollmar, MD, Institute for Experimental Surgery, Medical Faculty, University of Rostock, Schillingallee 69a, 18055 Rostock, Germany. brigitte.vollmar@med.uni-rostock.de
Telephone: +49-381-4942500 Fax: +49-381-4942502
Received: June 28, 2010 Revised: August 26, 2010
Accepted: September 2, 2010
Published online: December 28, 2010

Key words: Hepatic blood flow; Hepatic arterial buffer response; Liver

Peer reviewer: Fausto Catena, MD, PhD, Department of General, Emergency and Transplant Surgery, St Orsola-Malpighi University Hospital, Via Massarenti 9, Bologna 40139, Italy

Eipel C, Abshagen K, Vollmar B. Regulation of hepatic blood flow: The hepatic arterial buffer response revisited. *World J Gastroenterol* 2010; 16(48): 6046-6057 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6046.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6046

Abstract

The interest in the liver dates back to ancient times when it was considered to be the seat of life processes. The liver is indeed essential to life, not only due to its complex functions in biosynthesis, metabolism and clearance, but also its dramatic role as the blood volume reservoir. Among parenchymal organs, blood flow to the liver is unique due to the dual supply from the portal vein and the hepatic artery. Knowledge of the mutual communication of both the hepatic artery and the portal vein is essential to understand hepatic physiology and pathophysiology. To distinguish the individual importance of each of these inflows in normal and abnormal states is still a challenging task and the subject of ongoing research. A central mechanism that controls and allows constancy of hepatic blood flow is the hepatic arterial buffer response. The current paper reviews the relevance of this intimate hepatic blood flow regulatory system in health and disease. We exclusively focus on the endogenous interrelationship between the hepatic arterial and portal venous inflow circuits in liver resection and transplantation, as well as inflammatory and chronic liver diseases. We do not consider the hepatic microvascular anatomy, as this has been the subject of another recent review.

PHYSIOLOGY OF LIVER BLOOD FLOW AND HEPATIC MACROHEMODYNAMICS

Hepatic blood flow and hepatic pressures

The liver has the most complicated circulation of any organ. According to the anatomical peculiarity of the double afferent blood supply of the liver, 75%-80% of the blood entering the liver is partially deoxygenated venous blood supplied by the portal vein, which collects all the blood that leaves the spleen, stomach, small and large intestine, gallbladder and pancreas[1-3]. The hepatic artery accounts for the remaining 25% with well-oxygenated blood. Total hepatic blood flow ranges between 800 and 1200 mL/min, which is equivalent to approximately 100 mL/min per 100 g liver wet weight[4]. Although the liver mass constitutes only 2.5% of the total body weight, the liver receives nearly 25% of the cardiac output.

The valveless portal vein is a low pressure/low resistance circuit, while the hepatic artery supplies the liver with arterial blood in a high pressure/high resistance system[4]. The mean pressure in the hepatic artery is similar to that in the aorta, while portal vein pressure has been reported

to range between 6 and 10 mmHg in humans when determined by direct cannulation[5] or by splenic puncture[6]. Portal pressure depends primarily on the degree of constriction or dilatation of the mesenteric and splanchnic arterioles and on intrahepatic resistance. Both afferent systems merge at the sinusoidal bed, where the pressure is estimated to be slightly, namely, 2-4 mmHg above that in the smallest collecting veins or the inferior vena cava.

Hepatic blood volume

Although only limited data exist, it appears that hepatic blood volume ranges from 25 to 30 mL/100 g liver weight, and accounts for 10%-15% of the total blood volume[7]. Estimations of hepatic blood volume are highly variable, as indirect calculations of hepatic blood volume from red blood cell content of the liver and arterial hematocrit are inaccurate, and hepatic venous pressure largely influences hepatic blood volume[4]. Furthermore, rough estimation suggests that more than 40% of the hepatic blood is held in large capacitance vessels (portal vein, hepatic artery and hepatic veins), while the sinusoids accommodate up to 60% as small vessel content[4]. Of note is the high compliance of the hepatic vascular bed, calculated as the change in blood volume per unit change in venous pressure[8]. In cats, the hepatic blood volume increases in response to elevated venous pressure and is doubled when hepatic venous pressure is elevated to 9.4 mmHg[8]. Hepatic blood volume may expand considerably in cardiac failure and, in turn, serves as an important blood reservoir in case of bleeding episodes, and compensates up to 25% of the hemorrhage by immediate expulsion of blood from the capacitance vessels[9].

Hepatic oxygen consumption

As in any other artery of the body, oxygen saturation of the hepatic artery usually exceeds 95%. Oxygen saturation of portal blood during the fasting state ranges up to 85%, which is greater than that of other systemic veins; however, it substantially drops after food ingestion. It is generally accepted that 50% of the oxygen requirements of the liver are provided by portal venous blood and the other half derives from the hepatic artery[1]. If oxygen demand is increased, the liver simply extracts more oxygen from the blood in order to maintain oxygen uptake. In line with this, alterations of hepatic oxygen supply, attained by isovolemic hemodilution or stimulation of hepatic enzymes, lead to reduced oxygen content in the inflow and outflow vessels, but do not cause dilatation of the hepatic artery, which disproves the view that the hepatic artery might be regulated by the metabolic activity of the liver cell mass[10].

Hepatic blood flow control

Liver blood flow is controlled by mechanisms that are independent of extrinsic innervation or vasoactive agents that regulate (1) hepatic arterial inflow; (2) portal venous inflow; and (3) the interrelationship between hepatic arterial and portal venous inflow circuits. The relationship between arterial pressure and hepatic arterial blood flow has been analyzed in several species. However, there is disagreement as to whether the hepatic arterial vasculature exhibits autoregulation of blood flow. The term autoregulation is specifically used to describe the non-linearity of the arterial pressure-to-arterial flow relationship and comprises the tendency for local blood flow to remain constant in the face of pressure changes in the arteries that perfuse a given organ. Some studies have revealed evidence of pressure-dependent autoregulation of blood flow in the hepatic arterial bed[11-15]. In denervated dog liver preparations, Hanson and Johnson have shown that a step-wise reduction of hepatic artery pressure from 90 to 30 mmHg was accompanied by a substantial reduction in hepatic artery resistance[15]. Comparably, livers with intact peri-arterial nerve plexi showed a 60% decrease in arterial resistance upon a 63% pressure reduction[11]. Overall, however, the degree of autoregulation is considered small[11] and present in only about 60% of all preparations[15]. The fact that papaverine infusion can abolish hepatic artery dilatation indicates that the observed effects are primarily mediated by myogenic adaptation of the vasculature to changes in transmural pressure[11]. Besides that, a metabolic washout hypothesis is also tenable, where the hepatic artery washes out the endogenous adenosine, thereby completely accounting for autoregulation of the hepatic artery[16].

Less controversy exists concerning pressure-to-flow autoregulation of the portal venous vascular bed. Only a few studies have indicated autoregulation of portal venous blood flow[13], while the majority of studies have revealed a linear pressure-to-flow relationship with constant or increased portal venous resistance at low pressure gradients. In fact, there is even evidence for an opposite effect with (1) a partial passive collapse of the portal vascular bed taking place upon reduction of portal pressure; and (2) a reciprocal decrease in resistance upon a step-wise increase in portal venous pressure[15].

REGULATION OF LIVER BLOOD FLOW BY THE HEPATIC ARTERIAL BUFFER RESPONSE

Besides the intrinsic regulation of the hepatic artery by the classical arterial autoregulation, that is, the myogenic constrictive response of the hepatic artery if the arterial pressure rises, there is a second form of intrinsic regulation, termed the hepatic arterial buffer response (HABR). This unique mechanism represents the ability of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow. Although Burton-Opitz observed an increase in hepatic arterial blood flow upon reduced portal venous inflow in 1911[17], this intimate relationship between these two vascular systems was termed HABR for the first time in 1981 by Lautt[18]. If portal blood flow is reduced, the hepatic artery dilates, and the hepatic artery constricts, if portal flow is increased[19,20]. Using transit-time ultrasonic volume flowmetry, intraoperative

measurement of the hepatic artery and portal venous flows in anesthetized patients with carcinoma of the splanchnic area has revealed a sharp and significant increase in hepatic arterial flow of about 30% after temporary occlusion of the portal vein, while temporary occlusion of the hepatic artery did not have any significant effect on portal venous circulation[20]. The HABR seems to operate in each individual under physiological conditions regardless of age. In addition, by establishing a method for measuring fetal hepatic arterial blood velocity, it has been reported that HABR even operates prenatally[21].

The increase in hepatic arterial blood flow is capable of buffering 25%-60% of the decreased portal flow[22,23]. The physiological role of this response is to minimize the influence of portal venous flow changes on hepatic clearance and to maintain adequate oxygen supply to tissues[24]. The latter function, however, may be of minor importance, since the liver normally receives more oxygen than it requires, and it can extract more oxygen to compensate for reduced delivery[25]. Thus, metabolic activity of the hepatic parenchymal cells does not directly control the hepatic arterial flow[22,25]. Instead, hepatic arterial flow subserves the hepatic role as a regulator of blood levels of nutrients and hormones by maintaining blood flow and thereby hepatic clearance as steadily as possible[24,26]. Because the portal vein cannot control its blood flow, which is simply the sum of outflows of the extrahepatic splanchnic organs, there is no reciprocity of the HABR, that is, alterations of the hepatic arterial perfusion do not induce compensatory changes of the portal vascular flow[18,20] or resistance[27].

The current view is that the HABR can be accounted for by the adenosine washout hypothesis[23]. This hypothesis states that adenosine is released at a constant rate into fluid in the space of Mall that surrounds the hepatic resistance vessels and portal venules. The space of Mall is contained within a limiting plate that separates this space from other fluid compartments. The concentration of adenosine is regulated by washout into the portal vein and the hepatic artery. If portal blood flow is reduced, less adenosine is washed away from the space of Mall, and the elevation in adenosine levels leads to dilation of the hepatic artery with a subsequent increase in hepatic arterial flow[10].

There are several lines of evidence that adenosine mediates the HABR: (1) adenosine produces hepatic arterial dilation[23]; (2) portal venous application of adenosine exerts one-half to one-third the effect of the same dose infused directly into the hepatic artery, which indicates that portal blood has some access to the arterial resistance vessels[10]; (3) adenosine uptake antagonists potentiate the HABR[23]; and (4) pharmacological antagonists of adenosine produce competitive blockade of the buffer response[16,28-30]. However, it has been suggested that adenosine itself does not diffuse from the portal venous to hepatic arterial bed to elicit the arterial response[31,32]. Rather ATP is released from the portal venous vasculature as a response to hypoxia associated with portal flow reduction, and diffuses into the hepatic arterial vasculature. No difference in the degree of inhibition of HABR by an adenosine antagonist has been observed between intra-arterial and intraportal injection of ATP in a rabbit model. This suggests that only the adenosine produced from ATP catabolism in the hepatic arterial vasculature contributes to arterial dilation. The adenosine produced from ATP in the portal venous vasculature is taken up effectively by the endothelium and vascular smooth muscle cells as soon as it is formed, and it does not diffuse to the hepatic arterial vasculature[30]. Mathie and Alexander have pointed out that adenosine is unlikely to be the sole regulator of HABR[33]. Other vasoactive compounds, such as nitric oxide and carbon monoxide, may be potential candidates to affect hepatic arterial flow and contribute to the HABR. Nitric oxide participates in regulation of hepatic arterial blood flow with changes in portal venous blood flow *via* ATP-dependent stimulation of endothelial purinergic receptors in the hepatic artery, which results in vasodilation[30,34,35]. Although nitric oxide is an important regulator of hepatic arterial resistance[36], it does not mediate the HABR and it is not found to play any significant role in total hepatic capacitance regulation[37].

Although nitric oxide serves as a potent vasodilator in the hepatic arterial circulation and exerts only a minor vasodilatory effect in the portal venous vascular bed, carbon monoxide is reported to maintain portal venous vascular tone in a relaxed state and to exert no vasodilation in the hepatic artery[38]. Recently, a third gaseous mediator, H_2S, has been recognized as an important endogenous vasodilator and neuromodulator[39]. There is now major evidence that H_2S also contributes to the HABR and partly mediates the vasodilatory response of the hepatic artery. This conclusion is based on the fact that supplementation of H_2S increases hepatic arterial conductance and almost doubles the buffer capacity. In turn, inhibition of the H_2S function by application of a selective inhibitor of K_{ATP} channels, which mainly mediate the ability of H_2S to relax vascular smooth muscle cells, markedly decreases buffer capacity[40].

Next to vasoactive mediators, there is evidence that sensory innervation and sensory neuropeptides are, at least to some extent, involved in the HABR. Accordingly, sensory denervated rats[41] and pigs[42] have revealed a reduced HABR upon partial occlusion of the portal vein. Furthermore, pretreatment with antagonists of calcitonin gene-related peptide (CGRP) and neurokinin (NK)-1 receptors significantly reduce the hepatic arterial blood flow, which indicates that the observed vasodilation in the vascular bed of the hepatic artery is due to stimulation of CGRP and NK-1 receptors[41].

IMPLICATIONS OF THE HABR IN LIVER DISEASES

HABR in liver resection, transplantation and laparoscopic surgery

The ability of the liver to regenerate after major resection has been studied extensively, but the factors responsible

for regeneration are not fully understood[43]. Although a clear association between flow and regenerative response has been suggested, the exact role of hepatic blood flow in liver regeneration is still a matter of intense debate. The increased blood flow to liver mass ratio immediately after partial hepatectomy (pHx) and the resultant increased intrahepatic shear stress have been proposed to stimulate and regulate liver regeneration[44-47]. On the other hand, the failure of the liver to control directly the portal venous blood flow has the consequence of portal hyperperfusion of the reduced-size liver (Figure 1A and B), which has been shown to impair seriously postoperative recovery of patients who are undergoing living donor liver transplantation or extended pHx[48,49]. In humans, 60% pHx results in a doubling of the portal flow in the 40% of remnant liver tissue[50]. This extent of pHx is followed by a transient and minor degree of small-for-size syndrome that usually resolves spontaneously within a few days. In contrast, major liver resection (> 75%) is followed by a more pronounced and long lasting small-for-size syndrome with much higher morbidity and mortality[50].

With the increasing practice of living-donor liver transplantation and the enlargement of the resectable limit, the small-for-size syndrome has emerged as an important clinical problem[51]. Although the pathogenetic causes of the small-for-size syndrome are still debated, it is assumed that the syndrome is primarily linked to portal hyperperfusion with high intravascular shear stress[52-54]. As a consequence of portal venous hyperperfusion, however, HABR may lead to hepatic arterial hypoperfusion of reduced-size livers (Figure 1B). In line with this, Smyrniotis *et al*[55] have shown in a porcine study that portal flow to split grafts with a graft-to-recipient liver volume ratio of 2:3 and 1:3 showed an inverse relationship to graft size, for example, the smaller the graft, the higher portal blood flow. By contrast, arterial flow decreased proportionately to graft size. In addition, HABR, which is present in all split-liver transplanted pigs, has been found to be increase as the graft-to-recipient liver volume ratio decreases[55].

A comparable hemodynamic pattern of hepatic blood flow has been observed in living related liver transplantation, in which size disparity between graft and native liver is the rule and almost universal[56]. In patients with living right lobe living donor transplantation, the grafts are subjected to impressive, more than double, increases of portal blood flow (Figure 1A and B). In the absence of active regulation, arterial flow might be expected to double as well. On the contrary, striking decreases in arterial flow have been seen in right lobe grafts[57], which represent the HABR as a reflexive response to changes in portal blood flow, to maintain total blood flow within an acceptable physiological range[58]. Troisi *et al*[59] have reported mean recipient portal venous flow values in small liver grafts (graft-to-recipient body weight ratio < 0.8) at least three times higher than those recorded in donors. Simultaneously, hepatic artery flow is significantly reduced and results in a decreased contribution to the liver from 30% in donors to only 6% in the recipients. In a porcine small-for-size liver transplantation model, the portal-to-arterial flow ratio remains increased until 5 d after surgery, which is poorly tolerated by transplanted livers[60].

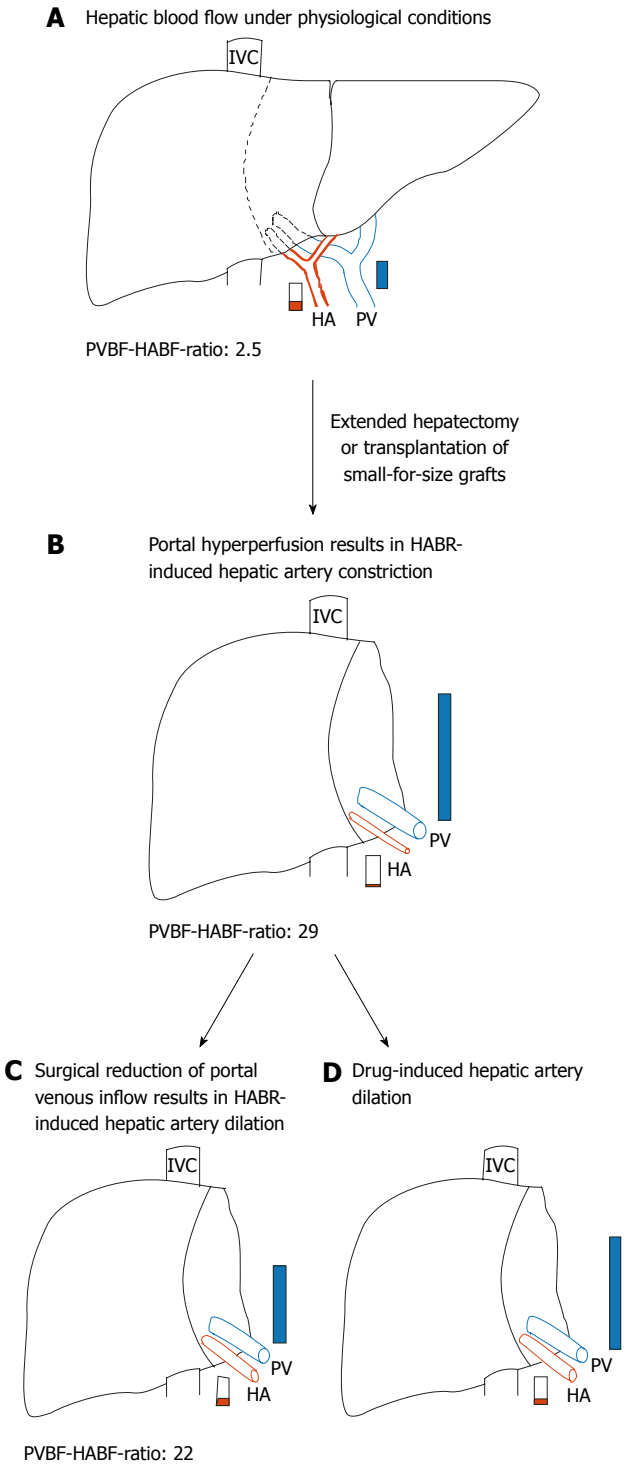

Figure 1 Hepatic hemodynamics in normal and reduced-size livers. A: Preoperative hepatic blood flow in a donor liver or before extended hepatectomy representing a normal portal vein blood flow-hepatic artery blood flow (PVBF/HABF) ratio of 2.5; B: As a consequence of portal hyperperfusion, hepatic arterial buffer response (HABR) leads to hepatic arterial hypoperfusion of reduced-size liver that is characterized by a dramatically increased PVBF/HABF ratio of 29; C: Surgical reduction of the portal venous inflow, for example, by splenectomy or hepatic artery ligation, leads to HABR-induced dilation of the hepatic artery and results in a reduced PVBF/HABF ratio of 22; D: Possible effects of pharmacological interventions to preserve hepatic artery supply. PVBF/HABF ratios are adopted from[59]. HA: Hepatic artery; IVC: Inferior vena cava; PV: Portal vein.

The HABR has been clearly demonstrated to be present also in patients after cadaveric liver transplantation[61]. Measurement of hepatic arterial and portal venous flow using ultrasound transit-time flow probes over the first 3 h after reperfusion has revealed a mean total liver blood flow of 2091 ± 932 mL/min, with a disproportionately high mean portal flow of 1808 ± 929 mL/min, which represents approximately 85% ± 10% of total liver blood flow. Correlation analysis has shown a positive correlation between cardiac output and portal venous flow, and a trend toward negative correlation between cardiac output and hepatic arterial flow[61]. In patients with a 50% reduced portal flow, Henderson and colleagues have reported a significant increase in hepatic artery flow, which indicates an intact HABR after cadaveric liver transplantation[61]. In line with this, Payen *et al*[62], by measuring hepatic arterial and portal venous blood flow during alternative clamping of both vessels every 12 h during 7 d in patients after orthotopic liver transplantation, have reported reciprocal increases of hepatic arterial flow only during selective clamping of the portal vein. By analysis of patients transplanted for liver cirrhosis, a high portal flow was present, together with an early increase of hepatic arterial resistance, which agrees with the HABR theory[63]. The presence of HABR in the transplanted liver is unequivocal[61,62,64], and because of liver denervation, it might be the only active mechanism that regulates liver arterial flow.

The consequences of inadequate hepatic arterial flow range from mild cholestasis to rapidly progressive graft failure[65,66]. In a porcine model of small-for-size syndrome, histological examinations of the grafts consistently confirm hepatic artery vasospasm and its consequences; namely, cholestasis, centrilobular necrosis and biliary ischemia[67]. In severe cases of small-for-size grafts, poor hepatic arterial flow and vasospasm lead to functional de-arterialization, ischemic cholangitis, and parenchymal infarcts[54,68]. Michalopoulos has concluded that the failure to regenerate is not different from the situation in which pHx is accompanied by ligation of the hepatic artery, which also results in failure to regenerate[68].

Prolonged CO_2 pneumoperitoneum in laparoscopic surgery reduces substantially the portal venous flow in humans, and the extent of the flow reduction is related to the level of intraperitoneal pressure[69,70]. HABR may serve for maintenance of total liver blood supply during laparoscopy-associated portal venous flow reduction. However, controversial data exist on the maintenance of HABR during high-pressure pneumoperitoneum. Yokoyama *et al*[71] have reported on activation of HABR in a rat model using fluorescent microspheres to measure splanchnic flow. Although portal venous flow decreased, the hepatic arterial flow was relatively preserved throughout all levels of intraperitoneal pressure studied. In contrast, Richter *et al*[72] have used ultrasonic flow probes in a rat model, and have shown reduced portal venous flow paralleled by a linear reduction of hepatic arterial flow during CO_2-pneumoperitoneum. HABR is also markedly impaired in cirrhotic rats undergoing CO_2 pneumoperitoneum[73]. Studies in large animals have revealed intact HABR with doubled hepatic arterial flow in neonatal lambs during abdominal distension[74], as well as loss of HABR with reduced hepatic arterial flow in pigs[75], or unchanged hepatic arterial flow in dogs upon CO_2 pneumoperitoneum[76]. In particular, head up body position leads to reduction in portal venous and arterial hepatic blood flow during elevated abdominal pressure[77]. Thus, head up position and intraperitoneal pressure elevation above 15 mmHg should be avoided during laparoscopic surgery to preserve hepatic blood flow[77,78].

HABR in inflammatory liver diseases

Owing to the scarcity of clinical studies on this subject, one must turn to experimental data, with reservations concerning their extrapolation to humans. In models of continuous intravenous infusion of *Escherichia coli* in rats, portal venous flow was reduced, and increased hepatic arterial flow resulted in unchanged total hepatic blood flow[79,80]. The increased hepatic arterial flow could be a result of an active HABR, although, in parallel, reports exist to demonstrate an increased hepatic artery flow without a reduction in portal venous flow during endotoxemia[81-83]. In a porcine model, it has been shown that endotoxin shock leads to time-dependent impairment of liver inflow beds, which results in increased portal venous back pressure and incremental resistance. The hepatic artery bed is dilated in the early phase of endotoxic shock but, over time, it is constricted[84]. There is ongoing discussion as to whether excessive production of nitric oxide is the cause of the endotoxin-induced alterations in hemodynamic homeostasis. While nitric oxide induces arterial hypotension and hepatic arterial vasodilation during endotoxic shock[85], ablation of the HABR has been shown to be independent of nitric oxide or an α-adrenergic-receptor agonist[84]. On the contrary, early administration of the nitric oxide donor sodium nitroprusside can reverse the negative effects on hepatic arterial flow induced by endotoxin[86,87]. Moreover, sodium nitroprusside partially reverses the detrimental effect of the nitric oxide synthase inhibitor L-NAME in experimental endotoxemia, which implies that the endotoxin-induced dysfunction of the HABR may be due to a selective inhibition of vascular endothelial function[87]. Furthermore, nitroprusside maintains mRNA levels of constitutive nitric oxide synthase in liver tissue that are decreased by endotoxin shock and tempers the burst in inducible nitric oxide synthase expression, thereby reestablishing the autoregulatory response of the hepatic artery following reduction of portal venous blood flow[86].

In turn, application of the vasopressin analog terlipressin during long-term hyperdynamic porcine endotoxemia significantly decreases portal venous flow, whereas hepatic arterial flow is markedly increased, which presumably reflects a restored HABR[88]. Furthermore, terlipressin attenuates the endotoxin-induced increase in exhaled nitric oxide[88], which points to the interaction between the vasopressin and the nitric oxide system in septic shock[89].

Almost no data exist on hepatic hemodynamics during conditions of acute or chronic viral hepatitis[90]. In addition, only a few studies have addressed hepatic hemodynamics under low-flow conditions, such as hemorrhagic shock. However, the data so far are consistent in that HABR is not abolished during sustained low abdominal blood flow[91-93]. In critically ill patients, mechanical ventilation has been found to decrease splanchnic perfusion. However, importantly, HABR is preserved and increased hepatic arterial blood flow compensates the decrease in portal blood flow under conditions of ventilation-associated positive end-expiratory pressure[94].

HABR in liver fibrosis and cirrhosis

The pathogenesis of liver fibrosis and cirrhosis is characterized by initial hepatocyte necrosis and inflammatory response, with subsequent activation of hepatic stellate cells and their transformation into myofibroblasts, which is responsible for excessive extracellular matrix synthesis and deposition. As a consequence, distinct alterations of the hepatic microvasculature, that is, rarefaction of sinusoids and structural changes of sinusoidal endothelia[95,96], result in deteriorated nutritive blood supply, increased total hepatic vascular resistance, and hence, portal hypertension and portosystemic collateralization[97]. Due to this increase in sinusoidal resistance, the capillarization of the hepatic microvasculature and the development of portocaval collaterals[98], portal venous blood flow progressively decreases in patients with cirrhosis[99,100]. An increase of hepatic arterial blood flow, that is, a decrease of hepatic arterial resistance, if it occurs, would indicate an activated HABR.

Studies in cirrhotic rats have underlined this hypothesis by demonstrating that, under baseline conditions, cirrhotic animals have higher hepatic arterial blood flow compared to controls[101]. Moreover, induction of HABR by a stepwise reduction of portal venous inflow causes a disproportionate increase in hepatic arterial flow in cirrhosis, which is further reflected by the significantly higher buffer capacity[101,102].

Although this concept has been well established, analyses in cirrhotic patients have produced conflicting results. Several studies have shown an increased hepatic arterial resistance in patients with cirrhosis. This is related to the degree of portal hypertension[103,104], portal resistance[104,105] and Child-Pugh score[104]. In contrast to these observations, a considerable body of evidence exists to indicate that, in cirrhosis, hepatic arterial vasodilatation occurs in response to reduced portal venous blood flow[106-109]. Accordingly, intraoperative measurements in patients with end-stage liver cirrhosis, who underwent living-donor liver transplantation, have revealed a continuously activated HABR under baseline conditions[109]. In these cirrhotic patients, the reduced portal venous blood flow is associated with an increased hepatic arterial blood flow (hepatic arterial to portal venous flow ratio = 0.88), which is in contrast to the relationship in healthy volunteers (hepatic arterial to portal venous flow ratio = 0.58)[109]. However, total clamping of the portal vein provokes a blunted response, as evaluated by the absolute and relative changes in hepatic arterial blood flow and by the buffer capacity[109]. In line with this, Iwao *et al*[110] have reported that the hepatic artery buffer index is significantly lower in cirrhotic than in control subjects. They have analyzed portal venous blood flow and hepatic artery pulsatility index as measures of hepatic artery resistance upon a 500-kcal mixed liquid meal consumption, which increases portal venous blood flow. They found an increase in hepatic artery resistance in all subjects, however, it was less pronounced in cirrhotic than control subjects[110,111]. Vice versa, the vasopressin-induced decrease of portal venous blood flow was met by a fall in hepatic arterial pulsatility index, which again was significantly lower in cirrhotic than control subjects[110]. In a large series of patients with advanced cirrhosis, who are undergoing transjugular intrahepatic portosystemic shunt (TIPS), Gülberg *et al*[108] have demonstrated that patients with hepatofugal blood flow show significantly lower resistance index before TIPS placement than patients with antegrade portal flow direction, and TIPS placement induces a significant decrease in the resistance index in patients with hepatopedal flow, but not in patients with hepatofugal flow. The fact that some degree of HABR is preserved even in patients with advanced cirrhosis and significant portal hypertension may further underline the biological need for this intrinsic mechanism. Although one might argue that the drop in resistance and thus increase in hepatic arterial flow may not fully compensate for the TIPS-induced reduction in portal blood flow, it has been shown that hepatic arterial vasodilatation provides substantial functional benefit in patients with cirrhosis, and that this effect does not depend directly on hepatic arterial microperfusion and is observed preferentially in patients with decompensated disease[107]. Thus, it is reasonable to state that the change in the ratio of portal venous to hepatic arterial blood flow in favor of the hepatic artery may sustain oxygen delivery and exert a protective effect on organ function and integrity. In line with this, portal vein occlusion does not cause deterioration in hepatic tissue pO_2 in the presence of HABR, although maximum buffer capacity of the hepatic artery was limited to 50%-60% in both cirrhosis and control animals, and total liver blood flow was found to be restored to only 71%-76% of baseline values[102].

MODIFICATIONS OF THE HABR

Surgical interventions for modification of the HABR

With the development of partial liver transplantation, either as living donation or as deceased donor split graft, much effort has been spent on improvement of surgical techniques. Full-right full-left splitting for two adult recipients is associated with risk of small-for-size syndrome, which manifests as a pattern of liver dysfunction associated with portal hypertension, diminished arterial flow,

delayed synthetic function, and prolonged cholestasis[112-115]. However, the present rates of splitting livers are too low in comparison with the calculated potential and it is to be expected that further improvement in the management of small-for-size grafts would bring splitting of the liver for two adult recipients within the reach of broad application[112]. Small-for-size syndrome is a clinical problem that is also observed after living donor liver transplantation and extended hepatectomy[52,116]. When the full portal vein flow has to transverse through a much reduced liver size, then the pressure building up in the portal vein effectively shuts down the flow through the hepatic arterioles and the liver becomes de-arterialized[68]. Arterial flow impairment appears as result of an active HABR, although in the past, reduced hepatic arterial flow has repeatedly been ascribed to the splenic artery steal syndrome[117-120]. This phenomenon describes the impaired hepatic artery flow by shifting of the main blood flow to the splenic or gastroduodenal artery in patients with hypersplenism. Quintini *et al*[121] have analyzed whole-organ liver recipients by Doppler ultrasonography, and have reported that hepatic artery vasoconstriction in response to portal hyperperfusion and exaggerated HABR produces a high resistive index with poor arterial perfusion. In all patients, splenic artery embolization reduces the resistance to distal hepatic artery flow by reducing flow in the splenic circulation and consequently in the portal vein. This has prompted the authors to revise the name of splenic artery steal syndrome to splenic artery syndrome, thereby underlining that the cause is portal hyperperfusion and not arterial siphon[121]. Most recently, a retrospective analysis of 650 orthotopic liver transplantations has revealed an incidence of 5.1% for splenic artery syndrome[122], which is well within the range of the estimated incidence of artery splenic syndrome of 3.1%-11.5% after orthotopic liver transplantation[117,118,123,124]. Prophylactic treatment with ligation of the splenic artery for all patients at risk for development of splenic artery syndrome is recommended and effectively prevents splenic artery syndrome[122]. In the case of postoperative diagnosis of splenic artery syndrome, coil embolization of the splenic artery can be recommended as the treatment of choice, with a low risk profile[122].

Clinical features of small-for-size syndrome are neither specific nor inevitable in low-weight livers, and many other factors than actual liver weight contribute to their occurrence. Among these, early elevation of portal venous pressure and persistent portal overperfusion most probably play a key role[48,49,125-128]. A reduction in the portal venous flow by means of splenic artery ligation, splenic artery embolization, or splenectomy has been shown to be efficient also in case of the small-for-size syndrome[48,59,127,129]. A case report by Lo *et al*[51] and prospective studies by Troisi *et al*[130] and Umeda *et al*[131] have shown that modulation of the recipient portal inflow by ligation or embolization of the splenic artery leads to an increase in recipient hepatic arterial inflow (Figure 1C), with improved liver function. The fact that splenic artery syndrome and small-for-size syndrome can be successfully

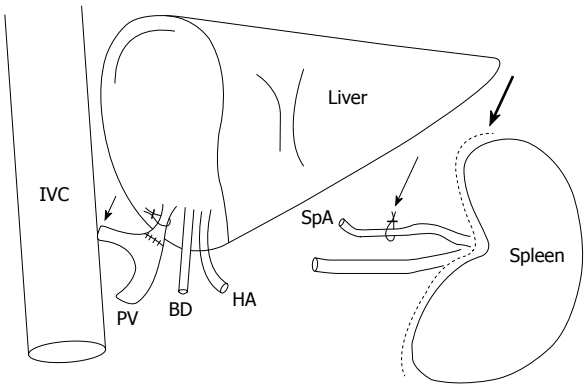

Figure 2 Surgical interventions for modulation of the hepatic inflow, showing the portocaval shunt (short arrow), ligation of the splenic artery (thin long arrow) and splenectomy (thick long arrow). BD: Bile duct; HA: Hepatic artery; IVC: Inferior vena cava; PV: Portal vein; SpA: Splenic artery.

treated by coil embolization of the splenic artery strongly underlines that both syndromes are pathophysiologically linked to the HABR. In line with this, detailed histopathological examination of sequential post-transplant biopsies and failed allografts with clinicopathological correlation has revealed that portal hyperperfusion, venous pathology, and the arterial buffer response make an important contribution to early and late clinical and histopathological manifestations of small-for-size syndrome[54]. In the most recent study of our group, we observed significantly increased survival of simultaneously splenectomized and hepatectomized rats compared to animals with 90% pHx alone[132]. It has been suggested that this effect is mainly caused by suppression of intrahepatic flow and less sinusoidal shear stress[49,55,133]. However, reduction of total hepatic inflow in simultaneously splenectomized and hepatectomized animals was marginal and not as pronounced as that required to improve survival by reduced shear stress. Instead, splenectomy before pHx caused a doubling of hepatic tissue pO_2 due to a HABR-induced rise of hepatic blood flow during extended pHx, which led to high tissue pO_2 values and reduced hypoxic stress. Supposedly, the increase of arterial inflow covers the oxygen demand and thereby improves organ regeneration and animal survival. Thus, improved arterial inflow rather than reduction of portal venous hyperperfusion is of great significance for the beneficial effect after inflow modulation in small-for-size livers[132].

Besides splenic artery ligation, established techniques such as portocaval or mesocaval shunts (Figure 2) may cause not only reduction of portal hyperperfusion, but also an increase of hepatic arterial inflow by reversion of the HABR (Figure 1C)[130,134-136].

In conclusion, the intraoperative measurement of both hepatic blood flows is important to predict the risk of small-for-size syndrome. The better ability to regulate finely the hepatic inflows would be useful in the treatment of liver dysfunction in settings of small-for-size transplantation, as well as extended hepatectomy, and necessitates further studies.

Pharmacological modifications of the HABR

The responsiveness of the hepatic artery to changes in portal flow is undoubtedly a desirable homeostatic mechanism under most circumstances, that is, the increase of hepatic arterial blood flow in case of reduced portal venous inflow. In contrast, the opposite situation, in which the dramatic excess of portal flow due to a smaller-than-average organ causes hepatic arterial constriction and hypoperfusion, might harm the liver (Figure 1A and B). Both extended hepatectomy and split-liver transplantation by fashioning two transplantable grafts from one liver result in small-for-size livers[50,116,137]. The regenerating liver requires an enormous amount of oxygen for its increased metabolic load and for DNA synthesis[138,139]. Suboptimal arterial inflow may be poorly tolerated in the reduced-size liver and increase the risk of organ dysfunction[60,68,138]. Possible pharmacological interventions could aim to enhance the hepatic arterial supply (Figure 1D). In line with this, in a porcine model of small-for-size syndrome, hepatic arterial infusion of adenosine significantly restored hepatic artery flow, reversed pathological changes in the graft, and finally improved survival[67]. In addition, an imbalance of vasorelaxing and vasoconstricting mediators is considered to be an important pathogenetic feature in reduced-size livers[140]. Maintenance of endothelin-1/nitric oxide balance by blocking endothelin A receptor reduces small-for-size injury by protecting the liver microcirculation and reducing hepatocellular damage[140]. Vice versa, substitution of nitric oxide has been shown to counteract the decline in hepatic arterial inflow in rats with 85% hepatectomy and cause a significantly greater increase in cell proliferation, with improvement of liver function[141].

Several programs in Japan have started clinical trials to reduce injury in small-for-size grafts by direct infusion of drugs such as prostaglandin E_1 and proteolytic enzyme inhibitors into the portal flow[126,142]. However, further experimental studies, including intraoperative measurement of both hepatic blood inflows, are warranted to clarify the precise strategies of pharmacological interventions and to select the appropriate drugs.

CONCLUSION

The crucial importance of the HABR as a regulatory mechanism to maintain adequate liver function and metabolic homeostasis has been recognized. Now, establishment of measures to modulate altered hemodynamics in small-for-size livers, as well as in cirrhotic and critically ill patients, warrants increased attention. Every effort for a timely diagnosis of altered or impaired HABR should be made in order to treat potential ensuing problems. Pharmacological and surgical interventions, which may be applied most easily, have to be proven in larger randomized clinical trials in order to improve the outcome of patients with liver disease with altered hepatic hemodynamics.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6058

World J Gastroenterol 2010 December 28; 16(48): 6058-6067
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Molecular mechanisms of liver preconditioning

Elisa Alchera, Caterina Dal Ponte, Chiara Imarisio, Emanuele Albano, Rita Carini

Elisa Alchera, Caterina Dal Ponte, Chiara Imarisio, Emanuele Albano, Rita Carini, Department of Medical Sciences, University of East Piedmont "A. Avogadro", Via Solaroli 17, 28100 Novara, Italy

Author contributions: Alchera E, Dal Ponte C, Imarisio C and Carini R performed the research and analyzed the data; Albano E and Carini R designed the research; Albano E critically revised the paper; Carini R wrote the paper.

Supported by The Regional Government of Piedmont, Italy (Carini, Fondi Ricerca Sanitaria Finalizzata, 2006, 2007; 2008, 2008 bis, 2009; Alchera, Fondi Ricerca Sanitaria Finalizzata, 2008 bis, 2009); and by the University "Amedeo Avogadro"

Correspondence to: Dr. Rita Carini, Department of Medical Sciences, University of East Piedmont "A. Avogadro", Via Solaroli 17, 28100 Novara, Italy. carini@med.unipmn.it
Telephone: +39-321-660685 Fax: +39-321-620421

Received: June 28, 2010 Revised: November 3, 2010
Accepted: November 10, 2010
Published online: December 28, 2010

Abstract

Ischemia/reperfusion (I/R) injury still represents an important cause of morbidity following hepatic surgery and limits the use of marginal livers in hepatic transplantation. Transient blood flow interruption followed by reperfusion protects tissues against damage induced by subsequent I/R. This process known as ischemic preconditioning (IP) depends upon intrinsic cytoprotective systems whose activation can inhibit the progression of irreversible tissue damage. Compared to other organs, liver IP has additional features as it reduces inflammation and promotes hepatic regeneration. Our present understanding of the molecular mechanisms involved in liver IP is still largely incomplete. Experimental studies have shown that the protective effects of liver IP are triggered by the release of adenosine and nitric oxide and the subsequent activation of signal networks involving protein kinases such as phosphatidylinositol 3-kinase, protein kinase C δ/ε and p38 MAP kinase, and transcription factors such as signal transducer and activator of transcription 3, nuclear factor-κB and hypoxia-inducible factor 1. This article offers an overview of the molecular events underlying the preconditioning effects in the liver and points to the possibility of developing pharmacological approaches aimed at activating the intrinsic protective systems in patients undergoing liver surgery.

Key words: Apoptosis; Hepatocyte; Hypoxia; Ischemia/reperfusion; Liver surgery; Necrosis; Pharmacological preconditioning; Preconditioning; Survival pathways

Peer reviewers: Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4, IV Piano, 09125 Cagliari, Italy; Hussein M Atta, MD, PhD, Professor, Department of Surgery, Faculty of Medicine, Minia University, Misr-Aswan Road, El-Minia 61519, Egypt

Alchera E, Dal Ponte C, Imarisio C, Albano E, Carini R. Molecular mechanisms of liver preconditioning. *World J Gastroenterol* 2010; 16(48): 6058-6067 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6058.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6058

INTRODUCTION

The understanding of the proteomic features associated with cell response to stresses is one of the present-day challenges in medical science. This knowledge is increasingly necessary to identify new molecular targets for therapeutic interventions. A turning-point on this matter has been the discovery that tissues already possess a number of inducible systems able to make them more resistant to a wide array of injuries. One of these adaptive responses is represented by the capacity of a non-lethal ischemia to modulate cell functions by increasing resistance to subsequent lethal ischemia/reperfusion[1,2]. Since its first description in the myocardium[1], this phenomenon, termed "ischemic preconditioning" (IP), has been the subject of

rising interest in the scientific and medical communities. The effects of IP can be differentiated into early effects and late effects. The former, immediately follows the transient ischemia and involves the direct modulation of specific cell functions, while late effects are evident within 12-24 h from the transient ischemia and require the simultaneous activation of multiple stress-responsive genes associated with the synthesis of several proteins[2,3].

ISCHEMIA-REPERFUSION INJURY OF THE LIVER

Hepatic ischemia/reperfusion (I/R) injury occurs as a consequence of trauma and hemorrhagic shock as well as temporary clamping of the hepato-duodenal ligament during liver resection (Pringle manoeuvre).

I/R is the main factor responsible for primary graft non-function or malfunction following liver transplantation[3,4]. Even moderate reperfusion damage, which does not severely affect the graft, can impair long-term hepatic recovery and enhance patient susceptibility to infections and multiple organ failure[3,4]. The shortage of organs for liver transplantation, forces consideration of cadaveric and steatotic grafts (marginal grafts) which have a higher susceptibility to I/R injury[4]. Living donor liver transplantation (LDTL) is a promising alternative approach aimed at increasing the number of donor livers[5]. A major concern over the application of LDTL in adults is graft size disparity which is responsible for the appearance of the life threatening effects of the "small for size syndrome"[6]. "Small for size syndrome" can occur even when the critical mass for safe LDTL (40% of standard liver volume) is transplanted and this effect is related to the impaired regeneration of the reduced liver mass[7] induced by I/R[7,8].

LIVER ISCHEMIC PRECONDITIONING

Beside the heart, IP effects have been demonstrated in many other tissues[2,3]. Studies performed in rats and mice, showed that interruption of liver blood supply for 5-10 min followed by 10-15 min of reperfusion reduced hepatic injury during a subsequent extended period of ischemia followed by reperfusion[9-13]. These beneficial effects were particularly evident in fatty livers in which preconditioning almost halved transaminase release and histological evidence of necrosis[11]. The application of preconditioning protocols to rodent liver transplantations showed that IP applied before cold preservation, decreased transaminase release and sinusoidal endothelial cell killing in the graft, improving rat survival[12,13].

A further feature of hepatic IP was the capacity to promote hepatocyte regeneration. Hepatocyte proliferation in rats subjected to 70% hepatectomy is significantly reduced by 45 min of hepatic ischemia. Such an effect was entirely reverted by pre-exposure to IP[14]. Consistently, preconditioning procedures significantly enhanced liver regeneration in the experimental model of reduced-size rat liver transplantation[15,16].

MOLECULAR SIGNALS OF HEPATIC ISCHEMIC PRECONDITIONING

Despite a significant number of studies on liver preconditioning, knowledge on the mechanisms responsible for the induction of the "preconditioned" phenotype is still incomplete. Studies from our and other laboratories have indicated that the process of preconditioning implies the production of complex proteomic modifications within liver cells which are now beginning to be characterized.

Adenosine, adenosine triphosphate and nitric oxide as molecular inducers of hepatic preconditioning

"*In vivo*" and "*in vitro*" studies have clearly established that the onset of IP is triggered by the production of adenosine and by the subsequent stimulation of adenosine A2a receptor (A2aR)[9,17-21]. In particular, Peralta *et al*[9,17] showed that adenosine treatment reproduced the protective action of IP and that IP was reverted by adenosine deaminase and by the adenosine A2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine. Pretreatment of rats with the adenosine A2 receptor agonist, CGS21680, but not with the adenosine A1 receptor agonist, *N*-phenylisopropyl adenosine, enhanced tolerance against IR damage[18]. By using primary rat hepatocytes preconditioned with 10 min of hypoxia plus 10 min of re-oxygenation, we confirmed that the extracellular release of adenosine induced hepatocyte protection by autocrine stimulation of the A2aR[20,21] (Figure 1). Indeed, studies in extra-hepatic and hepatic tissues have clearly shown that transient oxygen deprivation triggers the release of several metabolites including adenosine triphosphate (ATP)[2]. In the extracellular space, ATP is rapidly metabolized to adenosine *via* CD39 and CD73 ecto-nucleotidases[22,23] present on the extracellular portion of cell plasma membranes. In particular, ectoapyrase (CD39) converts ATP to adenosine monophosphate (AMP), while ecto-5'-nucleotidase (CD73) further degrades it to adenosine[24]. Thus, CD73 represents the major extracellular pathway for adenosine generation. Consistently targeted gene deletion or pharmacologic inhibition of CD73 was demonstrated to abolish hepatic protection by IP[19].

Recent observations from our group also suggested that ATP itself could act as an additional trigger of liver preconditioning. We observed that the release of ATP from hepatocytes enhanced their tolerance to hypoxia independently from the generation of adenosine[24] (Figure 1). Such an effect was mimicked by treatment with the non-hydrolyzable ATP analogue adenosine-5'-O-(3-thiotriphosphate) (ATPγS) and involved the stimulation of the P2Y2 purinergic receptor[25].

Further evidence indicated that during IP, hepatic endothelial cells responded to adenosine stimulation by generating nitric oxide (NO) which contributed to the modulation of hepatocyte tolerance to I/R[3,17,26-28]. Indeed, the administration of NO donors promoted tolerance to I/R in the absence of adenosine, while NO synthase inhibitors reverted IP[17,26]. Similarly, the treatment of primary

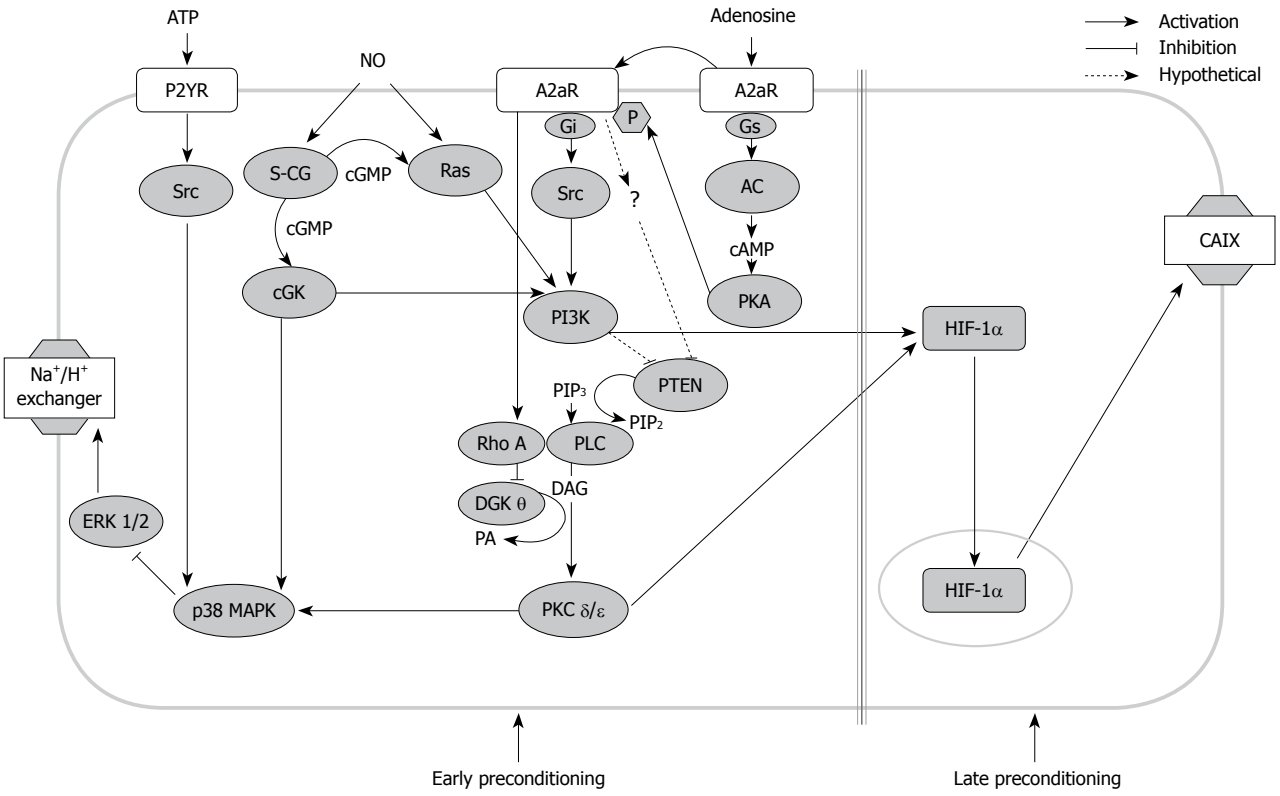

Figure 1 Signalling pathways involved in the development of ischemic preconditioning in rat hepatocytes. Adenosine triphosphate (ATP), adenosine and nitric oxide (NO) act as inductors of hepatocyte preconditioning by modulating a network of constitutive and newly synthesized signal mediators. Some of these mediators play a common central role in hepatocyte cytoprotection. p38 MAP kinase (p38 MAPK) is a mediator of the cytoprotective effects of all three preconditioning stimuli. Phosphatidylinositol 3-kinase (PI3K) mediates both adenosine and NO early resistance to hypoxia. PI3K together with protein kinase C (PKC) δ and ε, also induces hepatocyte late resistance to hypoxia contributing to the normoxic activation of hypoxia-inducible factor 1 (HIF-1). Diacylglycerol kinase theta (DGKθ) and the phosphatase tensin-homologues-deleted from chromosome 10 (PTEN) which metabolize diacyglycerol and phosphatidylinositol, respectively, are inhibited during preconditioning to sustain activation of the diacylglycerol (DAG)-dependent PKC δ and ε and the PI3K-dependent signals. See text and Refs[21,25,27,28,32,35,37,43]. P2YR: Purinergic P2Y receptors; A2aR: Adenosine 2A receptors; S-CG: Soluble guanylate cyclase; cGMP: Cyclic guanosine monophosphate; cGK: cGMP-dependent kinase; AC: Adenylate cyclase; cAMP: Cyclic adenosine monophosphate; PIP3: Phosphatidylinositol-3-phosphate; PKA: Protein kinase A; CAIX: Carbonic anhydrase IX; PLC: Phospholipase C; PA: Phosphatidic acid.

rat hepatocytes with the NO donor (Z)-1-{N-methyl-N-[6-(N-methyl-ammonio-hexyl) amino]} diazen-1-ium-1,2-diolate (NOC-9) reproduced hepatocyte resistance to hypoxic damage induced by IP, ATP or A2aR stimulation[27,28], suggesting that NO could act as an independent mediator of hepatic preconditioning[26].

Signalling pathways involved in adenosine and ATP-induced hepatoprotection

Using preconditioned rat hepatocytes, we observed that A2aR stimulation activated a cascade of intracellular signals involving Gi protein, phospholipase C (PLC), the novel isoforms of protein kinase C (PKC) δ and ε and p38 MAP kinase (p38 MAPK)[21] (Figure 1). The effective contribution of p38 MAPK in liver IP signalling was confirmed *in vivo* in mice where increased p38 MAPK phosphorylation was associated with tolerance against reperfusion injury[29]. Moreover, p38 MAPK inhibitors abolished resistance to I/R injury both "*in vitro*" and "*in vivo*"[21,30].

A2aRs are known to be typically coupled to Gs proteins that through adenylate-cyclase (A-C) stimulate protein kinase A (PKA)[31]. However, in an early study we excluded the involvement of PKA in mediating IP, as PKA pharmacological activation was devoid of protective action[21]. Subsequent research clarified this discrepancy, as we observed that A2aRs were actually coupled with Gs proteins and PKA[32]. PKA, however, by phosphorylating A2aR, shifted A2aR coupling from Gs proteins to Gi proteins and this led to the recruitment of the PLC-PKC pathway[32] (Figure 1). Interestingly, PKA phosphorylated A2aR only in the presence of its ligand (adenosine) and this explained why direct PKA activation in the absence of adenosine lacked protective activity[21,32] (Figure 1). The same research also highlighted the critical role of phosphatidylinositol-3-kinase (PI3K) in hepatic IP[32]. PI3Ks are a family of intracellular signal transducers that generate phosphatidylinositol (3,4,5)-triphosphate (PIP3), a second messenger that plays a central role in the regulation of cell proliferation, survival and metabolism[33]. In preconditioned hepatocytes, PI3K was activated upon A2aR engagement through Gi protein and Src kinase stimulation[32]. PI3K was shown to contribute to IP by promoting the activation of PLC and of PKC δ and ε (Figure 1)[32]. It is well known, however, that downstream of PI3K, protein kinase B (PKB/AKT) is a key modulator of a variety of pro-survival and pro-regenerative signals[33]. Thus, the PI3K-PKB/AKT pathway likely

represents an important pathway in the development of liver IP. Interestingly, PKB/AKT activation in connection with the development of tolerance to I/R was evident in rat hepatocytes and mouse livers[32,34] undergoing IP, as well as in preconditioned human liver grafts immediately after transplantation[35].

At present, the intracellular signals involved in ATP-dependent preconditioning are less well characterized. We reported that ATP-mediated activation of P2Y receptors was coupled with the phosphorylation of Src tyrosine kinase and of p38 MAP kinase that, in turn, inhibited the activation of ERK 1/2 consequent to hypoxic stress[25] (Figure 1).

Constitutive mediators of nitric oxide-induced cytoprotection

The signalling pathways responsible for the cytoprotective action of NO were investigated in rat hepatocytes treated with the NO donor, NOC-9, and then exposed to hypoxia. NOC-9-induced protection involved two parallel pathways. In one pathway, NO stimulated Ras GTPase, and in the other, NO directly activated the soluble guanylate cyclase (sGC) that by producing cyclic guanosine monophosphate (cGMP), stimulated the cGMP-dependent kinase (cGK) that also contributed to Ras GTPase activation[27,28]. Both the Ras and the cGK pathways then converged on the activation of PI3K, while only the sGC-cGK pathway was responsible for activating p38 MAPK (Figure 1)[27,28].

Negative regulators of liver preconditioning

It is increasingly clear that the development of hepatic IP requires the activation of a complex network of signals comprising cell-surface receptors, redox signals and a diverse array of protein kinases including PKCδ and PKCε. In preconditioned hepatocytes, the membrane recruitment and activation of PKCδ and PKCε was fully dependent on their direct interaction with diacylglycerol, generated by adenosine-induced activation of PLC-γ and diacylglycerol analogues which fully mimicked the activation of the signals that induce IP[3,21]. However, it is now clear that the accumulation of cellular diacylglycerol also depends on the rate of its metabolism to phosphatidic acid by diacylglycerol kinases (DGKs)[36]. In this regard, we recently observed that following IP or A2aR activation, the onset of hepatocyte tolerance to hypoxia was associated with a decrease in DGK activity[37]. Moreover, stimulation of A2aR specifically inhibited DGK isoform θ by activating RhoA-GTPase[37]. The pharmacological inhibition of DGKs has consistently led to a diacylglycerol-dependent activation of PKC δ/ε and of p38 MAPK. Moreover, both genetic and pharmacological inhibition of DGK θ induced cell tolerance to hypoxia[37]. Altogether these results unveiled a novel mechanism in the onset of hepatocyte preconditioning and demonstrated that the down-regulation of antagonist enzymes such as DGK was essential to obtain the diacylglycerol accumulation required to trigger PKC-mediated survival signals.

Similarly, preliminary data indicated that in parallel with the activation of PI3K, A2aR stimulation reduced the intracellular levels of the dual protein/lipid phosphatase tensin-homologues-deleted from chromosome 10 (PTEN) that inhibits PI3K-mediated signals by degrading phosphatidylinositol (3,4,5)-triphosphate[33]. We observed that PTEN inhibitors mimicked the induction of preconditioning (Cescon *et al*[35] unpublished results), while PKB/AKT activation and the clinical efficacy of IP in preconditioned human liver were fully explicated only in the presence of significant PTEN down-regulation.

Altogether these results demonstrated the importance of the down-modulation of key inhibitory enzymes for full activation of preconditioning responses. Moreover, these observations indicated the possible use of inhibitors of DGKs or PTEN as pharmacological inducers of hepatic preconditioning.

Nuclear transcription factors in liver preconditioning

As previously mentioned, the late effects of IP require the transcription of different stress-responsive genes and protein synthesis[2,3]. Growing evidence indicates that these responses are achieved by the coordinated activation of several transcription factors.

Nuclear factor-κB: Nuclear factor-κB (NF-κB) is typically devoted to the regulation of genes involved in inflammatory response and cell survival[38]. In experimental models of liver I/R, IP modifies NF-κB activity in different ways[29,39]. In one study, IP decreased NF-κB activity 1 h after reperfusion[39], and in another study, IP activated NF-κB during the ischemic period[29]. These contrasting results could be due to predominant NF-κB modulation in non-parenchymal *vs* parenchymal cells or to a differential regulation of NF-κB in the different phases of liver preconditioning. In addition, the NF-κB decrease during reperfusion was strictly related to a reduction in inflammatory cytokine expression[39], indicating a down-regulation of pro-inflammatory responses in Kupffer/sinusoidal endothelial cells. Conversely, NF-κB activation during ischemia was associated with the hepatoprotective action of IP[29], suggesting that, in hepatocytes, NF-κB-dependent genes contributed to survival responses.

Signal transducer and activator of transcription: The signal transducer and activator of transcription (STAT) transcription factors are a group of proteins implicated in the control of cell proliferation and survival processes[40]. IP induced the activation of the interleukin (IL)-6/STAT3 axis in liver and this pathway was involved in both cytoprotection and hepatic regeneration. On the one hand, as a result of hepatic preconditioning, NF-κB was shown to stimulate the expression of IL-6 and STAT3 that, in turn controlled cyclin beta1 synthesis and cell cycle progression[29]. On the other hand, studies with IL-6 null mice showed that the cytoprotective effects of IP against I/R injury depended on IL-6 signalling and were associated with hepatic STAT3 activation[41].

Hypoxia-inducible factor 1: Hypoxia-inducible factor 1

(HIF-1) is the main regulator of tissue adaptation to oxygen deprivation[42]. Active HIF-1 is a heterodimer consisting of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1α is extremely labile in normoxia, as it is continuously degraded in proteasomes following hydroxylation, catalyzed by the oxygen-dependent HIF-prolyl-4-hydroxylase and arginyl-hydroxylase[42]. The lowering of intracellular oxygen prevents HIF-1α hydroxylation allowing its nuclear translocation and binding to hypoxic response elements of a number of genes regulating erythropoiesis, angiogenesis, glucose transport, glycolysis and cell survival[42]. Using preconditioned hepatocytes, we showed that HIF-1 activation was associated with the induction of a long lasting tolerance to hypoxic injury[43]. Furthermore, Amador and co-workers reported an increase in HIF-1α in concomitance with a lowering of hepatocyte apoptosis in human transplanted livers exposed to IP[44]. HIF-1 activation by IP was not due to the transient hypoxia occurring during the induction of preconditioning, but required A2aR activation[43]. This implicated an oxygen-independent mechanism in the regulation of HIF-1. Indeed, several reports demonstrated that a number of non-hypoxic stimuli (i.e. growth factors, cytokines, hormones and endotoxins) can activate HIF-1 in an oxygen-independent manner[45]. This process implies a PI3K- and PKC-dependent increase in the translation of HIF-1α mRNA, a process that shifts the synthesis/degradation balance towards HIF-1α accumulation[46]. We found that in hepatocytes, adenosine-dependent HIF-1 activation required the stimulation of both PI3K and PKC pathways[43]. This indicated that preconditioning stimuli, acting through the same survival pathways, could contextually lead to the early and late phase of response against cell injury.

Changes in the pattern of protein expression following liver preconditioning

Information concerning the genes modulated in response to liver IP is still limited. In accordance with the role of NO production as a trigger of IP, increased nitric oxide synthase expression was detected in preconditioned rat liver[47]. Microarray analysis of preconditioned human liver confirmed a significant increase in the amount of inducible nitric oxide synthase and also showed an increase in the anti-apoptotic protein, Bcl-2[48]. These analyses also showed that IL-1 receptor antagonist (IL-1Ra) was the most over-expressed gene in human preconditioned livers[48], in accordance with the anti-inflammatory effects of IP. Parallel studies investigating the gene expression pattern in preconditioned rat hepatocytes showed changes in 43 genes including those of the anti-inflammatory IL-10 and the antioxidant enzyme superoxide dismutase 2 (SOD2)[49]. In another study, a marked increase in SOD as well other endogenous antioxidants such as catalase (CAT) and glutathione peroxidase was also observed[50].

As previously mentioned, HIF-1 controls the expression of a variety of genes implicated in erythropoiesis, angiogenesis, glucose transport, glycolysis and cell survival[42]. In this context, we observed that the A2aR-dependent activation of HIF-1 in hepatocytes was associated with the expression of carbonic anhydrase IX (CAIX)[43], a transmembrane enzyme that by catalyzing bicarbonate production was implicated in preventing hepatocyte death (see later).

MOLECULAR MECHANISMS OF CELL RESISTANCE TO INJURY FOLLOWING HEPATIC PRECONDITIONING

The hepatoprotective effects of liver preconditioning impact on a number of different mechanisms. These include several processes acting against ischemia-induced damage as well as against reperfusion injury[2,3,50].

Protection against ischemic damage

A decrease in hepatic energy state is the main cause of liver cell injury during ischemia. Oxygen deprivation causes loss of mitochondrial potential, ATP depletion and intracellular acidification which are turning points in the onset of irreversible liver cell injury[3,50,51]. In early research, we found that activation of the Na^+/H^+ exchanger in response to cellular acidosis combined with the inhibition of Na^+ extrusion by the Na^+/K^+ ATPase, resulted in Na^+ accumulation within hepatocytes[52] (Figure 2). Na^+ overload was a critical step in hepatocyte damage during warm and cold hypoxia, as well as at the beginning of re-oxygenation, and its prevention markedly delayed the appearance of necrotic cell death[52-60]. Indeed, increased Na^+ caused an irreversible influx of Ca^{2+} by activating the Na^+/Ca^{2+} exchanger[56] and deranged cell volume regulatory mechanisms that ultimately led to osmotic hepatocyte lysis[53,58]. In rat hepatocytes, IP or treatment with A2aR agonists, ATP analogues or NO donors all protected against hypoxia-induced Na^+ overload and such protection was causally associated with increased cell survival[20,21,25,27,28,43]. Interestingly, the maintenance of Na^+ homeostasis was achieved both in the early phase of hepatocyte preconditioning[20,21,25,27,28], as well as in the late effects[43] (Figure 2). In the early phase of IP, inhibition of the Na^+/H^+ exchanger and activation of the vacuolar ATPase (V-ATPase) were mainly involved (Figure 2). Indeed, in hepatocytes treated with ATPγS, activation of the P2Y receptors/Src/p38MAPK axis inhibited ERK 1/2-mediated activation of the Na^+/H^+ exchanger responsible for Na^+ influx during hypoxia[25] (Figure 2). Adenosine- and NO-dependent maintenance of Na^+ homeostasis in preconditioned hepatocytes depended on p38 MAPK and PI3K signalling and involved the neutralization of intracellular pH achieved by the activation and translocation on plasma membrane of the V-ATPase (Figure 2). V-ATPase, acting as an alternative pH buffering system, extruded protons thus avoiding the activation of Na^+-dependent transporters[20,27,28]. The mechanism of Na^+ maintenance during the late phase of IP involved the HIF-1-mediated expression of CAIX in hepatocyte plasma membranes. The bicarbonate generated by CAIX was transferred to the cytosol through the Cl^-/HCO_3^- exchanger and neutralized intracellular pH avoiding Na^+ influx[42] (Figure 2). Beside these effects on

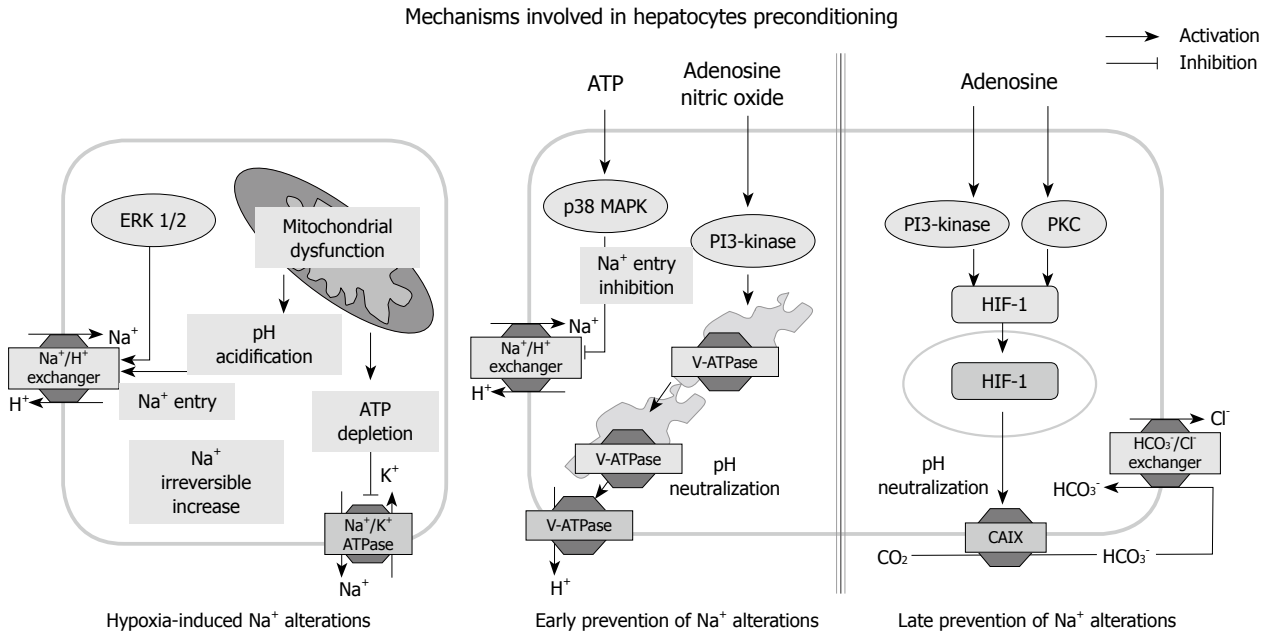

Figure 2 Na^+-dependent mechanisms involved in hepatocyte damage by hypoxia and their modulation by ischemic preconditioning. Hypoxia-induced adenosine triphosphate (ATP) depletion causes intracellular acidification, leading to inhibition of Na^+/K^+ ATPase and the activation of acid buffering systems (Na^+/H^+ exchanger). This leads to an increase in intracellular Na^+ that precipitates irreversible hepatocyte damage. ATP-dependent signalling through purinergic P2Y receptors prevents Na^+ accumulation by inhibiting the ERK 1/2-dependent activation of the Na^+/H^+ exchanger. Adenosine and nitric oxide (NO) activate the vacuolar proton ATPase that maintains intracellular pH avoiding activation of the Na^+/H^+ exchanger. Adenosine also induces the hypoxia-inducible factor 1 (HIF-1) target gene, carbonic anhydrase IX (CAIX), which converts CO_2 into bicarbonate, that once transported into the hepatocytes through the Cl^-/HCO_3^- exchanger, neutralizes the intracellular pH and prevents Na^+ accumulation. See text and Refs[20,21,25,27,28,43,52,53,58]. P38 MAPK: p38 MAP kinase; PI3-kinase: Phosphatidylinositol 3-kinase; PKC: Protein kinase C.

Na^+ homeostasis, during ischemia, IP also down-modulated hepatic energy metabolism by preserving the ATP and glycogen pools and limited lactate accumulation[61].

Protection against reperfusion damage

Mitochondria are a major target of the damaging effects of reperfusion[51]. Oxygen re-admission promotes free radical formation by uncoupled mitochondria with consequent mitochondrial oxidative damage and swelling[62]. IP protected mitochondria from oxidative reperfusion damage[63] and preserved mitochondrial redox-state[64], thus attenuating the impairment of ATP synthesis occurring at reperfusion. IP also improved hepatic intracellular oxygenation[65], preserved sinusoidal wall integrity and avoided liver microcirculatory failure induced by I/R[64]. Together these actions preserved aerobic ATP synthesis maintaining the hepatic energy status during re-oxygenation.

During reperfusion, preconditioned livers also showed a significant reduction in oxidative damage[66,67]. This effect could be ascribed to the increased content of antioxidant enzymes such as SOD, CAT and GSPx[49,50], as well as the reduced generation of reactive oxygen species by mitochondria and inflammatory cells. In the latter context, several studies have outlined the capacity of liver preconditioning to reduce inflammatory responses associated with reperfusion. IP decreased leukocyte adhesion to sinusoidal endothelial cells, lowering post-ischemic neutrophil infiltration[68,69]. IP also attenuated the production of pro-inflammatory cytokines/chemokines during reperfusion[10,68,69]. Finally, pharmacological stimulation of A2aR inhibited the activation of hepatic natural killer T lymphocytes, a process that was causally associated with the protective action of IP against hepatic reperfusion damage[70].

An important consequence of IP was the prevention of hepatocyte and sinusoidal endothelial cell apoptosis[3]. Such an effect can be ascribed to the amelioration of oxidative damage, to the reduced production of pro-apoptotic cytokines as well as to a direct interference with apoptotic mechanisms. Indeed, the increase in PKB/Akt observed in preconditioned hepatocytes[32] represents an important anti-apoptotic signal, since PKB/Akt blocks apoptosis by interfering with Bad, caspase-9 and cFLIP functions[71]. NF-κB might also be implicated in the regulation of hepatocyte response to pro-apoptotic stimuli and the increase in NF-κB nuclear binding observed as early as 30 min after liver IP[29] should be considered in this context. It cannot be excluded that NO-mediated signals might also contribute to the anti-apoptotic action of preconditioning by preventing loss of mitochondrial potential, cytochrome c release and caspase activation[72].

In conclusion, the combined effects of liver preconditioning on energy status, ion homeostasis, oxidative stress, pro-apoptotic responses and inflammation could explain the reduction in hepatocyte and sinusoidal endothelial cell death observed in preconditioned livers exposed to I/R[3,51].

Induction of hepatic regeneration

One of the key issues in the possible exploitation of preconditioning on LDTL is related to its effects on hepatocyte proliferation. The mechanisms involved in the pro-regenerative effects of liver preconditioning are beginning to be elucidated. Hepatocyte growth factor (HGF) and

transforming growth factor (TGF)-β are two cytokines that have opposite actions on liver regeneration, and promote and inhibit hepatocyte proliferation, respectively[73]. The capacity of IP to enhance liver regeneration after reduced-for-size transplantation was associated with increased HGF levels[15] and a lowering of TGF-β production[16]. These effects were causally related to a reduction in IL-1α and an increase in heat shock protein (HSP) 70 expression, respectively[15,16]. Furthermore, a recent study also associated the capacity of IP to attenuate injury in small-for-size liver grafts with the prevention of free radical production and mitochondrial dysfunction, through an increased expression of HSP90, a molecular chaperone that facilitates the mitochondrial import of Mn-SOD[74].

ISCHEMIC POST-CONDITIONING

The term ischemic post-conditioning refers to the capacity to prevent myocardial I/R injury by the application of brief cycles of ischemia during the reperfusion period after a sustained ischemic episode[75-77]. To date, the effects of post-conditioning in the liver have been reported in two studies. These studies showed that the application of brief ischemia in the early phase of reperfusion after rat liver transplantation, was associated with an amelioration of transaminase release and prevention of hepatocyte apoptosis[78-80]. These observations have new important clinical implications as these mechanisms may also act when hepatic damage has already started. In relation to the mechanisms involved in liver post-conditioning, preliminary results in our laboratory indicated that pharmacological post-conditioning with A2aR agonists induced PI3K activation and prevented post-ischemic damage in hepatocytes[81].

CLINICAL APPLICATIONS OF LIVER PRECONDITIONING

The clinical efficacy of hepatic preconditioning was clearly demonstrated in clinical trials performed in patients undergoing hemi-hepatectomy[4,82,83]. In these patients, IP obtained by 10 min of ischemia and 10 min of reperfusion before 30 min of inflow occlusion, significantly reduced transaminase release and ameliorated sinusoid endothelial cell apoptosis as compared to liver exposed to Pringle's manoeuvre only[82,83]. These effects were particularly evident in patients with mild or moderate steatosis, but were not observed in subjects older that 60 years[82]. Considering the possible impact that preconditioning may have in attenuating the effects of long-term graft exposure to cold and warm ischemia during liver transplantation procedures[2,4], the therapeutic use of IP in this setting should have important outcomes. The application of IP in human liver transplantation from deceased donors has, however, demonstrated conflicting results[44,84-88]. Indeed, some studies have shown the efficacy of IP in ameliorating transaminase release and in reducing primary graft malfunctions, whereas others have not observed significant differences[44,84-87]. In an attempt to gain some insight into the possible reasons for the failure of IP to protect liver grafts against reperfusion injury, we investigated the intracellular signals activated by IP in transplanted livers from heart-beating deceased donors. The data obtained indicated that IP stimulated PI3K-mediated signals in only half of the grafts and such variability correlated with the clinical effectiveness of IP. Our data also suggested that it was the failure of PTEN down-modulation that likely contributed to the lack of PI3K response to IP[35]. These observations indicated the necessity to explore alternative procedures to surgical IP to overcome the variability of human grafts in activating preconditioning responses. In this regard, the pharmacological induction of liver preconditioning likely represents a more reliable technique for stimulating the intrinsic systems of cytoprotection in humans.

PHARMACOLOGICAL INDUCTION OF HUMAN LIVER PRECONDITIONING

The clinical potential of pharmacological liver preconditioning is clearly suggested by animal studies, however, only two trials have so far addressed this aspect. In one study, Lang and co-workers reported that patients receiving volatile NO during orthotopic liver transplantation displayed an accelerated restoration of liver function as compared to the control group[89]. In the other report, Beck-Schimmer and co-workers showed that preconditioning with the halogenated anaesthetic, sevoflurane, in 64 patients undergoing liver surgery significantly ameliorated transaminase release and the incidence of severe post-operative complications[90]. These observations are consistent with increasing data regarding the efficacy of sevoflurane preconditioning in preventing myocardial ischemia/reperfusion injury[90]. Nonetheless, the availability of several liver specific NO donors and of a variety of effective adenosine A2A receptor agonists[91-93] offers the possibility of extending the number of studies aimed at directly evaluating new approaches to pharmacological liver preconditioning in humans.

CONCLUSION

In spite of a large number of studies on liver preconditioning, general knowledge on this phenomenon is far from complete. The available data give some insight into the signalling pathways responsible for both the early and late responses of IP, as well as some of the cellular modifications involved in the hepatoprotective effects of preconditioning. Additional extracellular inductors and constitutive or newly synthesized mediators are, however, likely to be involved. Little is known about the proteomic changes associated with inhibition of the inflammatory responses and the promotion of hepatic regeneration. Further research is thus needed to clarify these aspects. In particular, preclinical studies are necessary to identify a panel of the most suitable targets of liver preconditioning whose modulation by means of pharmacological or genetic therapies will allow effective activation of endogenous hepatoprotective systems in patients.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6068

World J Gastroenterol 2010 December 28; 16(48): 6068-6078
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Heme oxygenase system in hepatic ischemia-reperfusion injury

James A Richards, Stephen J Wigmore, Luke R Devey

James A Richards, Stephen J Wigmore, Luke R Devey, Centre for Inflammation Research, The University of Edinburgh, The Queen's Medical Research Institute, Edinburgh, EH16 4TJ, United Kingdom

Author contributions: Richards JA, Wigmore SJ and Devey LR all generated the ideas and contributed to the writing of this manuscript.

Supported by The Maurice Wohl Fellowship from the Royal College of Surgeons of Edinburgh and a Research Training Fellowship from The Wellcome Trust (to Richards JA); Tenovus Scotland and The Peel Medical Research Trust to support his current work (to Richards JA); A Clinician Scientist Fellowship from the Academy of Medical Sciences and the Health Foundation (to Devey LR)

Correspondence to: James A Richards, MBChB, MRCS, Centre for Inflammation Research, The University of Edinburgh, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ,
United Kingdom. jamesrichards@doctors.net.uk
Telephone: +44-131-2429100 Fax: +44-131-2429199
Received: June 28, 2010 Revised: September 7, 2010
Accepted: September 14, 2010
Published online: December 28, 2010

Abstract

Hepatic ischemia-reperfusion injury (IRI) limits access to transplantation. Heme oxygenase-1 (HO-1) is a powerful antioxidant enzyme which degrades free heme into biliverdin, free iron and carbon monoxide. HO-1 and its metabolites have the ability to modulate a wide variety of inflammatory disorders including hepatic IRI. Mechanisms of this protective effect include reduction of oxygen free radicals, alteration of macrophage and T cell phenotype. Further work is required to understand the physiological importance of the many actions of HO-1 identified experimentally, and to harness the protective effect of HO-1 for therapeutic potential.

Key words: Ischemia-reperfusion injury; Heme oxygenase; Transplantation; Ischemic pre-conditioning

Peer reviewer: Chi-Hin Cho, B.Pharm, PhD, Professor of Pharmacology and Chairman, Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China

Richards JA, Wigmore SJ, Devey LR. Heme oxygenase system in hepatic ischemia-reperfusion injury. *World J Gastroenterol* 2010; 16(48): 6068-6078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6068.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6068

THE CLINICAL IMPACT OF HEPATIC ISCHEMIA-REPERFUSION INJURY

The rate of liver failure is increasing in the UK population[1]. Liver transplantation is an effective treatment for patients with end-stage disease, giving an average of 17-22 years of additional life[2,3]. Access to liver transplantation is limited by donor availability; several innovations, including split liver, living donor transplantation, non-heart beating donation (NHBD) and the expansion of the donor criteria, have been attempted to tackle this disparity[4].

Ischemia-reperfusion injury (IRI) causes a spectrum of early organ dysfunction after transplantation; the most severe form, termed primary non-function, may result in patient death. "Marginal organs", including those from older donors, those affected by steatosis and those from donors with long pre-donation intensive care unit stay, may be judged to pose an excessive risk of IRI and be discarded, placing additional pressure on the already scarce donor resource[5].

Due to shifting patterns of organ donation, there is a tendency towards the increased use of marginal organs. Year on year, the mean donor age is increasing, in part due

to improvements in road safety and declining numbers of traumatic deaths. Furthermore, NHBD (also termed donation after cardiac death) is becoming an increasingly important component of the donor resource[6]. Compared with the "gold standard" of heart beating donation (HBD), NHBD is associated with a decreased quantity of donated organs (2.1 organs per donor compared with 3.4 organs per HBD). Albeit in small studies, NHBD liver transplantation is also associated with a higher risk of IRI leading to elevated incidence of primary non-function[7].

Our group has previously estimated that negating the effects of IRI in HBD would lead to a 6% increase in the donor supply through recruitment of these marginal organs back into the donor pool[4]. In the current climate of rapidly increasing NHBD and increasing donor age, the imperative to better understand and avert hepatic ischemia is becoming ever stronger.

DEFINITION OF IRI

Interruption of blood flow to any tissue results in inadequate tissue oxygenation and an increase in cellular anaerobic pathways: if adequate oxygenation is not restored then disruption of cellular functions and cell death results. On reperfusion, despite restoration of adequate cellular oxygenation, there is further damage caused both by direct cytotoxicity from oxygen free radicals (OFR)[8] and by a secondary immunological assault upon the injured organ involving components of both the innate and adaptive immune system[9]. The sequence of injuries resulting from interruption then reinstatement of blood flow is termed IRI.

HEME OXYGENASE-1

Heme oxygenase (HO) is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent microsomal enzyme, which catalyzes the breakdown of heme to biliverdin, iron and carbon monoxide (CO)[10] (Figure 1). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase while free iron is sequestrated by ferritin. Of the three heme oxygenase isoforms (HO-1, HO-2 and HO-3), only HO-1 (also known as heat shock protein 32) is inducible[11]. HO-1 is a 32 kDa enzyme encoded by the *hmox1* gene. It has been found to be upregulated during states of oxidative and cellular stress and plays an important role in maintaining oxidative/antioxidant homeostasis[12].

Induction of HO-1 and its metabolites is protective in a large number of seemingly unrelated pathologies, including sepsis, malaria, endotoxic shock, IRI, organ transplant rejection, induction of tolerance, myocardial infarction, type 2 diabetes and obesity[13]. This spectrum of protection is attributed to multi-level mechanisms of cytoprotection and inflammatory modulation.

Polymorphism in the (GT)n microsatellite of the *HMOX1* promoter is thought to be responsible for the variations seen in the human response of HO-1 to stimuli[14]. This may account for the differences in the susceptibility of individuals to certain pathologies and in the apparent longevity associated with increased HO-1 expression[15,16].

HO-1 in hepatic IRI

In the context of IRI and transplantation, HO-1 induction or supplementation with its metabolites has been shown repeatedly to be protective in both the liver and other organs.

In early experiments, HO-1 was induced by heat shock in donor livers prior to 44 h of cold ischemia preceding liver isogeneic transplantation in rats, resulting in marked improvements in recipient survival[17]. HO-1 upregulation with adenoviral HO-1 or cobalt protoporphyrin (CoPP) resulted in improved portal venous flow on *ex-vivo* perfusion, while on transplantation into syngeneic hosts, recipient survival doubled, histological injury was ameliorated and influx of macrophages and T cells was reduced[18]; findings confirmed in similar experiments using transplantation[19,20] and hepatic warm ischemia models[21]. More recently, targeted deletion of *Bach-1*, which normally suppresses HO-1 transcription, led to HO-1 upregulation and protection from myocardial ischemia[22], although these experiments have yet to be repeated in models of hepatic ischemia.

In our own laboratory we have shown that HO-1 is upregulated in Kupffer cells during ischemic preconditioning (IPC)[23], and that targeted deletion of *hmox-1* resulted in aberrant Kupffer cell differentiation and susceptibility to ischemia-reperfusion insults[24]. Our work has suggested that Kupffer cells are a likely site of HO-1 action: recent work from Kupiec Weglinski's laboratory has demonstrated that adoptive transfer of HO-1 overexpressing bone marrow-derived macrophages was capable of protecting mice from hepatic ischemic insults[25]. Developing this theme, other experiments have shown HO-1 induction with CoPP to protect mice from hepatic ischemia arising from liver transplantation: Kupffer cells recovered from HO-1-induced animals produced less tumor necrosis factor α (TNFα) and interleukin (IL)-6 under stimulation in *ex vivo* culture[26].

HO-1 in hepatic IPC

IPC is a surgical maneuvre in which an organ is paradoxically protected by a brief period of controlled ischemia and reperfusion immediately prior to a longer index ischemic event, which by itself would normally lead to injury[27,28]. Several small randomized controlled trials have looked at the effectiveness of IPC in both liver transplantation and resection; subsequent Cochrane reviews conclude that further trials are still required to evaluate its role in hepatic and transplant surgery[29,30].

Kanoria *et al*[31] demonstrated in their rodent model that the application of a hindleg tourniquet (remote IPC) led to a protective phenotype from hepatic IRI. Ischemic post-conditioning has also been described, in which injury is abrogated by modified reperfusion. This emphasizes that tissue damage from ischemia-reperfusion continues following the end of the ischemic insult[32].

The mechanisms behind IPC are poorly understood. Numerous candidate molecules, including nitric oxide, adenosine, protein kinase C, tyrosine kinase and mitogen-activated-protein kinase, have been identified as potential mediators of protection[33]. Given that IPC can act re-

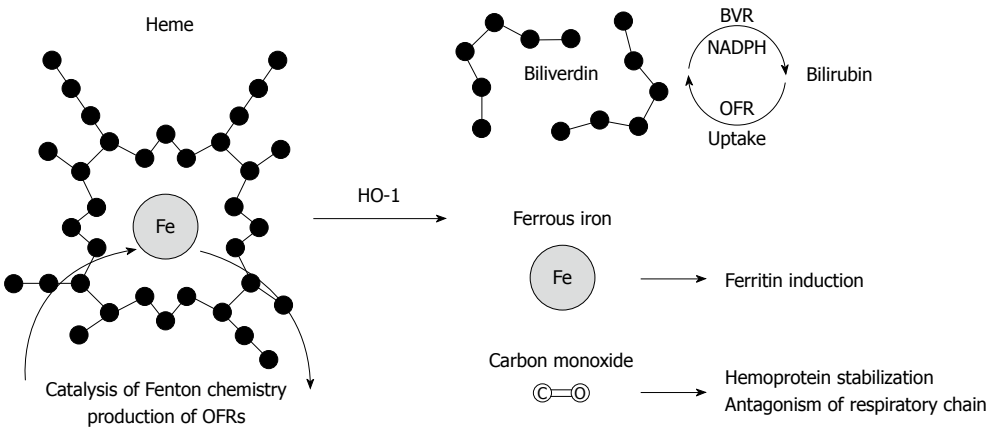

Figure 1 Putative mechanisms of heme oxygenase-1 antioxidant effect. HO-1: Heme oxygenase-1; BVR: Biliverdin; OFR: Oxygen free radicals; NADPH: Nicotinamide adenine dinucleotide phosphate.

motely, it may involve an immunomodulatory mechanism.

HO-1 is upregulated following IPC and may be responsible for the observed protection[23]. HO-1 is also upregulated in sites distant to the site of preconditioning. In a remote preconditioning model, renal ischemic insults led to cardiac HO-1 induction[34]. Likewise, HO-1 induction occurred in the liver following four 10 min episodes of femoral artery occlusion, and protection afforded by remote preconditioning was lost when HO-1 was inhibited with SnPP[35].

Given that HO-1 is upregulated in IPC and is known to be powerfully protective when upregulated, it is likely that HO-1 has a role in IPC together with other up- and downstream molecules.

Mechanisms of HO-1-mediated cytoprotection

As described above, it is now beyond doubt that HO-1 induction is protective in the context of hepatic ischemia and transplantation. HO-1's cytoprotective effects can be credited to a combination of removal of toxic metabolites (heme), and production of protective second messenger molecules in the forms of biliverdin (and subsequently bilirubin), CO, and free iron (which induces ferritin)[36]. HO-1 may also have other mechanisms of protection, independent of its enzyme activity[37].

Catabolysis of free heme

Heme consists of an iron atom contained within a porphyrin ring: heme moieties are usually contained within the "heme pockets" of hemoproteins. Although the most abundant hemoproteins are hemoglobin and myoglobin, heme moieties are contained within many other ubiquitous enzymes, for example iNOS and the mitochondrial electron transfer chain.

Under conditions of oxidative stress, hemoproteins may be oxidized, leading to the release of free heme which causes cellular injury by multiple mechanisms. Firstly, the central iron ion of the heme moiety catalyzes the production of free radicals by Fenton chemistry as follows: Step 1: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$; Step 2: $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$.

Heme-dependent free radical generation can cause direct cytotoxicity, including damage to the cytoskeleton, lipid bilayer, intermediary metabolic enzymes and DNA[38,39], while circulating heme can cause LDL oxidation leading to endothelial cell toxicity[40]. Heme sensitizes cells to pro-death signals including TNFα and Fas, an effect abrogated by treatment with antioxidants, implying dependence upon free radical production[36].

HO-1 reduces free heme concentrations by two mechanisms. Firstly, catabolysis of heme into metabolites biliverdin, iron and CO directly removes free heme. Secondly, binding of CO to hemoglobin, forming carboxyhemoglobin, prevents its oxidation to methemoglobin and ensuing release of free heme moieties[36].

Some authors have argued strongly that neutralization of free heme during cellular injury can account for a large proportion of the protection offered by HO-1. In experimental malaria, it was found that C57/Bl6J mice had reduced HO-1 induction compared with BALB/c animals, and consequently had higher levels of circulating free heme and greater disease severity. Administering exogenous heme to BALB/c animals caused worsening of their disease severity whereas conversely, CO treatment of C57Bl6J mice was protective[41]. In the same model, HO-1 protected animals from hepatic failure induced by overwhelming circulating heme[42].

Hepatic HO-1 expression is focused in Kupffer cells. This cell type is highly adapted to detect hemolysis: the hemoglobin-haptoglobin complex receptor CD163 is able to induce HO-1 in an IL-10-dependent manner[43,44].

Production of biliverdin

Biliverdin is produced by catalysis of the heme porphyrin ring and is almost immediately converted by biliverdin reductase to unconjugated bilirubin. This, in turn, is glucuronidated to render it water soluble. Bilirubin is a powerful antioxidant believed to be responsible for much of the antioxidant activity of serum[45]. At micromolar concentrations it is capable of scavenging large volumes of reactive oxygen species (ROS), protecting cells from high concentrations of peroxide, with enhanced efficacy in

hypoxic conditions, making this effect particularly relevant in the context of ischemia[46]. Bilirubin's extraordinary antioxidant capacity may arise from an active cycle in which biliverdin reductase undertakes NADPH-dependent reduction of biliverdin to bilirubin: in turn bilirubin is oxidized by free radicals back to biliverdin, before enzymatic reduction back to bilirubin. Although controversial[47], this cycling mechanism has been proposed as a highly adapted mechanism through which cells and whole organisms maintain redox status[45].

Bilirubin's effects may extend beyond its antioxidant action. It has been identified as an endogenous ligand of the aryl hydrocarbon receptor (AHR), present on many immune cell populations[48]. In some experiments AHR ligands have suppressed T cell proliferation through expansion of T regulatory (Treg) cells[49], although expansion of either Treg or their polar opposite, Th17 cells, has been shown depending upon which experimental AHR ligand is used[50].

Patients with persistent hyperbilirubinemia (Gilbert's syndrome) have been observed to have significantly lower incidence of atherosclerotic disease in several studies, an effect attributed to enhanced antioxidant potential of serum[51]. The effect of chronic hyperbilirubinemia upon T cell phenotype is unclear, although immunosuppression has been reported in patients with cholestatic jaundice[52].

Work using *ex vivo* liver perfusion models in which explanted livers were stored for 16[53] or 24 h[54] at 4℃ in University of Wisconsin solution, prior to mechanical perfusion, found that addition of low concentrations of bilirubin to the graft perfusate mimicked the protective effect of heme oxygenase induction. Graft function was improved in terms of portal venous flow and bile production, and hepatocellular injury was ameliorated in terms of histological injury scoring and transaminase release. Biliverdin has also been shown to have an immunomodulatory effect. In an MHC-mismatched cardiac allograft model, twice or three times daily injections of biliverdin for 2 wk increased graft survival, and led to complete allograft tolerance in 50% of animals[55].

To test whether bilirubin was protective in the clinical setting, our group hypothesized that hyperbilirubinemia would protect transplant recipients from IRI, in which case there would be an inverse correlation between preoperative bilirubin and post-operative transaminase measurements. In a small retrospective study, no relationship was observed, although given the heterogeneity of the study population an effect could not be ruled out[56].

Production of Fe^{2+}

Paradoxically for an antioxidant enzyme, one of HO-1's reaction products, free iron, is a powerful oxidant. Free iron catalyzes the generation of OFR by Fenton chemistry in a manner comparable to free heme as described above. However, it is more effectively neutralized than when contained within a heme ring, being actively exported from the cell[57] and chelated by iron-binding proteins, including ferritin. Ferritin sequesters Fe^{2+} by oxidation and placement of iron ions within a "core" in which they are unable to catalyze Fenton reactions[58]. HO-1 induction leads to increased ferritin expression[59], through a mechanism dependent upon the production of free iron[60,61].

Since other HO-1 metabolites had been shown to be capable of substituting for HO-1 induction in protecting animals from IRI, Berberat *et al*[62] hypothesized that ferritin overexpression could also confer protection. Using an *ex vivo* perfusion model of hepatic IRI, this group identified that transfection with adenoviral heavy chain ferritin conferred protection in terms of bile flow, portal blood flow, histological injury scoring and transaminase release, and improved survival of syngeneic liver recipients.

Ferritin induction has been observed in retinal[63] and cardiac IPC[64] in a manner dependent upon an iron signal[65]: although there are no published studies of ferritin expression in hepatic preconditioning, it would be reasonable to hypothesize that similar results would be obtained.

Although ferritin is capable of protecting cells and organs from oxidative stress, it is worth noting that its induction is unlikely to be the only mechanism of cellular protection by HO-1. Sheftel *et al*[61] compared heme and sodium arsenite (a non-heme inducer of HO-1) *in vitro*, and found sodium arsenite to confer protection through HO-1 induction without parallel ferritin induction.

Production of CO

CO is best known for its toxicity, causing death at atmospheric concentrations of 500-1000 ppm, however, at lower doses CO has been shown to have important cytoprotective and immunomodulatory functions: it is released by HO-1 during the catabolysis of heme and can substitute for the protective effect of HO-1.

CO cannot have a specific receptor since it binds only to transition metals and is not thought to be capable of direct interaction with amino acids. Therefore, its pharmacology as a signaling molecule is necessarily novel, and subject to ongoing debate. An important hypothesis is that CO effects must be mediated by interactions with proteins which contain transition metal cores, for example in heme rings contained within a range of enzymes including soluble guanylate cyclase (sGC), cytochromes, hemoglobin, myoglobin and nitric oxide synthase[66]. It has been suggested that CO protects from oxidative stress by preventing hemoprotein oxidation and subsequent release of heme rings[36], diminishing production of free radicals and subsequent apoptosis[67]. Others have suggested that the protection may be mediated by antagonism of respiratory chain enzymes, reducing cellular oxygen requirements[66], by inducing vasodilatation *via* sGC[68] or by opening calcium-sensitive ion channels[69].

Using an *ex-vivo* liver perfusion model, Amersi *et al*[70] demonstrated enhanced portal blood flow and bile production, and amelioration of hepatic IRI, in terms of histological injury score and transaminase release when perfusate was supplemented with CO at 300 ppm. Blockade of heme oxygenase using zinc protoporphyrin (ZnPP) did not obliterate the protective effect, indicating that CO

was capable of substituting for HO-1 function. Inhibitor studies have demonstrated that the CO effect was independent of iNOS and cGMP but dependent upon p38 MAPK.

In a cardiac transplantation model, no hearts stored at 4℃ for 24 h prior to implantation into syngeneic recipients functioned after implantation, whereas 5 out of 6 grafts survived when HO-1 was induced with CoPP, an effect lost with HO-1 inhibition. Administration of 400 ppm CO to the heart donor during cold storage resulted in survival equivalent to that achieved with HO-1 induction[71]. In xenotransplant models, HO-1 inhibition with tin protoporphyrin (SnPP) caused rejection, whereas this effect was overcome by recipient CO inhalation[72].

Although the finding that CO can be protective at low doses is scientifically exciting, the potential therapeutic use of inhaled CO can be limited by practical difficulties, both in control of dosage, and spillage of the gas into the environment around the patient. For this reason, Motterlini has developed transition metal carbonyl "CO releasing compounds" (CORMs). Of these (CORM-A1, CORM-2, CORM-3), the most commonly used has been CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)], which releases CO when dissolved in saline but not water[73]. To control for the presence of the ruthenium compound, a CO depleted substance (iCORM3) can be prepared by dissolving CORM-3 in PBS prior to the experiment.

Using a coronary occlusion model in which mice were subjected to 30 min of ischemia followed by 24 h of reperfusion, investigators administered CORM-3 during the first hour of reperfusion, which halved the area of myocardial infarct compared to iCORM control[74]. In a further study, the same group compared the effects of CORM-3 with a late-phase IPC protocol. Animals received either IPC or intravenous CORM-3 infusion lasting 60 min, with appropriate controls. Animals then underwent 30 min coronary ischemic insults 24 to 120 h later. The reperfusion phase lasted for 24 h post-operatively before animals were killed and hearts examined for infarct size. Both IPC and CORM-3 infusion resulted in cardioprotection at between 24 and 72 h compared with appropriate control groups[75].

In the context of transplantation, the potential for using CO to enhance graft function has been explored in various transplantation models. In the kidney, several studies have successfully protected grafts using storage media supplemented with CORM-3/CORM-A1[76] or CORM-2[77] for extended periods of cold ischemia prior to isogenic transplantation. In a vascular allograft model, donor HO-1 induction or CORM treatment reduced subsequent CD8 influx and intimal hyperplasia lasting until animals were sacrificed at 6 wk[78].

In the liver, CO persufflation of University of Wisconsin (UW) storage solution improved subsequent graft function[79], a result replicated using CORM-3 supplemented UW[80,81] after cold ischemic times of 48 h. Use of CO treatment in experimental liver transplant recipients has also been explored. Tomiyama *et al*[82] transplanted livers from wild-type donors into E-GFP transgenic rat recipients who received inhaled CO prior to and for the 24 h post transplantation. Grafts within CO-treated recipients were found to have reduced numbers of infiltrating CD45+ host leucocytes, while purified donor CD68+ Kupffer cells produced less IL-6 and TNFα: primary cultured *ex vivo* Kupffer cells from CO-treated animals secreted less IL-6 and TNFα in response to lipopolysaccharide (LPS) stimulus.

CO clearly has powerful antioxidant and anti-inflammatory effects in a variety of systems. For this reason, despite the difficulties presented by administering it in its inhaled form, phase 2 clinical trials are underway in renal transplantation (www.clinicaltrials.gov identifier NCT00531856), chronic obstructive pulmonary disease (NCT00094406) and post-operative intestinal ileus (NCT01050712). Further development of CORMs should make CO therapies more convenient, thereby widening their potential clinical applications: on the basis of the preclinical data presented above such applications would be expected to include hepatic IRI and transplantation.

Other mechanisms of HO-1-mediated cytoprotection

The bulk of data exploring mechanisms of HO-1-induced cytoprotection supports the concept that it is its antioxidant function which protects cells and animals from injury. Two separate pieces of data suggest that other mechanisms should be considered. Firstly, Ponka's laboratory have demonstrated that the amounts of intracellular heme available for degradation may be insufficient to account for HO-1's powerful antioxidant effects[61]. Secondly, Dennery's laboratory has shown HO-1 translocation to the nucleus after cellular hypoxia or heme treatment, suggesting a role in transcriptional regulation[37]. Developing this work, this group has published research using a reporter system with catabolically inactive HO-1 which suggests HO-1 may have a forward-acting positive effect upon its own transcription[83], although as yet there has been no work demonstrating a protective effect of catabolically inactive HO-1.

HO-1 AND IMMUNOMODULATION IN IRI

It is likely that HO-1 protects organs from IRI by modulation of both direct cellular injury and both the innate and adaptive immune systems[84]. Deficiency of HO-1 in both humans and mice is associated with a pro-inflammatory phenotype[85,86].

According to Matzinger's "Danger Model" of the initiation of immune responses to injured organs, injured parenchymal cells release "Danger-Associated Molecular Patterns" (DAMPs) which stimulate antigen presenting cell (APC) activation through pattern recognition receptors[87,88]. Activated APCs subsequently activate and recruit immune effector cells through the secretion of pro-inflammatory mediators (cytokines, chemokines and adhesion molecules) and antigen presentation[9], resulting in

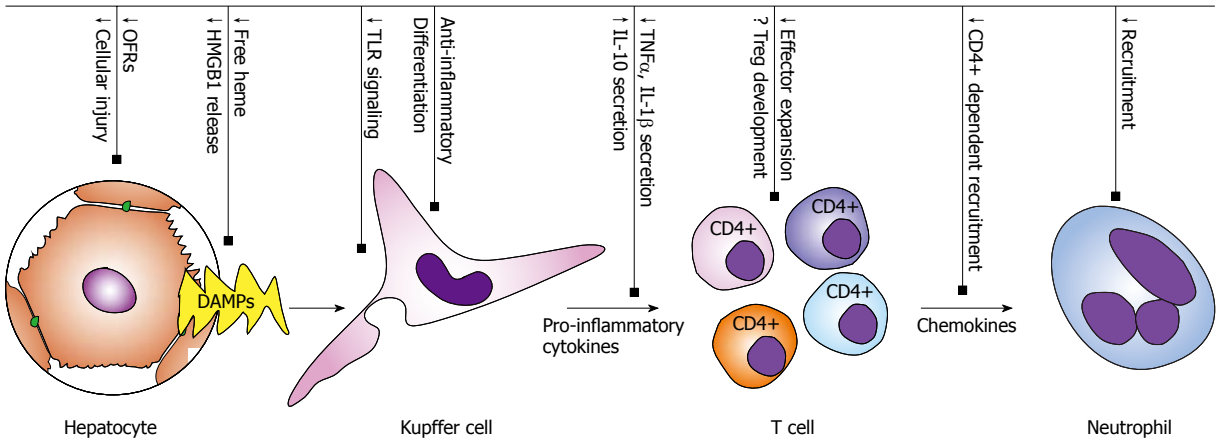

Figure 2 Heme oxygenase-1-mediated suppression of the inflammatory response in hepatic ischemia-reperfusion injury. HMGB1: High Mobility Group Box-1; TLR: Toll-like receptor; DAMPs: Danger-associated molecular patterns; TNFα: Tumor necrosis factor α; IL: Interleukin; OFR: Oxygen free radicals.

the activation and recruitment of T cells and neutrophils (Figure 2). This recruitment of leucocytes into the tissue is further facilitated by increased endothelial permeability and adhesion molecule expression resulting from endothelial activation.

Danger-associated molecular patterns

Hypoxic cellular injury can be regarded as the initiating event in a cascade of immunological activation. "Danger-Associated Molecular Patterns" or "alarmins" spilt by dying cells act on tissue resident immune populations *via* various molecular sensors including the toll-like receptor (TLR) family and purinergic receptors. A prototypic "alarmin", High Mobility Group Box-1 (HMGB1) is released passively from injured cells, a signal amplified by active secretion from surrounding viable immune and non-immune cells[89]. Several authors have published studies demonstrating that circulating HMGB1 levels rise after experimental hepatic ischemia and that anti-HMGB1 neutralizing antibodies are capable of lowering circulating TNFα and IL-6 levels, and ameliorating injury[90-92]. Human clinical studies have confirmed the synthesis of HMGB1 during early reperfusion after liver transplantation, an effect exaggerated in steatotic livers, and with levels correlating with injury severity as measured by peak ALT[93]. Recombinant HMGB1 has been found to worsen the severity of experimental hepatic ischemic injury, an effect neutralized by targeted deletion of its receptor TLR-4[92].

HO-1 is capable of modulating the HMGB1 alarmin system. In sepsis[94] and acute lung injury[95] models, macrophage LPS-mediated HMGB1 release was suppressed by HO-1 induction by CORMs resulting in improved survival. Oxidative stress can initiate macrophage HMGB1 synthesis which occurs in a dose response relationship with peroxide stress *in vitro*[96]. It is therefore unclear whether HO-1's modulation of HMGB1 secretion arises simply by reducing the extent of parenchymal cell injury or whether it is dependant upon another mechanism.

Free heme is another potential DAMP, being released from damaged cells and acting on TLRs[97]. Heme has also been shown to induce neutrophil chemotaxis using *in vitro* transmigration assays and after intraperitoneal injection *in vivo*[98]. By metabolising free heme, HO-1 induction would be expected to reduce this immune stimulus, further contributing to the possible mechanisms of protection from injury.

Intriguingly, HO-1 and CO also modulate TLR signaling upon which many DAMPs converge[99]. CO treatment of cultured RAW264.7 macrophages was found to reduce TLR4 signaling by reducing TLR4/Myd88 interactions and movement of TLR4 receptors to the cell surface[100].

It is likely that much of the immune modulation offered by HO-1 is owed to its antioxidant action which quietens the initial immune stimulus by reducing cellular injury and spillage of DAMPs.

Kupffer cells

Hepatic tissue resident macrophages (Kupffer cells) are the first immune cells to be activated by IRI, being acted upon by DAMPs released from surrounding parenchymal cells as well as being subject themselves to cellular hypoxia. Subsequently, they coordinate an appropriate influx of other immune cells by secretion of chemokines and cytokines. Kupffer cells have the ability to harm surrounding parenchymal cells by secretion of pro-inflammatory cytokines including TNFα, IL-6 and IL-1, which may be directly toxic[101]. On the other hand, Kupffer cells may protect the tissues by secretion of anti-inflammatory cytokines including IL-10. Furthermore, as the principle HO-1-expressing cells of the liver[24] they may secrete diffusible CO which may act in a paracrine manner upon surrounding cells to protect them from oxidative stress.

In our work, we have identified that HO-1 acts as a powerful switch on resting macrophage differentiation. We found HO-1-deficient Kupffer cells *in vivo* and bone marrow-derived monocytes (BMDMs) *in vitro* to differentiate down a Ly6c+ MARCO+ F4/80- pathway associated with macrophage inflammatory protein (MIP)-1 responsive pro-inflammatory monocytes[102]. HO-1 deficiency was associated with marked susceptibility to hepatic IRI measured in terms of ALT release and histological injury

score[24]. Parallel work elsewhere using an *in vitro* migration assay has demonstrated enhanced migration of *Hmox-1*$^{-/-}$ BMDMs towards MIP-1α[95]. In an experimental liver transplant model, inhaled CO conferred protection from injury and reduced secretion of pro-inflammatory cytokines from recovered CD68+ cells cultured *ex vivo*[82], reducing the subsequent influx of CD45+ leucocytes. *In vitro* and *in vivo,* low dose CO downregulates the production of macrophage pro-inflammatory cytokines [TNFα, IL1β and MIP-1β (CCL4)] and increases the expression of the anti-inflammatory cytokine IL10[103].

Data from injury models showing modulation of pro-inflammatory cytokine secretion by HO-1 induction is subject to the criticism that the changes observed are merely the downstream effects of parenchymal cellular protection, and suppression of DAMPs. However, data from our laboratory has shown HO-1 effects upon the resting state of macrophage differentiation implying that HO-1 may modulate immune responses themselves.

Adaptive immune system

There is growing evidence from a variety of animal IRI models (T cell-deficient nude rats, severe combined immunodeficiency mice, RAG 1$^{-/-}$ mice, CD4+/CD8+$^{-/-}$ mice and CD4+ depleted mice) that there is a significant decrease in biochemical and histopathological evidence of injury in the absence of T cells (reviewed by Linfert *et al*[9]). More specifically, Khandoga *et al*[104] demonstrated it was a CD4+ rather than CD8+ T cell-dependent phenomenon; depletion of CD4+ T cells leads to a significant reduction in the observed injury. This influx of CD4+ cells on reperfusion is rapid and may determine the mode of neutrophil activation and subsequently the extent of the observed tissue damage[105,106]. In a series of elegant adoptive transfer experiments with CD4+ cells in a renal model, Burne *et al*[107] found IRI to be an interferon-γ-dependent process; this may imply the importance of the T helper 1 lineage in the pathology of IRI.

HO-1 has a number of immunomodulatory effects on effector and Treg cells. HO-1 and CO appear to inhibit T cell proliferation and activation through the suppression of IL-2 secretion[108]. HO-1 induction has also been shown to induce the apoptosis of activated T helper cells through activation-induced cell death[109].

Treg cells suppress the activation of the immune system and inhibit the activation of autoreactive T cells[110]. In clinical studies, Treg/Th17 "imbalance" has been associated with acute coronary syndromes[111]. HO-1 is induced by FoxP3: inhibition of HO-1 function *in vivo* reduced the suppressive ability of naturally occurring Tregs[112]. Furthermore, studies from Fritz Bach's group have shown tolerogenesis to require HO-1[109]. Recently, however, these studies have been challenged by showing that *Hmox-1*$^{-/-}$ mice have normal numbers of Treg cells[113] and that HO-1 is not necessary or sufficient for T reg function[114]. These controversies may be resolved by work from the Agarwal group which showed that although *Hmox-1*$^{-/-}$ mice had normal (or elevated) numbers of circulating Treg cells, their suppressive function was dependant upon HO-1 expression by antigen-presenting cells[115].

In the context of hepatic ischemia, further work is needed firstly to establish which T cell populations are responsible for IRI and IPC, and secondly to resolve the controversy concerning the role of HO-1 in the expansion of different T cell subsets.

Neutrophils

Severe IRI is associated with significant influx of neutrophils[9]. Their recruitment from the vascular compartment into the liver is mediated by the expression of chemokines, cytokines and adhesion molecules[9]. Accumulation of activated neutrophils directly injures hepatocytes and the vascular epithelium through the release of proteases and the generation of ROS[116]. The initial recruitment of neutrophils appears to be a CD4+-dependent process[105]; continued neutrophil recruitment and activation may be dependent on the neutrophil production of IL-17A, which is important in a positive feedback mechanism[117]. As described above, heme is also capable of initiating neutrophil chemotaxis[98].

Although neutrophil influx is enhanced in *Hmox-1*$^{-/-}$ animals after ischemia-reperfusion insults[24], it is unclear whether this is due to a direct effect upon neutrophils or merely as a result of reduced stimulation due to upstream effects.

CONCLUSION

A wealth of data has now proven that HO-1, and its metabolites iron, bilirubin and CO, protect the liver from IRI. HO-1 appears to ameliorate ischemic injury through synergistic actions at many levels in the danger pathway. Powerful effects have been identified upon neutralization of OFR, and apparently at every step of the ensuing danger pathway. What remains is to understand the true physiological importance of each of these effects, and to attempt to separate apparent immunomodulation from suppression of upstream danger signals. Further understanding of the detailed pathophysiology of IRI and the mechanisms underlying IPC would also be invaluable in identifying the full range of potential therapeutic targets.

The protective effect of HO-1 upregulation in IRI was first discovered over a decade ago, and is proven beyond doubt in preclinical studies. Work to date has highlighted multiple potential therapeutic strategies ranging from CORM infusions to cell therapies with HO-1-transduced macrophages. In light of the severity of the many clinical problems caused by IRI including the liver donor shortage, there is now some urgency to translate this body of scientific knowledge into viable treatments which will benefit patients.

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S- Editor Shi ZF **L- Editor** Logan S **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6079

World J Gastroenterol 2010 December 28; 16(48): 6079-6086
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Role of nitric oxide in hepatic ischemia-reperfusion injury

Arunotai Siriussawakul, Ahmed Zaky, John D Lang

Arunotai Siriussawakul, Ahmed Zaky, John D Lang, Department of Anesthesiology and Pain Medicine and the VA Puget Sound Healthcare System, the University of Washington School of Medicine, Seattle, WA 98108, United States
Author contributions: Siriussawakul A, Zaky A and Lang JD all significantly contributed to the content of this paper.
Correspondence to: John D Lang, MD, Associate Professor, Department of Anesthesiology and Pain Medicine and the VA Puget Sound Healthcare System, the University of Washington School of Medicine, Seattle, WA 98108, United States. jdlang@u.washington.edu
Telephone: +1-206-7642854 Fax: +1-206-7642914
Received: June 28, 2010 Revised: September 7, 2010
Accepted: September 14, 2010
Published online: December 28, 2010

Abstract

Hepatic ischemia-reperfusion injury (IRI) occurs upon restoration of hepatic blood flow after a period of ischemia. Decreased endogenous nitric oxide (NO) production resulting in capillary luminal narrowing is central in the pathogenesis of IRI. Exogenous NO has emerged as a potential therapy for IRI based on its role in decreasing oxidative stress, cytokine release, leukocyte endothelial-adhesion and hepatic apoptosis. This review will highlight the influence of endogenous NO on hepatic IRI, role of inhaled NO in ameliorating IRI, modes of delivery, donor drugs and potential side effects of exogenous NO.

Key words: Nitric oxide; Liver; Ischemia-reperfusion injury; Drug delivery

Peer reviewer: Ana J Coito, Associate Professor of Surgery, Department of Surgery, The Dumont, UCLA Transplant Center, 77-120 CHS, Box 957054, Los Angeles, CA 90095-7054, United States

Siriussawakul A, Zaky A, Lang JD. Role of nitric oxide in hepatic ischemia-reperfusion injury. *World J Gastroenterol* 2010; 16(48): 6079-6086 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6079.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6079

INTRODUCTION

Ischemia-reperfusion injury (IRI) is a series of multifaceted cellular events that takes place on the resumption of oxygen delivery after a period of hypoxia. This injury could be severe enough to lead to a significant morbidity and mortality.

The liver may be involved in IRI in procedures that are associated with sequential vascular impediment and restoration of blood flow; for example hepatic resections and orthotopic liver transplantation. During these procedures, unclamping of the vascular inflow to the liver after a temporary period of cross clamping results in major hepatocellular damage.

Nitric oxide (NO) has various protective effects on cells during IRI. NO has been demonstrated to inhibit oxidative stress, cytokine release, leukocyte endothelial adhesion and apoptosis[1]. On a cellular-signaling level, NO effects are mediated *via* redox-sensitive sites, and include: inhibition of protein kinase C, activation of tyrosine kinase, inactivation of nuclear factor (NF)-κB and activation of G proteins[2]. Previous studies have demonstrated that a reduction of NO during hepatic IRI, generally *via* a reduction in endothelial nitric oxide synthase activity, leads to liver injury[3]. Inhaled NO or NO donor drugs are novel treatments that have been used clinically to attenuate liver IRI[4]. This review will discuss the pathophysiology of liver involvement during IRI, and the clinical use of nitric oxide in ameliorating the impact of liver IRI.

BRIEF REVIEW OF THE PATHOPHYSIOLOGY OF IRI

The complex mechanisms of IRI have been revealed by advanced molecular biology[5] (Figure 1). During the isch-

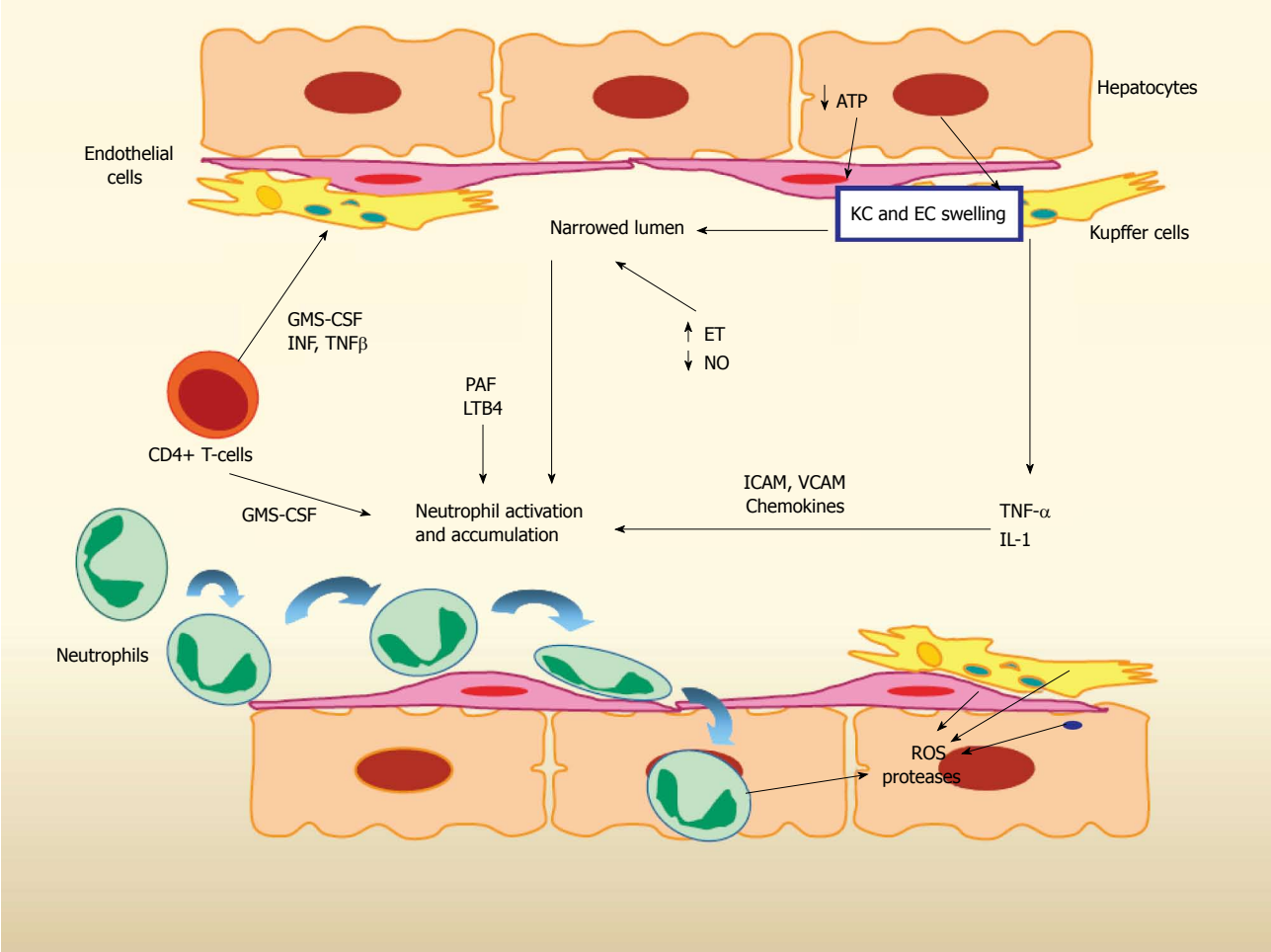

Figure 1 Multifaceted hepatic ischemia-reperfusion injury. Kupffer and endothelial cells produce cytokines and chemokines, recruiting neutrophils that further accentuate injury. EC: Endothelial cell; KC: Kupffer cell; ATP: Adenosine triphosphate; TNF: Tumor necrosis factor; IL: Interleukin; ICAM: Intercellular adhesion molecule; VCAM: Vascular adhesion molecule; PAF: Platelet activation factor; LTB4: Leukotriene B4; GMS-CSF: Granulocyte macrophage colony stimulating factor; INF: Interferon; ROS: Reactive oxygen species (Courtesy of Dr. Joan Rosello-Catafau, Barcelona, Spain).

emic phase, anaerobic metabolism ensues and produces an inadequate amount of high-energy phosphates which are fundamental to most cellular functions. Low levels of high-energy phosphates affect a myriad of cellular functions: homeostasis, signaling interactions, cellular proliferation and processing of the apoptotic death cycle. Adenosine triphosphate (ATP) depletion impairs sodium/potassium ATPase (Na^+/K^+-ATPase) function, resulting in an impairment of the efflux of sodium from the cell. Additionally, toxic metabolites, which are generated during ischemia, attract free water into ischemic cells and organelles leading to the formation of cellular edema[6]. If the ischemic insult lasts greater than 24 h, it is likely that ATP-synthase activity becomes irreversible after blood restoration, leading to cellular necrosis, apoptosis or neuroapoptosis[7]. Ischemia also causes an increased expression of adhesion molecules that leads to endothelial cell and neutrophil adhesion, resulting in vascular studding and occlusion[8]. Furthermore, disequilibrium between NO and endothelin (ET) induces vasoconstriction and subsequent microcirculatory failure even though blood circulation has been re-established[9]. Re-establishment of blood flow will serve to amplify inflammation with consequent injury that is highly variable but dependent on numerous variables including the extent of mediators produced (i.e. reactive oxygen species), the degree of endothelial and neutrophil adhesive responses and the degree of Kupffer cell activation.

PRINCIPAL PARTICIPANTS IN LIVER IRI

Sinusoidal endothelial cells

Injury to these cells is initiated during cold ischemia whereby Ca^{2+}-ATPase results in the accumulation of intracellular calcium[10]. Following this event, a series of actions occur making the endothelium more susceptible to platelet adhesion and reduced sinusoidal flow.

Kupffer cells

Kupffer cells are crucial in liver injury orchestration. Metabolic alterations of these cells occur during no-flow ischemia leading to the formation of reactive oxygen species during early reperfusion[11]. Additionally, at the onset of reperfusion, Kupffer cells undergo further activation by Toll-like receptor 4 signaling and/or by complement. Subsequently, Kupffer cells release pro-inflammatory cytokines such as TNF-α and interleukin-1 which them-

selves can perpetuate inflammatory injury by such means as leukocyte activation.

Hepatocytes

While major participants in the promotion of injury, during cold ischemia hepatocytes undergo intracellular bioenergetic perturbations that reduce ATP stores due to mitochondrial dysfunction and predispose these cells to injury during reperfusion[12].

Leukocytes and lymphocytes

As a result of IRI, cellular adhesion molecules (i.e. intercellular adhesion molecule-1 or ICAM-1, vascular adhesion molecule-1 or VCAM-1), selectins and integrins are activated and upregulated on the surface of endothelial cells, neutrophils and platelets. The activated neutrophils adhere to endothelial cells at the initial stages of reperfusion, and subsequently transmigrate across the endothelium where they continue to injury orchestration. The accumulation of activated neutrophils contributes to microcirculatory disturbances both locally and remotely. Activated neutrophils release reactive oxygen species, specifically superoxide radical (O_2^-•), proteases and various cytokines[13]. Monocytes and macrophages are also activated shortly following reperfusion[14]. Recent studies propose an important role for lymphocytes, especially $CD4^+$ T cells, in augmenting injury responses after IRI. However, lymphocytes may also play a protective role, but this is probably dependent on cell type and time course of injury[15].

Reactive oxygen species and reactive nitrogen species

During periods of ischemia, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated which can promote intracellular damage. Due to electron transport chain alterations, mitochondrial dysfunction ensues leading to reductions in ATP production and with subsequent loss of inner membrane stability resulting in mitochondrial swelling and rupture. With the reintroduction of oxygen during reperfusion, ROS are produced due to reactions of oxygen introduced during reperfusion with xanthine oxidase. ROS serve to stimulate other cell lines including Kupffer cells to produce proinflammatory cytokines[16]. The major ROS are hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2). Reactions of ROS such as O_2^-• with NO yield products such as peroxynitrite ($ONOO^-$), a RNS which can be an extremely aggressive oxidant.

Cytokines

Cytokines play a vital role in IRI, both by inducing and sustaining the inflammatory response, and by modulating IRI severity. Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are the two cytokines most commonly implicated in liver IRI. TNF-α is a pleiotropic cytokine generated by various different cell types in response to inflammatory and immunomodulatory stimuli. TNF-α modulates leukocyte chemotaxis and activation, and induces ROS production in Kupffer cells[17]. Additionally, IL-1 is known to promote production of ROS, induce TNF-α synthesis by Kupffer cells and induce neutrophil recruitment[18].

Complement

The complement system also contributes significantly to IRI and is composed of approximately 30 soluble and membrane-bound proteins. This system can be stimulated in three pathways: (1) the antibody-dependent classical pathway; (2) the alternative pathway; or (3) the mannose-binding lectin pathway[19]. Complement, when activated, acts as a membrane-attacking complex that stimulates the production of proinflammatory cytokines and chemotactic agents. Furthermore, it can regulate adaptive immunity[20].

THE INFLUENCE OF ENDOGENOUS NO ON LIVER IRI

Damage to the liver due to IRI is a culmination of inflammatory cross talk with the principal participants mentioned previously. IRI is the main cause of liver injury in response to vascular clamping during hepatic procedures such as hepatectomy and liver transplantation. This insult on the liver results in disturbances of the sinusoidal microcirculation and the generation of a variety of mediators such as ROS, cytokines, activation of chemokines and other cell signaling molecules previously mentioned.

Hepatic IRI can cause severe hepatocellular injury that contributes to morbidity and mortality after liver surgery. As briefly mentioned previously, reductions of NO during liver IRI occur and are associated with increased liver injury[3]. This is now appreciated to be due to decreases in NO steady state production resulting from low concentrations of endothelium-derived nitric oxide synthase (eNOS). This event coupled with NO inactivation due to reactions with abundant ROS, such as O_2^-•, results in reduced NO bioavailability. The consequences of this reduced bioavailability include, but are not exclusive to, increased oxidative stress, increased apoptosis, increased leukocyte adhesion, increased microcirculatory tone, and perturbed mitochondrial function. Interestingly, restoration of NO to more "physiologic" concentrations serves to diminish the liver ischemic injury *via* countering of the adverse actions mentioned previously. Studies have demonstrated findings that are consistent with the premise that eNOS is crucial for minimizing injury during liver IRI. For example, liver injury was demonstrated to be less in wild type mice compared to eNOS knockouts ($eNOS^{-/-}$)[21] (Figure 2), in addition to the findings that agents given to increase eNOS expression or donate NO afford greater liver IRI protection[22,23]. It is also well established that the NO concentrations during various inflammatory states are significantly increased by increasing expression of inducible nitric oxide synthase (iNOS). However, the influence of iNOS and its true contribution in conferring liver protection (or not) deserves additional studies. In a rat model of liver IRI, iNOS expression was significantly increased correlating with increases in iNOS RNA at 1 and 5 h[24]. This is consistent with other studies measuring iNOS expression in conditions of liver IR. In

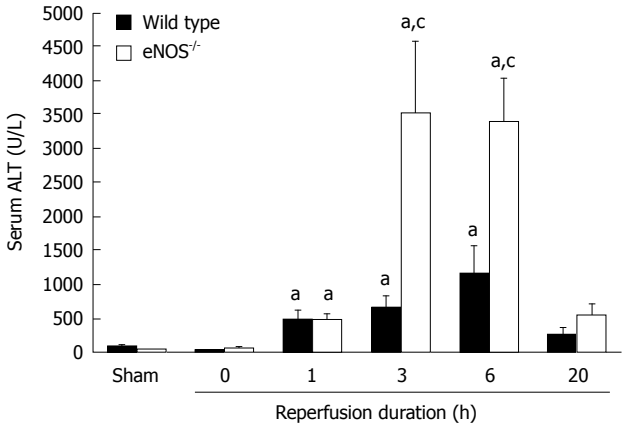

Figure 2 Increased liver injury as assessed by serum alanine aminotransferase in endothelium-derived nitric oxide synthase knockout mice compared with their wild type controls. $^{a}P < 0.05$ *vs* sham-operated controls. $^{c}P < 0.05$ *vs* time-matched wild type control. ALT: Alanine aminotransferase; eNOS: Endothelium-derived nitric oxide synthase (Courtesy of Dr. Ianes N. Hines, Chapel Hill, NC).

a porcine model of IRI, intraportal injection of the selective iNOS inhibitor, aminoguanidine, was demonstrated to decrease injury[25]. In an intriguing study, iNOS knockout mice (*iNOS*$^{-/-}$) exposed to warm liver IRI demonstrated a much greater magnitude of injury compared to wild type mice. Of notable interest was the finding that even though injury was greater in the iNOS knockout mice, little to no iNOS RNA was detectable in the wild type mice. It would appear that for now, the true influence of iNOS on liver injury during IR remains unclear.

A number of other endogenous NO-mediated mechanisms thought to confer protection have been published. For example, NO has been shown to inhibit caspase proteases *via S*-nitrosylation, thereby inhibiting apoptosis[26]. This appears to be somewhat concentration-dependent. Low physiological concentrations of NO may inhibit apoptosis. In contrast, higher concentrations may lead to the formation of toxic products such as $ONOO^{-}$ or other ROS which lead to cell necrosis and apoptosis[27]. Other published mechanisms of NO-mediated protection include inhibition of NF-κB[28], reversible inhibition of mitochondrial complex I, and decreased mitochondrial calcium accumulation[29]. As to be expected, controversy exists concerning "if" and "how" NO exerts cellular protection. For instance, in a study by Jaeschke *et al*[11], administration of a NO synthase inhibitor did not attenuate or accentuate liver injury during the initial reperfusion period. Inhibition of NO was observed not to influence neutrophil migration to the injured sites. While this contradicts a number of other studies, based on their findings, the authors concluded that NO availability was unlikely to be involved in the post-ischemic oxidant stress and reperfusion injury[30]. Nevertheless, the majority of published literature has demonstrated the beneficial effects of NO during liver IRI. These conflicting results might be explained by the fact that the mechanism of NO-mediated protection varies depending on cell type, quantities supplied, laboratory methods applied, timing and duration of NO exposure.

While iNOS was shown to be protective against hepatic IRI in some studies, it was shown to be deleterious in others. In a rat model of hepatic IRI, Takamatsu *et al*[31] observed increased hepatic expression of iNOS mRNA, ALT, and plasma iNOS at 3, 12, and 24 h after hepatic reperfusion. Concomitantly, there was evidence of histologic damage and nitrotyrosine formation in the liver sampled post-reperfusion. These changes were absent in the control group given the selective iNOS inhibitor, ONO-1714. The authors concluded that peroxynitrite may be involved in iNOS-mediated hepatic injury following IR[31].

In another model of hepatic IR in rats, Wang *et al*[32] observed an increase in iNOS protein and mRNA expression on the first day following hepatic reperfusion. Higher levels of iNOS correlated with evidence of increased hepatic injury in the form of elevated serum levels of ALT and AST. Administration of the non-selective nitric oxide synthase (NOS) inhibitor, L-NAME, significantly increased AST and ALT, while administration of the selective iNOS inhibitor, AE-ITU, significantly decreased AST and ALT levels, respectively[32]. The authors postulated that the deleterious effects of L-NAME were due to inhibition of eNOS, while the protective effects of AE-ITU were due to inhibition of injury-provoking iNOS. In a rat model of hepatic IR and small-for-size living-related liver transplantation, Jiang *et al*[33] observed increased iNOS mRNA and protein expression post-reperfusion from a warm ischemic insult with peak expression at 3 h post-reperfusion. This was accompanied by significant increases in concentrations of AST, ALT, malondialdehyde (MDA) and histologic evidence of damage compared to controls. The authors postulated that iNOS-induced hepatic damage was *via* significant production of ROS[33]. We summarize some key studies investigating endogenous NO and NOS in hepatic IRI in Table 1[3,21,25,31-37].

THE USE OF EXOGENOUS NO ADMINISTRATION IN ATTENUATING HEPATIC IRI

Inhaled nitric oxide

Inhaled NO was approved by the US Food and Drug Administration in December of 1999 for the treatment of persistent hypertension of the newborn. Over the last decade, the primary advantage of inhaled nitric oxide (iNO) was seen to be its ability to selectively decrease pulmonary vascular resistance with minimal effects on systemic blood pressure; however, there is currently much interest in exploring its other benefits, including its antioxidant properties and its cytoprotective abilities[4]. In many animal studies, iNO decreased infarct size and left ventricular dysfunction after IRI, increased coronary artery patency after thrombosis, increased blood flow in brain, kidney and peripheral vasculature, decreased leukocyte adhesion in bowel during ischemia-reperfusion, and decreased platelet aggregation[38]. Date *et al*[39] reported the use of iNO in 15 out of 32 patients who suffered from immediate severe allograft dysfunction, with iNO administered at 20 to 60 ppm. The mortality was significantly lower in the

Table 1 Effect of endogenous nitric oxide and nitric oxide synthase on liver ischemia-reperfusion injury

Species	Experimental methods	Ischemic time (min)	NO or NOS effects	Ref.
Pigs	Aminoguanidine, 5 min before ischemia	120	NO derived from iNOS, antioxidant	[25]
Dogs	FK 409, 30 min before ischemia and15 min before and 45 min after reperfusion	60	NO, improves hepatic microcirculation	[34]
Rats	L-arginine, 7 d before IRI	60	NO, antioxidant	[35]
Rats	L-NAME 60 min before ischemia	30	NO, antioxidant	[3]
Mouse	Gadolinium chloride 24 h before ischemia L-NAME methyl ester 15 min prior to ischemia	45	NO derived from eNOS, antioxidant, suppresses Kupffer cell function, regulated basal hepatic blood flow, but did not affect blood flow after reperfusion, attenuated neutrophils infiltration	[21]
Rats	L-arginine or Sodium nitroprusside or L-Name prior to ischemia	60	NO, improves peripheral liver blood flow after reperfusion, cytoprotective	[36]
Male rats	Arginine or L-NAME or 8-bromo guanosine 3′ 5′-cyclic monophosphate or rat atrial natriuretic peptide (ANP 1-28) 30 min before ischemia	45	NO, antioxidant, antiproinflammatory cytokines, improves microcirculation by the cGMP pathway, inhibits neutrophil infiltration and platelet aggregation	[37]
Male rats	IRI group: had partial clamping of portal vein and hepatic artery ONO-1714 group: as above plus ONO-1714 just prior to reperfusion and 6 h thereafter Control group: sham operation	90	iNOS expression peaked at 3 h and diminished at 24 h post reperfusion in IRI and ONO-1714 groups ONO-1714 significantly inhibited plasma nitrates at 24 h post reperfusion ONO-1714 significantly inhibited plasma ALT at 12 h post reperfusion, together with inhibiting histological damage and peroxynitrate expression in liver	[31]
Male rats	Microvessel clamping of portal vein and left hepatic artery L-NAME and AE-ITU given to each of 6 rats exposed to microvessel clamping (time unknown)	60	L-NAME worsened, elevated levels of ALT/AST in IRI groups AE-ITU mildly and significantly decreased levels of AST	[32]
Male rats	Portal vein, hepatic artery and bile ducts clamped by microvessel clamp followed by reperfusion	60	Significant elevation of AST/ALT, MDA/SOD in IRI and small-for-size liver transplantation groups	[33]

NO: Nitric oxide; NOS: Nitric oxide synthase; iNOS: Inducible nitric oxide synthase; eNOS: Endothelium-derived nitric oxide synthase; cGMP: Cyclic guanosine monophosphate; L-NAME: L-nitroarginine; ANP: Atrial natriuretic peptide; IRI: Ischemia-reperfusion injury; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AE-ITU: Aminoethyl-isothiourea; MDA: Malondialdehyde; SOD: Superoxide dismutase.

iNO group (7% and 24%, respectively). The gross benefits reported were that iNO improved oxygenation, decreased pulmonary artery pressure, shortened the period of postoperative mechanical ventilation, and reduced airway complications and mortality[39]. Likewise, a recent retrospective study also presented a picture of improvement of overall respiratory functions. The authors encouraged the administration of iNO for the prevention and treatment of early graft failure in lung transplant recipients[40]. Varadarajan *et al*[41] were the first group to study the relationship between NO metabolism and IRI in human liver transplantation[41]. From their study, they concluded that reduced bioavailability of eNOS contributed to IRI one hour after portal reperfusion. On the other hand, iNOS did not contribute to early IRI after human liver transplantation. Clinical and mechanistic reports on therapeutic use of iNO demonstrated action well beyond vascular relaxation, subsequently inactivated by oxy- or deoxyhemoglobin in the red blood cells. iNO has various positive effects on extrapulmonary systems. However, how iNO mediates these extrapulmonary effects remains unclear. Evidence supporting stable forms of iNO is probably strongest for *S*-nitrosothiols (SNOs) and nitrite[38]. In a prospective, blinded, placebo-controlled study, 80 ppm of iNO was administered to patients undergoing orthotropic liver transplantation[42]. Many advantages were reported in the iNO group, including reduced platelet transfusion, an improvement in the rate at which liver function was restored post-transplantation, and a decrease in the length of hospital stay. Most interesting was the finding of an approximate 75% reduction of hepatocellular apoptosis in patients treated with iNO[42] (Figure 3). Possible biochemical intermediates of iNO include plasma and red blood cell nitrate, nitrite, SNOs, C- or N-nitrosamines and red blood cell ferrous nitrosylhemoglobin. In this study, a detailed analysis indicated that the most likely candidate transducer of iNO in liver IRI was nitrite.

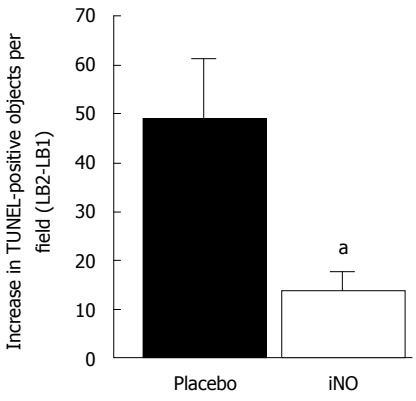

Figure 3 Decreased apoptosis indicated by TUNEL staining in patients treated with inducible nitric oxide compared to controls (Courtesy of John D. Lang, MD, Seattle, WA). $^{a}P < 0.05$. iNO: Inducible nitric oxide.

iNO delivery systems

An iNO delivery system should allow for constant and accurate measurements of NO and nitrogen dioxide (NO_2)

Table 2 Nitric oxide donors

Model	Drugs	Outcomes	Ref.
Canine liver IRI	FK-409	Promoted hepatic tissue blood flow, decreased serum endothelin-1, cytoprotection	[34]
Isolated hepatocytes	S-nitroso-N-acetylpenicillamine	Drug induced the expression of heat shock protein 70 mRNA and protein resulting in cytoprotection from TNFα	[2]
Murine liver IRI	Sodium nitroprusside	Promotes hepatic tissue blood flow after reperfusion-cytoprotection	[36]
Murine liver IRI	PEG-poly SNO-BSA, a sustained release of NO	Decreased neutrophil accumulation, prevented the excessive production of iNOS	[54]
Murine liver IRI	Macromolecule S-nitrosothiols	Prevented hepatocellular injury	[55]

NO: Nitric oxide; SNO: S-nitrosothiol; iNOS: Inducible nitric oxide synthase; IRI: Ischemia-reperfusion injury; TNF: Tumor necrosis factor.

concentration in inspired gas, as well as minimization of the contact time between oxygen and NO, in order to decrease the feasibility of producing high NO_2 concentrations. The measurement of iNO and NO_2 concentrations can be undertaken using chemiluminescence or electrochemical devices. There are some drawbacks of chemiluminescence devices such as cost, the need for a relatively high sample volume, noise and maintenance difficulties[43]. However, an electrochemical analyzer is relatively insensitive, and these measurements may be affected by pressure, humidity, temperature and the presence of other gases in the environment[44]. The delivery system should display the pressure of iNO in the cylinder and should have a backup power supply to avoid sudden discontinuation of iNO. Inhaled NO is usually supplied in nitrogen at various concentrations. The gas mixture concentration should be sampled downstream of the input port just proximal to the patient manifold. iNO also can be administered *via* nasal cannula, oxygen mask and oxygen hood[45]. Finally, the exhausted gas should be scavenged by passing it through carbon and filters, soda lime or activated charcoal[46].

POTENTIAL TOXICITIES DURING INHALATION

In the presence of high concentrations of O_2, NO oxidizes to nitrogen dioxide (NO_2). NO_2 reacts with the alveolar lining fluid to form nitric acid. NO dissolved in the alveolar lining fluid reacts with O_2^- yielding $OONO^-$, then decomposes into a hydroxyl anion[47]. Nitration of tyrosine residues of proteins is used as a marker of oxidative stress[48]. The rate at which NO is oxidized to NO_2 depends on the square of NO concentration and fractional concentration of oxygen to which it is exposed. The Occupational and Health Administration recommend 5 ppm exposure to NO per 8 h per 24-h-interval as the upper safe limit of human exposure[49]. In order to protect against NO_2 toxicity, iNO should be given with the least possible O_2 concentration. Inhaled NO and NO_2 concentrations should be monitored, exhaled gases should be scavenged, and a soda lime canister should be placed in the inspiratory limb of the breathing circuit.

Nitrite

The simple molecule nitrite had been thought to be just an index of NO production for decades[3]. Recently, a number of lines of evidence suggest that nitrite is a pro-mediator of NO homeostasis[50]. Administration of nitrite at near physiological concentrations (< 5 μg) leads to vasodilatation in animal and human studies[46]. Shiva *et al*[51] observed that nitrite was metabolized across the peripheral circulation. In addition, nitrite caused an increase in peripheral forearm blood flow when 80 ppm iNO was administered[51]. Under distinct conditions such as hypoxia and acidosis, nitrite can be reduced to NO by a number of deoxyhemeproteins (hemoglobin, myoglobin, neuroglobin and cytoglobin), enzymes (cytochrome P_{450} and xanthine oxidoreductase), and components of the mitochondrial electron transport chain[4]. Since nitrite can be converted back to NO during hypoxia, nitrite therefore is expected to be utilized during IRI. Furthermore, nitrite shows more potential benefits than NO in terms of safety and ease of administration. In other words, nitrite concentrations administered need only to be a small dose in order to increase plasma and tissue nitrite levels several folds. Routes of administration are oral, intravenous injection or infusion, intraperitoneal, *via* nebulizer or topical[52]. Nitrite has now been demonstrated to have cytoprotective effects in animal models of ischemia-reperfusion in organs. Duranski *et al*[52] evaluated the effects of nitrite therapy in *in vivo* murine models of hepatic and myocardial IRI, and showed that nitrite was associated with cytoprotective effects. In that setting, nitrite reduced cardiac infarct size by 67% and limited elevations of liver enzymes in a dose-dependent manner. These workers also demonstrated that nitrite was reduced to NO regardless of eNOS and heme oxygenase-1 enzyme activities[52]. The exact mechanisms as to how nitrite protects against this particular condition are being explored, but it appears that the benefit is mediated through the modulation of mitochondrial function by involving the posttranslational *S*-nitrosation of complex I to attenuate reperfusion oxygen radical generation and prevent cytochrome-C release[51].

NO donor drugs

Since nitric oxide is not considered to be an ideal gas for the treatment of IRI, NO donor drugs are now being explored as an alternative to the parent compound. Novel drugs have been developed and used for the delivery of NO in order to compensate for the very short half-

life of NO *in vivo*. However, there are only two types of NO donor drugs that are currently used clinically: organic nitrates and sodium nitroprusside. Organic nitrates are the most commonly used NO donor drug treatment for coronary artery disease and congestive heart failure because the drugs produce clear clinical responses through their vasodilatory effects. Preparations of drugs include slow release oral forms, ointments, transdermal patches, nebulizers and traditional intravenous forms. The main limitation of organic nitrates is the induction of drug tolerance with prolonged continuous use. NO release from nitroglycerin is likely *via* the enzyme, mitochondrial aldehyde dehydrogenase[53]. The mechanism of NO release from sodium nitroprusside, on the other hand, is more complex, as demonstrated by Yang *et al*[53] in a murine model of hepatic IRI. Sodium nitroprusside is thought to down-regulate the mRNA expression of several enzymes related to hepatic injury[54]. We summarize other novel NO donor drugs in Table 2[2,34,36,54,55].

CONCLUSION

Ischemia-reperfusion injury is a well-defined threat to the liver during periods of interruption and restoration of oxygen delivery, as occurs in certain procedures such as hepatic resections and orthotopic liver transplantations. Relative NO deficiency seems central in the pathogenesis of this injury. Replacing NO *per se* either by inhalation, nitrate anion or *via* donor drugs represents a novel means for ameliorating IRI. Further randomized controlled trials are needed to evaluate this therapy in patients undergoing operative procedures causing IRI.

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S- Editor Shi ZF **L- Editor** Logan S **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6087

World J Gastroenterol 2010 December 28; 16(48): 6087-6097
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Hepatoprotective actions of melatonin: Possible mediation by melatonin receptors

Alexander M Mathes

Alexander M Mathes, Department of Anesthesiology, Critical Care and Pain Medicine, Saarland University Hospital, Kirrberger Straße, D-66421 Homburg (Saar), Germany
Author contributions: Mathes AM made all the contributions of this paper.
Supported by (in part) Grants from the European Society of Anesthesiology and the HOMFOR Homburger Forschungsförderung
Correspondence to: Alexander M Mathes, MD, Department of Anesthesiology, Critical Care and Pain Medicine, Saarland University Hospital, Kirrberger Straße, D-66421 Homburg (Saar), Germany. alexander.mathes@uks.eu
Telephone: +49-6841-1662271 Fax: +49-6841-1622833
Received: June 28, 2010 Revised: August 8, 2010
Accepted: August 15, 2010
Published online: December 28, 2010

Key words: Antioxidant enzymes; Hemorrhagic shock; Hepatoprotection; Ischemia; Liver; Liver function; Melatonin; Melatonin receptor; Ramelteon; Reperfusion; Sepsis; Toxic liver injury

Peer reviewer: Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

Mathes AM. Hepatoprotective actions of melatonin: Possible mediation by melatonin receptors. *World J Gastroenterol* 2010; 16(48): 6087-6097 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6087.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6087

Abstract

Melatonin, the hormone of darkness and messenger of the photoperiod, is also well known to exhibit strong direct and indirect antioxidant properties. Melatonin has previously been demonstrated to be a powerful organ protective substance in numerous models of injury; these beneficial effects have been attributed to the hormone's intense radical scavenging capacity. The present report reviews the hepatoprotective potential of the pineal hormone in various models of oxidative stress *in vivo*, and summarizes the extensive literature showing that melatonin may be a suitable experimental substance to reduce liver damage after sepsis, hemorrhagic shock, ischemia/reperfusion, and in numerous models of toxic liver injury. Melatonin's influence on hepatic antioxidant enzymes and other potentially relevant pathways, such as nitric oxide signaling, hepatic cytokine and heat shock protein expression, are evaluated. Based on recent literature demonstrating the functional relevance of melatonin receptor activation for hepatic organ protection, this article finally suggests that melatonin receptors could mediate the hepatoprotective actions of melatonin therapy.

INTRODUCTION

It has been suggested that the substance melatonin (5-methoxy-N-acetyltryptamine), discovered by Aaron Lerner in 1958, exists in almost every animal species, and possibly even in all plants[1,2]. Its physiological functions are said to be diverse; while melatonin may be involved in modifications of vasomotor tone[3,4] and thermoregulation[5], it is primarily known as the signal of darkness[6].

In vertebrates, melatonin is synthesized in the pineal gland and secreted during darkness as a hormonal message of the photoperiod[7]. The rhythm of melatonin synthesis is mainly driven by an oscillator which is situated in the hypothalamic suprachiasmatic nucleus (SCN)[8]. This oscillator is usually entrained to a 24-h rhythm by environmental lighting conditions, which are perceived in the retina by rods, cones and intrinsically photosensitive retinal ganglion cells[9].

Based on the photoperiodic information transduced from the retina *via* the SCN to the pineal gland, melatonin is secreted during darkness after *de-novo* synthesis from tryptophan[10]. This nocturnal melatonin signal is proportional to the length of the night, thus encoding not only

circadian, but also seasonal variations in the photoperiod[11]. In so-called photoperiodic animals, like the Siberian hamster, these seasonal variations in melatonin output may have a profound influence on the regulation of reproduction[12,13], prolactin secretion[14], as well as coat color[15]. The nocturnal secretion of melatonin is generally independent of an animal's active period: in both nocturnal and diurnal species, melatonin levels rise during darkness[6].

Melatonin synthesis is not exclusively located in the pineal gland, but has also been described in numerous peripheral organs, such as the retina[16], bone marrow[17], skin[18], Harderian gland[19], platelets[20], lymphocytes[21], testes[22], and in the gastrointestinal tract[23]. Data on messenger RNA expression of two key enzymes responsible for melatonin synthesis, arylalkyamine-N-acetyltransferase and hydoxyindole-O-methyltransferase, suggest that even more peripheral organs may be able to produce this hormone[24].

So far, the physiological significance of extrapineal sites of melatonin synthesis remains unclear. However, besides its relevance in the time-keeping system, melatonin has been demonstrated to be a powerful radical scavenger[25]; it is tempting to assume that extrapineal melatonin may serve as a tissue protective agent.

MELATONIN AS AN ANTIOXIDANT

Processes of acute inflammation, e.g. sepsis, hemorrhagic shock or ischemia/reperfusion, typically result in an imbalance of oxidative homeostasis with excess generation of reactive oxygen species (ROS) and a relative deficiency of endogenous antioxidants; this state is called oxidative stress. ROS include oxidants, such as peroxynitrite, and free radicals, such as hydroxyl radicals and superoxide; these substances are toxic and may induce lipid peroxidation (LPO), as well as protein, sugar and DNA degradation[26].

The powerful antioxidant capacity of melatonin is usually attributed to its potential to eliminate free radicals by the donation of electrons[27,28]. For example, melatonin may neutralize hydroxyl radicals by forming 3-hydroxymelatonin, which is excreted in the urine[29]. Furthermore, melatonin was demonstrated to interact with toxic reactants like peroxyl radicals[30], singlet oxygen species[31], and hydrogen peroxide[32]. Metabolites of melatonin, including the major hepatic metabolite 6-hydroxymelatonin, as well as N-acetyl-N-formyl-5-methoxykynuramine and N-acetyl-5-methoxykynuramine have been shown to detoxify radicals themselves[32-34]. This powerful pyramid scheme of radical scavenging has been named "the antioxidant cascade of melatonin"[1,34].

In addition to these direct interactions with ROS, melatonin may induce upregulation of the activity of antioxidants and antioxidant enzymes, such as superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GSR), in the environment of oxidative stress[35,36]. In addition, the pineal hormone may induce downregulation of pro-oxidant enzymes like nitric oxide synthase (NOS)[37,38] and lipoxygenases[39], thus reducing the formation of nitric oxide (NO), superoxide anions, and subsequently peroxynitrite anions.

Both the direct detoxification of radicals, as well as the modification of pro- and antioxidative enzyme activities are thought to be relevant for the pineal hormone to act as a protective substance, for example when administered in models of oxidative stress. This valuable effect appears to be independent of the type of injury and the species investigated. Exogenous melatonin may exhibit beneficial actions in a myriad of models of organ damage; this is especially true for the liver.

HEPATOPROTECTION BY MELATONIN ADMINISTRATION

With respect to its hepatoprotective effects, countless publications have demonstrated that exogenous melatonin may be used successfully to treat a great variety of different pathophysiological conditions[40-146]: Table 1 gives an overview of the hepatoprotective effects of exogenous melatonin administration, without the pretension of being complete. Included in this summary are investigations mainly presenting a model of liver damage *in vivo*, evaluating parameters of hepatic integrity as a major endpoint, and the administration of melatonin as the primary therapeutic agent. Studies on chronic disease development, aging, investigations on nutritional or dietary changes, exercise-induced stress, remote organ injuries with the liver as a secondary target, as well as investigations on tumor development, cancer progression and liver metastases were excluded.

Based on this extraordinary pool of data, treatment with melatonin appears to be a versatile hepatoprotective strategy in models of experimental liver injury as demonstrated *in vivo* for rats, mice and chicks. There are remarkable variations concerning both the route of melatonin administration, as well as the dose given, the latter ranging a thousand-fold from 100 μg/kg[93,124] to 100 mg/kg[77] melatonin. Only limited data are available on dose-response relationships, and most studies did not include measurements of plasma melatonin levels. Furthermore, it should be mentioned that in some investigations, melatonin was given either as a single dose or repetitively - in some publications for weeks - as a pretreatment, before or while the damage was induced. Unfortunately, not all researchers used melatonin as a therapeutic substance following the infliction of damage, although this would be of high relevance for the evaluation of its clinical use.

Nevertheless, all these studies show similar or even identical results concerning the hepatoprotective effects of treatment with melatonin. Improvements are consistently demonstrated for - but not limited to - parameters of antioxidant enzymes, hepatocellular integrity, interleukin response, NO signaling, and survival.

Antioxidant effects

A strong antioxidant effect of melatonin seems evident as almost all investigators describe that in liver homogenates,

Table 1 Hepatoprotective effects of melatonin in different models of stress

Model	Induction/type	Melatonin treatment	Hepatoprotective effects of melatonin	Species	Ref.
Septic shock	CLP/LPS/LPS + BCG	0.25-60 mg/kg ip/iv/*po* 1-10 ×	hLPO↓, AST/ALT/GGT/ALP/BIL↓, hGSH/hGPx/hSOD/hCAT↑, hNEC↓, hPMN infiltration↓, hTNF-α/hIL-1/hNO↓, 72-h survival rate↑	Rats, mice	[40-49]
Hemorrhagic shock	90 min (MAP 35)/40%	10 mg/kg iv 1 dose	AST/ALT/LDH↓, liver function PDR-ICG↑, hepatic perfusion↑, hNEC↓	Rats	[50-52]
Ischemia/reperfusion	40-60 min ischemia/ischemia + resection	10-20 mg/kg ip/im 1-5 ×	hLPO↓, AST/ALT/LDH↓, hGSH↑, hNEC↓, hMPO↓, hPMN infiltration↓, hTNF-α/hCAS/hAPO/hiNOS↓, 7-d survival rate↑	Rats	[53-62]
Surgical trauma	70% hepatectomy	10 mg/kg per day ip for 7 d	hLPO↓, hGSH↑, histological alterations↓	Rats	[63]
Toxic liver injury	δ-Aminolevulinic acid	10 mg/kg per day ip 7-14 d	hLPO↓, hepatic DNA damage↓	Rats	[64,65]
	Acetaminophen	10-100 mg/kg ip/*po*/sc 1 ×	hLPO↓, AST/ALT↓, hGSH↑, hMPO↓, hNEC↓, 72-h survival rate↑	Mice	[66-68]
	Adriamycin	2-6 mg/kg ip/sc 1-7 ×	hLPO↓, hGSH/hGPx/hCAT↑, hHSP 40/60/70↓	Rats, mice	[69-71]
	Aflatoxins	5-40 mg/kg per day ig/ip for 3-8 wk	hLPO↓, hGSH/hGPx↑, hCAS/hNO↓, hHSP-70↓, hNEC↓	Rats, chicks	[72-76]
	Allyl alcohol	100 mg/kg ip 1 ×	hLPO↓, AST/ALT/LDH↓, hGSH↑, hNEC↓	Rats	[77]
	Arsenic	10 mg/kg ip for 5 d	hLPO↓, hGSH/hSOD/hCAT↑	Rats	[78]
	Cadmium	10-12 mg/kg per day ip/*po* for 3-15 d	hLPO↓, hGSH/hGPx↑, hNEC↓	Rats, mice	[79-82]
	Carbon tetrachloride	10-100 mg/kg ip/sc 1-30 ×	hLPO↓, AST/ALT/ALP/LDH/BIL↓, hGSH/hSOD/hCAT↑, hXO↓, hNO↓, hTNF-α/hIL-1b/hNF-κB↓, hNEC↓	Rats, mice	[77,83-92]
	Cyclophosphamide	100 μg/kg per day *po* for 15 d	hLPO↓, hGSH↑	Mice	[93]
	Cyclosporin A	715 μg/kg per day ip for 14 d	hLPO↓, AST/ALT/GGT↓, hNEC↓	Rats	[94-96]
	Diazepam	5 mg/kg per day sc for 30 d	hLPO↓, hSOD/hGSH↑	Rats	[97]
	Dimethylnitrosamine	50-100 mg/kg per day ip for 14 d	hLPO↓, AST/ALT/ALP/BIL↓, hSOD/hGSH/hGPx/hHO-1↑, hTNF-α/hIL-1b/hIL-6/hNF-κB↓	Rats	[98,99]
	Diquat	20 mg/kg ip 1 ×	ALT↓, hepatic content of F2-isoprostane↓, 24-h survival rate↑	Rats, mice	[100,101]
	Doxorubicin	10 mg/kg sc for 7 d	hLPO↓, GGT/LDH↓	Rats	[102]
	Endosulfan	10 mg/kg ip for 5 d	hLPO↓, AST/ALT/LDH↓, hGSH↑, hMPO↓, hTNF-α/IL-1b↓	Rats	[103]
	Iodine	1 mg/kg per day ip for 14 d	Hepatic content of Schiff's bases↓	Rats	[104]
	Kainic acid	4-10 mg/kg ip 1 ×	Hepatic DNA damage↓	Rats	[105]
	Lead	10-30 mg/kg per day ig for 7-30 d	hLPO↓, hGSH/hGPx/hSOD↑, hNEC↓	Rats	[106,107]
	Methanol	10 mg/kg ip 2 ×	hLPO↓, hGSH/hGPx/hSOD/hCAT↑, hMPO/hNO↓	Rats	[108]
	Methotrexate	10 mg/kg per day ip for 5 d	hLPO↓, hGSH↑, hNEC↓	Rats	[109]
	Mercury-(Ⅱ)	10 mg/kg ip 2 ×	hLPO↓, hGSH↑, hMPO↓	Rats	[110]
	α-Naphthyliso-thiocyanate	10-100 mg/kg ip/*po* 1-4 ×	hLPO↓, AST/ALT/LDH/GGT/ALP/BIL↓, hSOD/hCAT↑, hMPO↓	Rats	[111-114]
	Nodularin	5-15 mg/kg per day ip for 7 d	hGPx/hSOD/hCAT↑	Mice	[115]
	Ochratoxin A	5-20 mg/kg ig/*po* 1-28 ×	hLPO↓, GGT/ALP↓, hGSH/hGPx/hSOD/hCAT↑, hNEC↓	Rats	[116-120]
	Paraquat	1-10 mg/kg ip 5-6 ×	hLPO↓, hGSH↑, LD50 of paraquat↑	Rats	[121,122]
	Phosphine	10 mg/kg ip 1 ×	hLPO↓, hGSH↑	Rats	[123]
	Safrole	0.1-0.2 mg/kg sc 2 ×	Hepatic DNA damage↓	Rats	[124]
	Thioacetamide	3 mg/kg ip 3-5 ×	hLPO↓, AST/ALT/LDH/ammonia↓, hGSH/hCAT↑, hiNOS/hNEC↓	Rats	[125-127]
	Zymosan	5-50 mg/kg ip 1-7 ×	hLPO/hMPO↓	Rats	[128,129]
Cholestasis	Bile-duct ligation	0.5-100 mg/kg per day ip/*po* for 7-13 d	hLPO↓, AST/ALT/GGT/ALP/BIL↓, hGSH/hGPx/hSOD/hCAT↑, hMPO↓, hNO↓, hNEC↓, iron disturbances↓	Rats	[130-140]
Ionizing radiation	Full-body; 0.8-6.0 Gray	5-50 mg/kg ip 1-5 ×	hLPO↓, AST/ALT/GGT↓, hGSH/hSOD/hGPx↑, hMPO/hNO↓, hepatic DNA damage↓	Rats	[141-145]
Malaria	Schistosoma mansoni	10 mg/kg per day ip for 30 d	hLPO↓, AST/ALT↓, hGSH/hSOD↑, 56-d survival rate↑	Mice	[146]

↑: Upregulation/increase/improvement; ↓: Downregulation/decrease/deterioration; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; BCG: Bacillus Calmette-Guérin; BIL: Bilirubin; CLP: Cecal-ligation and puncture; GGT: γ glutamyl transferase; hAPO: Hepatic apoptosis; hCAT: Hepatic catalase; hCAS: Hepatic caspase; hGPx: Hepatic glutathione peroxidase; hGSH: Hepatic glutathione; hHSP: Hepatic heat shock protein; hHO-1: Hepatic heme oxygenase 1; hIL: Hepatic interleukin; hiNOS: Hepatic inducible nitric oxide synthase; hLPO: Hepatic lipid peroxidation; hMPO: Hepatic myeloperoxidase; hNEC: Hepatocellular necrosis; hNF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; hNO: Hepatic nitric oxide; hPMN: Hepatic polymorphonuclear granulocytes; hSOD: Hepatic superoxide dismutase; hTNF-α: Hepatic tumor necrosis factor α; hXO: Hepatic xanthine oxidase; ig: Intragastrically; im: Intramuscularly; ip: Intraperitoneally; iv: Intravenously; LD: Lethal dose; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; MAP: Mean arterial pressure; PDR-ICG: Plasma disappearance rate of indocyanine green; *po*: *Per os*; sc: Subcutaneously.

melatonin strongly attenuated hepatic LPO[40-49,53-99,102,103,106-114,116-123,125-146], usually measured by means of malondialdehyde quantification. Furthermore, melatonin appears to increase the activity and/or expression of hepatic antioxidant enzymes, such as GSH, GPx and SOD, after most types of injury[40-49,53-63,66-93,97-99,103,106-123,125-127,130-146]. Many in-

vestigators also report an increase in hepatic catalase after melatonin treatment[44,71,78,83-85,89,108,111,115,116,118,125,132,135,139].

Hepatocellular integrity

Administration of the pineal hormone appears to reduce the rise in serum enzyme levels of aspartate transaminase, alanine transaminase, lactate dehydrogenase, alkaline phosphatase, γ glutamyl transferase and bilirubin after almost all types of injury, indicating that the extent of cell damage was reduced[40-62,66-68,77,83-92,94-96,98-103,111-114,116-120,125-127,130-146]. This is supported by histopathology results when performed, showing that animals treated with melatonin typically presented with reduced hepatocellular necrosis or attenuated infiltration of polymorphonuclear granulocytes. Reduced hepatic levels of myeloperoxidase further indicate that neutrophil granulocyte infiltration was strongly reduced by the pineal hormone[41,55,67,108,109,111,134,143].

Interleukin response

With respect to interleukin signaling, melatonin was reported to suppress the formation of pro-inflammatory cytokines such as tumor necrosis factor α, interleukin (IL)-1, IL-1β, IL-6, as well as the cellular interleukin response protein, nuclear factor κ-light-chain-enhancer of activated B cells[42,43,53,62,88,99]. This was demonstrated in sepsis and after ischemia/reperfusion, as well as after carbon tetrachloride and dimethylnitrosamine toxicity. Thus, parts of the hepatoprotective actions of the pineal hormone could be based on its suppressive effects on the pro-inflammatory pathway of the immune response.

NO signaling

A large number of studies have investigated the relevance of the NO pathway in the protective effects of melatonin treatment[40,42,43,45,47,49,53,56,57,60,72,73,75,108,125,128,129,142,146]. Melatonin seems to reduce NO release in the vasculature and attenuate the expression of inducible NOS in the liver, as was demonstrated in models of sepsis, ischemia/reperfusion, cholestasis, ionizing radiation, and toxic liver injury with aflatoxins, carbon tetrachloride, methanol, and thioacetamide. As NO reacts with superoxide to form the potentially toxic oxidant peroxynitrite, the reduction in the expression of iNOS may well be another key element in the antioxidant potential of melatonin.

Survival

When investigated, the observed hepatoprotective effects of melatonin were associated with an improvement in survival rate or mean survival time, which was observed in models of sepsis, ischemia/reperfusion, acetaminophen and diquat toxicity, and malaria[41-43,49,53,60,68,101,146].

Taken together, the results from more than 100 experimental studies included here, show convincingly that various regimens of melatonin treatment may be used to reduce hepatic damage in acute liver injury *in vivo*[40-146]. However, this overview is likely to be incomplete: many other studies indicate similar results for chronic disease development and tumor therapy.

So far, only one investigation has been published regarding hepatoprotection by melatonin in humans: in a prospective study, increased survival, attenuated liver damage and reduced immunological activity after transcatheter arterial chemoembolization (TACE) and melatonin treatment were reported in patients with inoperable advanced hepatocellular carcinoma, compared with control patients who underwent TACE but were not given melatonin[147].

Limitations of melatonin

Despite the enormous amount of data supporting the idea of melatonin as a liver protective agent, it should be noted that there are reports which show no hepatoprotective effect of melatonin in a few models of stress. Daniels *et al*[148] were unable to demonstrate any benefit of melatonin administration with respect to carbon tetrachloride-induced liver injury *in vivo*, although ten other studies unanimously showed the value of such a treatment[83-92]. Furthermore, melatonin had no effect on 2-nitropropane-induced LPO in rat liver[149].

Equally interesting and disappointing, melatonin does not appear to be a protective agent with respect to hepatic ethanol toxicity. In a model of acute or chronic ethanol exposure, melatonin administration did not influence hepatic LPO, or GSH and GPx activities in rat[150]. El-Sokkary *et al*[151] demonstrated that administration of ethanol for 30 d did not increase hepatic LPO in the same species. Yet, a recent study showed that melatonin may reduce ethanol-induced liver injury in terms of reduced hepatocellular injury and inflammatory response in a rodent model[152]. As a consequence, further data are required to resolve the issue on whether melatonin may be helpful in reducing ethanol-associated liver damage.

Both positive and negative findings raise the question of how melatonin's intense hepatoprotective potential may be mediated. With respect to this matter, it has been suggested that the activation of membrane-bound melatonin receptors may be an important step in the induction of the antioxidant properties of the pineal hormone[35,36].

HEPATIC MELATONIN RECEPTORS

Melatonin receptors in mammals are classified as membrane-bound, high-affinity G-protein coupled receptors, officially named MT_1 and MT_2 (previous terminology: Mel_{1a} and Mel_{1b}, respectively)[153]. Both receptors are coupled to heterotrimeric G-proteins, and involve signaling through inhibition of cyclic adenosine-monophosphate (cAMP) formation, protein kinase A activity and phosphorylation of cAMP responsive element binding, as well as effects on adenyl cyclases, phospholipase A2 and C, and calcium and potassium channels[154-158]. A third receptor, named MT_3, was demonstrated to be equivalent to intracellular quinone-reductase-2[159]. Non-mammalian species express yet another receptor subtype named Mel_{1c}, which is the first type of melatonin receptor to be discovered[160].

In the liver, the presence of MT_1, MT_2 and MT_3 has been reported in various species[161-171]; Table 2 gives an

Table 2 Melatonin receptors in the liver of various species

Species	MT1	MT2	MT3/QR2	Technique	Ref.
Wistar rat	+	+	NT	RT-PCR	[161,162]
CH3/He mouse	+	+	NT	RT-PCR	[163]
Swiss mouse	+	-	NT	RT-PCR	[164]
Sprague-Dawley rat	-	+	NT	RT-PCR	[165]
Golden rabbitfish	+	+	NT	RT-PCR	[166,167]
European sea bass	-	+	NT	RT-PCR	[168]
Senegalese sole	+	-	NT	RT-PCR	[169]
Syrian hamster	NT	NT	+	Iodine ligand	[170,171]
CD-1 mouse	NT	NT	+	Iodine ligand	[170]
Dog	NT	NT	+	Iodine ligand	[170]
Cynomolgus monkey	NT	NT	+	Iodine ligand	[170]

+: Detected; -: Not detected; MT1: Melatonin receptor type 1; MT2: Melatonin receptor type 2; MT3/QR2: Melatonin receptor type 3/quinone reductase-2; NT: Not tested; RT-PCR: Reverse transcription-polymerase chain reaction.

overview on the current literature demonstrating hepatic melatonin receptor expression or specific iodine ligand binding. So far, there are no original research publications showing proof of hepatic MT_1 or MT_2 receptors in humans. Some evidence points to the possibility that melatonin receptor expression may exhibit circadian variations; this has also been demonstrated for hepatic MT_1 and MT_2[163,166-168].

The physiological significance of hepatic melatonin receptors is mostly unknown. Two studies indicated that hepatic melatonin receptors may be involved in regulating blood glucose[164,172]. Melatonin receptor double knock-out mice do exist, and they appear to have a generally unaltered phenotype. So far, there are no reports showing disadvantages regarding the lack of hepatic melatonin receptors under physiological conditions.

Unfortunately, there are currently no reliable antibodies available for MT_1 and MT_2 receptors[154]. Only a few publications have demonstrated data on the MT_1 or MT_2 protein[162,173]; the results are either non-specific or cannot easily be reproduced. Thus, additional techniques will be required to convincingly demonstrate melatonin receptor protein in the liver.

Nonetheless, our own laboratory was able to generate preliminary results concerning the immunohistochemical distribution of MT_1 in the liver[173]. It appeared that MT_1 was primarily localized in the pericentral area of liver lobules. Due to their metabolic state, pericentral fields of the liver are particularly sensitive to ischemic stress, compared to slightly better oxygenated periportal areas. Thus, this differential distribution of melatonin receptors could provide a way of focusing melatonin receptor-dependent liver protection to areas in need. It is tempting to speculate that this pattern of MT_1 expression might allow the preferential protection of centrolobular hepatocytes.

Further studies, using different techniques or improved antibodies, will be required to support this idea of differentially distributed hepatic melatonin receptors. Thus, the presence and distribution of both melatonin receptor protein subtypes in the liver remain to be determined.

RECEPTOR-MEDIATED ACTIONS OF MELATONIN IN THE LIVER

Only a few studies have analyzed the significance of melatonin receptors in the hepatoprotective effects of melatonin administration *in vivo*[50,51,174]. In a model of hemorrhage and resuscitation, the melatonin receptor antagonist luzindole was able to attenuate the protective effects of melatonin pretreatment and therapy with respect to liver function as measured by plasma disappearance rate of indocyanine green[50,51]. However, not all of the beneficial effects of melatonin were abolished. The use of this antagonist may not clarify all aspects of the effects of melatonin administration, as luzindole itself has been demonstrated to have a strong direct antioxidant potential[175], and to reduce LPO *in vitro*[176].

In the same model of hemorrhagic shock, therapy with the selective melatonin receptor agonist ramelteon improved liver function and hepatic perfusion in rats[174]; this melatonin receptor agonist does not possess any relevant radical scavenging properties[174]. These results point to the possibility that although beneficial, the radical scavenging capacity of melatonin may not be necessary for its protective actions.

This hypothesis is supported by the observation that in other organ systems, the protective potential of melatonin may also be antagonized by luzindole: this antagonist has been reported to abolish the protective capacity of melatonin after myocardial ischemia/reperfusion injury[177], after cyclosporine-A cardiotoxicity[178], in a model of neonatal brain injury[179], and with respect to stress-induced gastric lesions[180].

The following preliminary data from our own research laboratory may have even more impact: in a murine model of sepsis, we were able to demonstrate that the improvements in survival seen after melatonin therapy were not present in melatonin receptor double knock-out mice. This finding indicates once more that membrane-bound melatonin receptors may be responsible for the beneficial effects of melatonin administration.

As a consequence, if (1) no radical scavenging properties are necessary to provide organ protection *via* melatonin receptor activation[174]; (2) the melatonin receptor antagonist luzindole may abolish almost all protective effects of melatonin[177-180]; and (3) the absence of melatonin receptors impedes the protective action of melatonin administration, then it appears reasonable to conclude that melatonin receptors are necessary to mediate at least some of the beneficial effects of the pineal hormone in peripheral organs.

POTENTIAL INFLUENCE ON HEPATIC GENE EXPRESSION

The specific intracellular signal transduction cascade leading to hepatoprotective effects after melatonin receptor activation is presently unknown. However, a number of

hypotheses have been published, suggesting that cAMP responsive element- or estrogen responsive element-containing genes may be regulated by melatonin receptor activation[35,181]. Most certainly, melatonin has a profound influence on hepatocellular gene expression; this has been demonstrated in heat shock protein expression by various investigators[69,73,95]. Our research group was able to present preliminary data showing that melatonin influences different pathways of hepatocellular transcription, including modifications of a variety of heat shock proteins, as well as intense regulation of other membrane-bound receptors and signal transduction factors, in a rat model of hemorrhagic shock[182]. These findings allow the assumption that melatonin therapy may induce beneficial changes with respect to gene transcription in hepatocytes, in the environment of oxidative stress. However, it remains to be determined whether these modifications of hepatic gene expression are indeed mediated by melatonin receptor activation.

FROM BENCH TO BEDSIDE

While the current literature leaves little doubt that melatonin administration may induce hepatoprotective actions[40-146], many questions remain on how this effect may be transduced. The putative signaling cascade, leading from melatonin receptor activation to specific hepatoprotective gene expression profiles, remains to be determined. Based on the evidence available, it appears possible that melatonin receptors mediate the intense protective effects of the pineal hormone in the liver.

To bring this experimental knowledge into clinical use, a pilot study was initiated by Schemmer *et al*[183] in Germany to evaluate the use of melatonin in patients undergoing major liver resections. Should this investigation be successful, this would open the door for yet another important indication for the use of melatonin in human liver surgery: as an adjunct to reduce ischemia/reperfusion injury in liver transplantation. The research group of Freitas and Vairetti has already demonstrated in two studies that melatonin may reduce cold ischemic injury in rat liver[184,185], and suggested that the pineal hormone may be useful in the event of liver transplantation. This idea was supported by Casillas-Ramírez in a review on liver transplantation[186]. Thus, melatonin administration could be beneficial in patients not only to reduce damage to the transplant, but also to serve as a protective agent for the attenuation of reperfusion injury.

Future studies will demonstrate whether melatonin will meet our high expectations not only in the laboratory, but also for our patients. However, the currently available literature allows us to believe that melatonin will successfully continue its way from bench to bedside as a powerful hepatoprotective agent.

ACKNOWLEDGMENTS

The author would like to thank Dr. Larsen R, Professor, Dr. Rensing H, Professor, Dr. Volk T, Professor, Fink T and Wolf B for their encouragement and support.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6098

World J Gastroenterol 2010 December 28; 16(48): 6098-6103
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Current protective strategies in liver surgery

Kurinchi S Gurusamy, Hector D Gonzalez, Brian R Davidson

Kurinchi S Gurusamy, Hector D Gonzalez, Brian R Davidson, HPB and Liver Transplantation, Royal Free Campus, University College London Medical School, Royal Free Hospital, London, NW3 2QG, United Kingdom
Author contributions: Gurusamy KS wrote this manuscript; Gonzalez HD and Davidson BR revised the manuscript.
Correspondence to: Dr. Kurinchi S Gurusamy, MBBS, MRCS, MSc, Clinical Research Fellow, HPB and Liver Transplantation, Royal Free Campus, University College London Medical School, Royal Free Hospital, 9th Floor, Pond Street, London, NW3 2QG, United Kingdom. kurinchi2k@hotmail.com
Telephone: +44-207-8302757 Fax: +44-207-8302688
Received: June 28, 2010 Revised: August 9, 2010
Accepted: August 16, 2010
Published online: December 28, 2010

Abstract

During liver resection surgery for cancer or liver transplantation, the liver is subject to ischaemia (reduction in blood flow) followed by reperfusion (restoration of blood flow), which results in liver injury [ischemia-reperfusion (IR) or IR injury]. Modulation of IR injury can be achieved in various ways. These include hypothermia, ischaemic preconditioning (IPC) (brief cycles of ischaemia followed by reperfusion of the organ before the prolonged period of ischaemia i.e. a conditioning response), ischaemic postconditioning (conditioning after the prolonged period of ischaemia but before the reperfusion), pharmacological agents to decrease IR injury, genetic modulation of IR injury, and machine perfusion (pulsatile perfusion). Hypothermia decreases the metabolic functions and the oxygen consumption of organs. Static cold storage in University of Wisconsin solution reduces IR injury and has prolonged organ storage and improved the function of transplanted grafts. There is currently no evidence for any clinical advantage in the use of alternate solutions for static cold storage. Although experimental data from animal models suggest that IPC, ischaemic postconditioning, various pharmacological agents, gene therapy, and machine perfusion decrease IR injury, none of these interventions can be recommended in clinical practice. This is because of the lack of randomized controlled trials assessing the safety and efficacy of ischaemic postconditioning, gene therapy, and machine perfusion. Randomized controlled trials and systematic reviews of randomized controlled trials assessing the safety and efficacy of IPC and various pharmacological agents have demonstrated biochemical or histological improvements but this has not translated to clinical benefit. Further well designed randomized controlled trials are necessary to assess the various new protective strategies in liver resection.

Key words: Liver; Hepatectomy; Liver transplantation; Ischemia-reperfusion injury; Hypothermia; Ischaemic preconditioning

Peer reviewer: Marco Vivarelli, MD, Assistant Professor, Department of Surgery and Transplantation, University of Bologna, S. Orsola Hospital, Bologna 40123, Italy

Gurusamy KS, Gonzalez HD, Davidson BR. Current protective strategies in liver surgery. *World J Gastroenterol* 2010; 16(48): 6098-6103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6098.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6098

INTRODUCTION

Approximately 11 000 liver transplantations and an estimated 7000 to 10 000 liver resections are performed every year in US[1-3]. During liver resection and transplantation, the liver is subject to ischaemia (reduction in blood flow). A period of ischaemia is unavoidable in organ transplantation between the time the donor heart stops pumping blood through the circulation and the circulation to the organ is restored in the recipient. When the blood flow is restored (reperfusion), the liver is subjected to further injury. The damage caused by ischemia and then reperfusion in an organ is called ischemia-reperfusion injury (IR injury).

MECHANISM OF IR INJURY

The mechanisms involved in the production of the tissue damage by the IR injury are complex. Overviews of the mechanisms involved in liver IR injury have been described by various authors[4-6]. In simple terms, the sequence of ischaemia followed by reperfusion results in the activation of Kupffer cells (liver macrophages) and polymorphonucleocytes resulting in the production of reactive oxygen species (ROS), cytokines, and adhesion molecule activation leading to liver parenchymal damage.

PROTECTIVE STRATEGIES TO DECREASE LIVER IR INJURY

Modulation of IR injury can be achieved in various ways. These include hypothermia[7,8], ischaemic preconditioning (IPC)[9,10], ischaemic postconditioning[11], pharmacological agents to decrease IR injury[12,13], genetic modulation of IR injury[14], and machine perfusion[7,15]. Systematic reviews of well designed randomized controlled trials (with homogeneity) are currently considered the highest level of evidence to assess the effects of interventions[16]. A well designed randomized controlled trial is the next highest level of evidence[16]. The safety and effectiveness of the different interventions based on randomized controlled trials and systematic reviews of randomized controlled trials in humans is discussed under each of the methods.

Hypothermia

Hypothermia decreases the metabolic functions and the oxygen consumption of organs[17]. Although the organ can be preserved by warm perfusion, hypothermia has been used to decrease IR injury in the transplantation setting for several decades.

Invasive cooling of the donor liver: Ischaemic injury to the liver begins when the donor heart stops pumping blood through the circulation. During the liver retrieval operation, current standard practice involves perfusion of the liver through the aorta with or without perfusion through the portal vein using cold solution[18]. There are no randomized controlled trials comparing hypothermic with normothermic perfusion of the donor organ. Currently, there is evidence from one randomized controlled trial that the incidence of primary graft non-function decreases when double perfusion (aortic and portal vein perfusion) is used compared with single perfusion (aorta alone perfusion) in marginal donors (sub-optimal donors)[18]. In the optimal donor, there is currently no evidence of difference in clinical outcomes between single perfusion and double perfusion[18]. Apart from this comparison of the donor perfusion technique, there is currently no evidence for any difference in the graft or patient survival between the different solutions used for donor perfusion or different pressures used for perfusion[19-21].

Surface cooling of donor liver: There is currently no evidence that surface cooling of the donor liver in addition to invasive cooling by aortic and portal vein perfusion improves liver transplant outcomes.

Static cold storage and storage solutions: After removal of the liver from the cadaver, the liver is stored for a few hours till it can be transplanted to the recipient. This is the time required for the transport of the liver from the retrieval site to the transplant site. During this time, preservation injury occurs. This is because of lack of adequate oxygenation of the tissues. The current standard method for preservation is static cold storage. There have been no randomized controlled trials comparing static cold storage with other methods of organ preservation during transport of the liver. However, static cold storage remains the standard against which all other organ preservation methods can be compared. The introduction of University of Wisconsin (UW) solution in 1988[22] increased the capability of long distance procurement and sharing and decreased the costs associated with long distance procurement by decreasing the preservation injury[23,24]. Although the efficacy of UW solution compared with other solutions available at that time (Collin's solution) was not assessed by randomized controlled trial, the evidence for the benefits of UW solution over Collin's solution was so overwhelming[23,24] that a randomized controlled trial would have been considered unethical. To date, UW solution has remained the gold standard solution against which all other solutions are compared[25]. There is no evidence from randomized controlled trials that any of the other solutions such as Celsior solution or histidine-tryptophan-ketoglutarate solution result in a better graft or patient survival than UW solution[26-30].

Hypothermia in liver resections: While hypothermia has been used as the standard method of decreasing IR injury in liver transplantation, the role of hypothermia as a method of decreasing IR injury in liver resection surgery has not been established. The only randomized controlled trial assessing the impact of in-situ hypothermia in liver resections failed to demonstrate any major clinical benefits of in-situ hypothermia[8].

IPC

IPC is the mechanism by which brief periods of ischaemia followed by reperfusion of the organ results in the ability of the organ to withstand a subsequent prolonged period of ischaemia[31]. Overviews of the mechanisms of IPC have been provided by various authors[5,6,32,33]. Adenosine and nitric oxide play a pivotal role in the IPC response.

IPC can be achieved by a local preconditioning stimulus (direct IPC)[9,10] or by a remote stimulus (remote IPC)[6,34,35]. Remote IPC (RIPC) is the mechanism by which IPC of one vascular bed (area supplied by one artery) protects another vascular bed (area supplied by another artery) from IR injury[35]. The mechanisms involved in RIPC have been reviewed previously[6,34]. Currently, both neural and humoral pathways are believed to be involved in RIPC.

There is experimental evidence that direct IPC and RIPC protects against liver IR injury in the animal model[36-38]. In humans, a systematic review of randomized controlled trials showed that direct IPC decreases the enzyme markers of liver parenchymal injury after liver resections performed under vascular control (i.e. temporary occlusion of blood vessels supplying the liver)[9]. However, this did not translate into any clinical benefit[9]. One randomized controlled trial of remote IPC demonstrated a similar finding i.e. a decrease in the enzyme markers of liver parenchymal injury after liver resections without demonstrating any clinical benefit[39]. There is no evidence for benefit from direct IPC in liver transplantation based on a systematic review of randomized controlled trials[10]. Currently, there are no published randomized controlled trials of RIPC in liver transplantation. Thus, routine IPC (direct IPC or remote IPC) cannot be recommended in either liver resection or transplantation.

Ischaemic postconditioning

As opposed to IPC where the conditioning stimulus is applied prior to the prolonged period of ischaemia, ischaemic postconditioning (IPost) involves the application of the conditioning stimulus (brief intermittent cycles of IR) after the prolonged period of ischaemia but prior to permanent reperfusion i.e. ischaemia followed by conditioning stimulus followed by permanent restoration of blood flow[11]. Overviews of the mechanisms of ischaemic postconditioning have been reviewed previously[40,41]. As with IPC, adenosine and nitric oxide play a pivotal role in ischaemic postconditioning. As in the case of IPC, ischaemic post-conditioning can also be achieved by a local postconditioning stimulus (direct IPost)[11,42-44] or by a remote postconditioning stimulus (RIPost)[45].

In animal models, there is experimental evidence that IPost protects against liver IR injury[42-44]. There are currently no randomized controlled trials of ischaemic postconditioning (direct or remote) in either liver resection or liver transplantation. So, routine ischaemic postconditioning (direct IPost or RIPost) cannot be currently recommended in either liver resection or liver transplantation.

Pharmacologic interventions to decrease IR injury

Various pharmacologic interventions have been attempted with an intention of decreasing IR injury. Considering that ROS and inflammatory mediators play significant roles in IR injury[4-6], pharmacological interventions to neutralise or modulate the pathways using antioxidants and steroids are a subject of significant research[13].

There is experimental evidence that some pharmacological interventions[46,47] protect against liver IR injury in the animal model. In humans, a systematic review of randomized controlled trials assessing the role of pharmacologic interventions in decreasing IR injury after liver resections showed that some interventions such as methyl prednisolone decrease the enzyme markers of liver parenchymal injury after liver resections but without demonstrating evidence of clinical benefit[13]. The role of numerous pharmacological interventions in decreasing IR injury in liver transplantation has been investigated[48-77]. None of the interventions have shown any benefit in graft or patient survival.

Genetic modulation of IR injury

As the molecular mechanisms of IR injury are increasingly understood, more research is being performed on the genetic modulation of the pathways in IR injury both for better understanding of the mechanisms involved in IR injury and for potential therapeutic applications[78]. Experimental evidence to demonstrate the potential role of genetic modulation of liver IR injury exists[14]. There are no randomized clinical trials assessing the impact of genetic modulation of IR injury in liver resections or liver transplantation.

Machine perfusion

Machine perfusion involves pulsatile perfusion of the liver using a machine as opposed to static cold storage. This can be performed by perfusing the liver with a hypothermic perfusate[79] or with a normothermic perfusate[80]. There is experimental evidence in animal models that machine perfusion protects against liver IR injury[80,81]. The safety and efficacy of machine perfusion compared to static cold storage to decrease liver IR injury is yet to be assessed in humans by randomized controlled trials.

DIFFERENCES IN RESULTS BETWEEN ANIMAL MODELS AND HUMAN TRIALS

As discussed above, there are major differences in the results of the role of the different interventions in decreasing liver IR injury between animal models and clinical results. Some possible reasons for this include the lack of fidelity of the model used (i.e. how truly are the results transferable from the model to humans)[82], the use of unvalidated surrogate outcomes, and the use of inadequate sample size in human trials.

FUTURE TRIALS

Future trials of adequate sample size and low risk of bias (low risk of prejudice towards the treatment arm or the control arm)[83] should be performed to decrease the random errors (arriving at wrong conclusions because of pure chance, usually due to inadequate sample size) and systematic errors (arriving at wrong conclusions because of prejudice towards the treatment or the control arm). Measurement of meaningful differences in clinical outcomes requires a large trial. Development and validation of composite outcomes and surrogate outcomes will enable evaluation of the interventions using a smaller sample size.

CONCLUSION

Currently, the only intervention that has shown to be beneficial in the protection of the liver during liver transplantation is hypothermia. In liver resection surgery, there is currently no established intervention targeted at modulating

IR injury that provides any major clinical benefit. However, many new therapies and targets are being discovered. Well designed randomized controlled trials are necessary to assess the new protective strategies in liver resection and liver transplantation.

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S- Editor Sun H **L- Editor** O'Neill M **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6104

World J Gastroenterol 2010 December 28; 16(48): 6104-6110
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Promoter polymorphism of MRP1 associated with reduced survival in hepatocellular carcinoma

Jing Zhao, Bing-Yun Yu, Dao-Yuan Wang, Jin-E Yang

Jing Zhao, Bing-Yun Yu, Jin-E Yang, Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, Guangdong Province, China
Dao-Yuan Wang, Department of Medical Oncology, The First Affiliated Hospital of Guangzhou Medical College, Guangzhou 510120, Guangdong Province, China
Author contributions: Zhao J designed and performed most of the research and wrote the paper; Yu BY performed the experiments; Wang DY performed the statistical analysis; Yang JE designed experiments, analyzed the data, and wrote the paper.
Supported by The Scientific and Technological Program of Guangdong Province, China, No. 2003B30102
Correspondence to: Dr. Jin-E Yang, PhD, Associate Professor, Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen (Zhongshan) University, Xin Gang Xi Road 135#, Guangzhou 510275, Guangdong Province, China. lssyje@mail.sysu.edu.cn
Telephone: +86-20-84115532 Fax: +86-20-84112169
Received: June 9, 2010 Revised: August 30, 2010
Accepted: September 7, 2010
Published online: December 28, 2010

Abstract

AIM: To investigate the effect of the G-1666A polymorphism in the multidrug resistance related protein-1 (*MRP1*) on outcome of hepatocellular carcinoma (HCC).

METHODS: A cohort of 162 patients with surgically resected HCC who received no postsurgical treatment until relapse was studied. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism analysis. Electrophoretic mobility shift assay (EMSA) was used to evaluate the influence of the G-1666A polymorphism on the binding affinity of the *MRP1* promoter with its putative transcription factors.

RESULTS: Kaplan-Meier analysis showed that patients with GG homologues had a reduced 4-year disease-free survival compared with those carrying at least one A allele (P = 0.011). Multivariate Cox regression analysis indicated that the -1666GG genotype represented an independent predictor of poorer disease-free survival [hazard ratio (HR) = 3.067, 95% confidence interval (CI): 1.587-5.952, P = 0.001], and this trend became worse in men (HR = 3.154, 95% CI: 1.604-6.201, P = 0.001). A similar association was also observed between 4-year overall survival and the polymorphism in men (HR = 3.342, 95% CI: 1.474-7.576, P = 0.004). Moreover, EMSA suggested that the G allele had a stronger binding affinity to nuclear proteins.

CONCLUSION: The *MRP1* -1666GG genotype predicted a worse outcome and was an independent predictor of poor survival in patients with HCC from Southeast China.

Key words: Multidrug resistance related protein-1; Single nucleotide polymorphism; Hepatocellular carcinoma; Prognosis

Peer reviewer: Yasuhiro Matsumura, MD, PhD, Research Center for Innovative Oncology, National Cancer Center Hospital East, Investigative Treatment Division, Kashiwa, Chiba 277-8577, Japan

Zhao J, Yu BY, Wang DY, Yang JE. Promoter polymorphism of MRP1 associated with reduced survival in hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(48): 6104-6110 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6104.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6104

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer death[1]. Optimal surgical resection is regarded as the best treatment for a curative outcome of HCC. However, long-term survival remains poor because of high rates of tumor recurrence or progression. Substantial effort has been made to identify prognostic factors

that can be used for improving therapeutic regimens and survival prediction. However, only a few factors, such as TNM stage or patient performance status, are consistent predictors, and their accuracy remains limited. Therefore, molecular markers that can accurately predict patient outcome are urgently needed.

The human multidrug resistance protein-1 (MRP1), also known as ABCC1, belongs to the ATP-binding cassette superfamily of cell-surface transport proteins. It participates in the transport of a wide variety of endogenously produced and exogenously administered molecules in an adenosine-triphosphate (ATP)-dependent manner[2,3]. Besides its well-known roles in drug resistance, MRP1 is proposed to contribute to the cellular antioxidative defense system by actively extruding glutathione (GSH)-conjugated xenobiotics and GSH-conjugated metabolites from cells[4]. Recent studies have also revealed that MRP1 is involved in inflammatory reactions, such as, dendritic cell differentiation and function[5]. MRP1 is expressed at moderate levels in most normal tissues, including lung, muscle, and kidney, but is barely detectable in normal liver[6-8]. However, in several liver diseases including HCC, its expression in the basolateral membrane is upregulated, which suggests a significant role for this transport protein during carcinogenesis[8,9].

Single nucleotide polymorphisms (SNPs) in the *MRP1* gene have been extensively studied in the past few years, and several genetic variants in the coding region have been shown to affect the function of MRP1[10-13]. For example, G2168A (Arg723Gln) can affect patients' sensitivity to chemotherapy in ovarian cancer[11]. G1299T (Arg433Ser) confers resistance to doxorubicin by reducing intracellular drug accumulation in HeLa cells that stably express mutant MRP1, whereas the G3173A (Arg1058Gln) variation increases the response to etoposide in HEK293 and CHO-K1 cells[12,13]. Recently, it has been observed that SNPs in the gene promoter can affect expression by disturbing the binding affinity of transcription factors, and are associated with disease prognosis[14]. However, whether SNPs in the *MRP1* promoter region have any clinical significance remains obscure. The expression level of *MRP1* is upregulated in HCC, therefore, we hypothesized that sequence variants in the promoter region potentially affect the expression of the *MRP1* gene and the prognosis of cancer, by modulating the efflux of toxins. To test this hypothesis, we investigated the potential of the *MRP1* G-1666A polymorphism (rs4148330) as a prognostic marker in a cohort of patients with HCC in Guangdong province of Southeast China.

MATERIALS AND METHODS

Study population

The study included 162 patients with HCC at the Cancer Center of Sun Yat-sen University (Guangzhou, China) from 2001 to 2005. All patients underwent hepatectomy as initial therapy, and did not receive chemotherapy or radiotherapy as follow-up treatment before recurrence. All samples were histologically confirmed. After surgical resection, the tissue samples were immediately frozen in liquid nitrogen and then stored at -80℃ until use.

Clinicopathological details and follow-up information were obtained from hospital records. The patients enrolled in the study were residents of Guangdong Province. Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) was diagnosed when HBV surface antigen or HCV antibody was detected by enzyme linked immunosorbent assay in the serum isolated from peripheral blood. The TNM criteria and the Edmondson and Steiner grading system were used to classify tumor stages and differentiation grades, respectively. Informed consent was obtained from each patient. This study was approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer Center.

DNA isolation and genotyping

Total genomic DNA was isolated with a standard protocol that included proteinase digestion, phenol-chloroform extraction, and ethanol precipitation. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to detect the genotype. A 160-bp fragment that covered the G-1666A polymorphism was generated using sense primer 5'-GCAACAGCATAACTGGCATT-3' and reverse primer 5'-GAGACCTCCCCCCAATCA-3'. PCR was performed as follows: 20 ng genomic DNA was amplified in a 20-μL reaction mixture that contained 2 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs, 0.2 μmol/L each primer, and 0.5 U *Taq* polymerase (Promega, Madison, WI, USA). After a total of 36 cycles of amplification at an annealing temperature of 58℃, 3 μL PCR products was then incubated overnight at 37℃ with 15 U *Hpa*Ⅱ (MBI Fermentas, Hanover, MD, USA). Digested products were analyzed by 2% agarose gel. PCR fragments that demonstrated altered electrophoretic patterns were purified and characterized by direct DNA sequencing. Results represent two independent experiments.

Cell lines and nuclear protein extraction

Liver cancer cell lines Huh7 and Hep3B were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in a humidified environment of 37℃ that contained 50 mL/L CO_2. Nuclear protein extracts from Hep3B and Huh7 cells were prepared according to the manufacturer's protocol (NucBuster Protein Extraction Kit; Novagen, Darmstadt, Germany).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with the Gel Shift Assay System (Promega), according to the manufacturer's instructions. The following oligonucleotides that corresponded to the promoter region of *MRP1* and covered the G-1666A polymorphism were synthesized (underline letters indicate polymorphism): -1666A allele, 5'-GGGGGACCCGGGCCAATAAAAAAATCA-3'; -1666G allele, 5'-GGGGGACCCAGGCCAATAAAAAAATCA-3'; nonspecific (scrambled) oligonucleotide, 5'-GAAGCGGTGACACGGAACATCACGAAA-3'. Oligonucleotides were annealed and end-labeled with [γ-^{32}P]-ATP. Five micrograms of Hep3B or

Huh7 nuclear extracts were added in each binding reaction. For the competition assay, a 10-, 50- or 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture as a competitor. The products were separated on pre-electrophoresed 5% polyacrylamide gels at 4℃. The gels were then dried at 80℃ for 4 h and exposed to a Storage Phosphor Screen (Amersham Bioscience, Sunnyvale, CA, USA), which was subsequently read with a Typhoon Phosphor Imager (Amersham Bioscience). The putative transcription factors that recognized the sequences that overlapped the G-1666A site were predicted with Alibaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and the transcription element search software (TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess).

Statistical analysis

The χ^2 and Fisher's exact tests were used for the analysis of the relationship between the genotypes and clinicopathological characteristics. Disease-free survival (DFS) was calculated from the day of surgery to either relapse or death without relapse, and it was censored only for patients who were alive and recurrence-free at the last follow-up. Overall survival (OS) was measured from the date of hepatectomy to the time of death or the last follow-up. Survival curves were obtained by the Kaplan-Meier method, and the statistical significance of the differences in survival among subgroups was evaluated with the log-rank test. The Cox proportional hazards model was employed to assess the independent prognostic values of the polymorphisms. Statistical analyses were all performed with SPSS software package (version 13.0; SPSS, Inc., Chicago, IL, USA). All statistical tests were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics and genotype

Demographic and clinicopathological characteristics of the 162 patients with HCC are summarized in Table 1. The mean age at first diagnosis of HCC was 48 years. Consistent with our previous study[15], most patients showed excessive γ-glutamyl transpeptidase and α-fetoprotein, along with liver cirrhosis, and > 80% of the enrolled patients were infected with HBV (140/161, 87.0%), which implicated HBV infection as a leading cause of HCC in Southeast China. In contrast, only a small number of patients were infected with HCV.

Genotyping was performed by PCR-RFLP. A 160-bp *MRP1* promoter region that covered the G-1666A variant was digested with *Hpa*Ⅱ. After full digestion of the amplified PCR products, those from AA homozygotes still existed as a single 160-bp fragment, whereas those from the GG homozygotes had been divided into two fragments of 71 bp and 89 bp, respectively. The allele frequency of patients with HCC was 0.61 for *MRP1* -1666A and 0.39 for -1666G. However, no significant correlations were found between the nucleotide variants and clinical variables (data not shown).

Table 1 Physiological characteristics of hepatocellular carcinoma patients (n = 162)

	n (%)
Sex	
Female	15 (9.3)
Male	147 (90.7)
Age (yr)	
< 48	77 (47.5)
⩾ 48	85 (52.5)
HBV infection[1]	
-	21 (13.0)
+	140 (86.4)
HCV infection	
-	158 (97.5)
+	4 (2.5)
GGT (U/L)[2]	
< 50	45 (27.8)
50-99	48 (29.6)
⩾ 100	67 (41.4)
AFP (ng/mL)	
< 20	53 (32.7)
20-399	45 (27.8)
⩾ 400	64 (39.5)
Tumor size (cm)	
< 5	53 (32.7)
⩾ 5	109 (67.3)
Ascites[3]	
-	148 (91.4)
+	14 (8.6)
Cirrhosis	
Total	19 (11.7)
Mild	80 (49.4)
Moderate	49 (30.2)
Severe	14 (8.6)
Edmondson grade	
Ⅰ	11 (6.8)
Ⅱ	71 (43.8)
Ⅲ	77 (47.5)
Ⅳ	3 (1.9)
TNM stage	
Ⅰ	106 (65.4)
Ⅱ	7 (4.3)
Ⅲ	49 (30.3)
G-1666A genotype	
AA	55 (34.0)
AG	89 (54.9)
GG	18 (11.1)

[1](-) absence, (+) presence, one case unconfirmed; [2]Two cases unconfirmed; [3](-) absence, (+) presence. HBV: Hepatitis B virus; HCV: Hepatitis C virus; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein.

Association analysis between the G-1666A polymorphism and survival

Growing evidence suggests that SNPs are closely related to the risk and outcome of cancer[14,16,17]. To investigate the impact of the G-1666A polymorphism on the prognosis of patients with HCC, we next analyzed the 4-year DFS and OS of patients with different genotypes. A significant correlation between the -1666 polymorphism and postoperative survival was found. The mean survival times of patients with the AA, AG and GG genotypes were 30.4 ± 18.2, 30.7 ± 17.4 and 24.8 ± 17.3 mo, respectively. The survival curves showed that the 4-year rate of DFS among patients who carried GG decreased significantly compared with those who carried the AA or AG allele (P = 0.031,

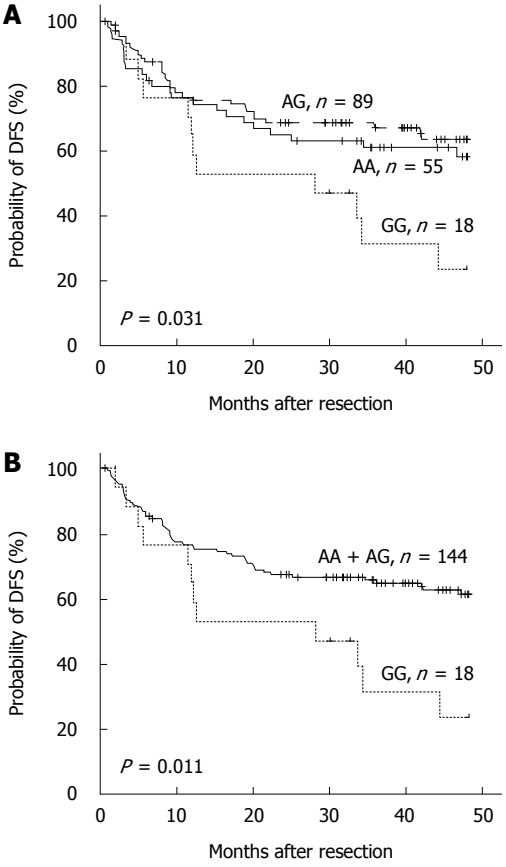

Figure 1 Kaplan-Meier disease-free survival curves for hepatocellular carcinoma patients who carried different *multidrug resistance related protein-1* -1666 genotypes. A: Comparison between three genotypes; B: GG genotype compared with the other two genotypes. Log-rank *P* values are indicated. Tick marks represent censored data. DFS: Disease-free survival.

Figure 1A). Moreover, if the patients with AA and AG genotypes were combined, the discrepancy became more obvious ($P = 0.011$, Figure 1B). Further analysis revealed a similar, albeit non-significant, trend between the -1666 polymorphism and 4-year OS (Table 2). Multivariate Cox proportional hazard analysis was then performed, and the variables that showed significance by univariate analysis were adopted as covariates (Table 2). The results revealed that the *MRP1* G-1666A polymorphism was an independent prognostic factor for 4-year DFS [hazard ratio (HR) = 3.067, 95% confidence interval (CI): 1.587-5.952, $P = 0.001$, Table 3].

One of the key features of HCC is the much higher incidence in men than in women[1]. In our study cohort, the male/female ratio was 9.8:1. Further stratification of the patients by sex revealed an even more pronounced association of the -1666GG genotype with poorer survival ($P < 0.05$, Figure 2). Multivariate analysis suggested that the -1666GG genotype was an especially powerful independent prognostic factor of 4-year DFS (HR = 3.154, 95% CI: 1.604-6.201, $P = 0.001$) and OS (HR = 3.342, 95% CI: 1.474-7.576, $P = 0.004$) in the men with HCC (Table 3).

Influence of the G-1666A polymorphism on the affinity of binding with nuclear proteins

We performed EMSA to evaluate the influence of the

Table 2 Determination of prognostic factors for disease-free survival and overall survival of patients with hepatocellular carcinoma, by univariate analysis ($n = 162$)

Variable	DFS		OS	
	HR (95% CI)	*P*[a]	HR (95% CI)	*P*[a]
Sex				
Female	1		1	
Male	4.231 (1.035-17.299)	0.045	7.522 (1.041-54.337)	0.045
GGT (U/L)[1]				
< 50	1		1	
50-99	2.064 (0.920-4.632)	0.079	1.771 (0.734-4.275)	0.203
⩾ 100	3.639 (1.761-7.519)	0.001	3.728 (1.734-8.014)	0.001
AFP (ng/mL)				
< 20	1		1	
20-399	1.852 (0.954-3.595)	0.068	2.004 (0.989-4.061)	0.054
⩾ 400	1.997 (1.070-3.725)	0.030	2.102 (1.079-4.094)	0.029
Tumor size (cm)				
< 5	1		1	
⩾ 5	2.230 (1.233-4.030)	0.008	2.089 (1.126-3.875)	0.019
Ascites[2]				
-	1		1	
+	2.562 (1.301-5.044)	0.007	2.81 (1.375-5.741)	0.005
Cirrhosis				
No	1		1	
Mild	2.005 (0.710-5.661)	0.189	2.505 (0.763-8.225)	0.130
Moderate	1.832 (0.623-5.387)	0.271	2.287 (0.670-7.806)	0.187
Severe	3.230 (0.993-10.505)	0.051	4.796 (1.297-17.738)	0.019
TNM stage				
Ⅰ	1		1	
Ⅱ + Ⅲ	3.165 (1.940-5.163)	< 0.001	3.424 (2.038-5.752)	< 0.001
Genotypes				
AA	1		1	
AG	0.830 (0.479-1.439)	0.507	0.818 (0.463-1.447)	0.490
GG	1.988 (0.982-4.024)	0.056	1.491 (0.682-3.258)	0.317
AA + AG	1		1	
GG	2.223 (1.185-4.172)	0.013	1.678 (0.822-3.422)	0.155

[a]Hazard ratio (HR) and *P* values were calculated using univariate Cox regression. $P < 0.05$ was considered to indicate statistical significance; [1]Two cases unconfirmed; [2](+) presence, (-) absence. DFS: Disease-free survival; OS: Overall survival; CI: Confidence interval; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein.

G-1666A polymorphism on the binding affinity of the *MRP1* promoter with putative transcription factors. The radiolabeled -1666G probe showed strong DNA-protein binding ability in the presence of nuclear proteins extracted from the Hep3B cell line, whereas the -1666A probe barely showed any interaction (Figure 3A, lane 2 and lane 10, respectively). In order to assess the binding specificity and the differences in binding affinity between the G and A alleles, competition assays were performed with unlabeled -1666A and -1666G oligonucleotides. A 50-fold excess of unlabeled -1666A oligonucleotides only partially disrupted the binding of the radiolabeled -1666G probe with nuclear extracts (Figure 3A, lane 7 and Figure 3B, lane 6), whereas this amount of unlabeled -1666G oligonucleotides almost completely abolished the binding (Figure 3A and B, lane 4). In contrast, a non-specific competitor had no effect (Figure 3B, lane 8). Similar results were obtained when using the nuclear extracts from Huh7 cells (data not shown). These data suggested that the G-1666A polymorphism could affect the binding affinity of the *MRP1* promoter with transcription factors, and

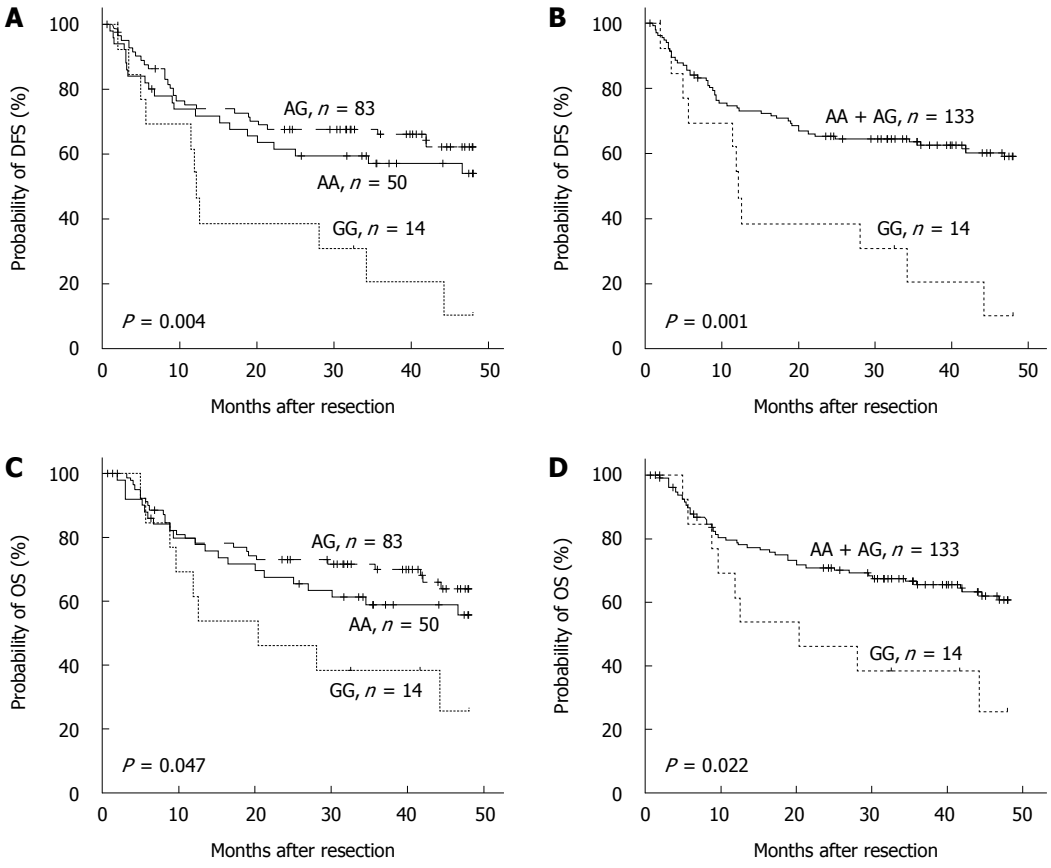

Figure 2 Kaplan-Meier curves for disease-free survival (A and B) and overall survival (C and D) for male patients with hepatocellular carcinoma and different *multidrug resistance related protein-1* -1666 genotypes. A: Comparison of disease-free survival (DFS) between three genotypes; B: AA and AG grouped together and compared to GG genotype; C: Comparison of overall survival (OS) between three genotypes; D: OS of AA and AG genotypes compared with GG genotype. Log-rank *P* values are indicated. Tick marks represent censored data.

Table 3 Multivariate analysis for prognostic value of *multidrug resistance related protein-1* G-1666A polymorphism in patients with hepatocellular carcinoma

Genotypes	DFS[1]		OS[2]	
	HR (95% CI)	*P*[a]	HR (95% CI)	*P*[a]
All (*n* = 162)				
AA+AG	1			
GG	3.067 (1.587-5.952)	0.001		
Men (*n* = 147)				
AA + AG	1		1	
GG	3.154 (1.604-6.201)	0.001	3.342 (1.474-7.576)	0.004

[a]Hazard ratio (HR) and *P* values were calculated using multivariate Cox regression. $P < 0.05$ was considered to indicate statistical significance; [1]Multivariate analysis of disease-free survival (DFS) in all patients was adjusted for sex, γ-glutamyl transpeptidase (GGT), α-fetoprotein (AFP), tumor size, ascites, and TNM stage; in male patients, it was adjusted for GGT, tumor size, and TNM stage; [2]Multivariate analysis of OS in male patients was adjusted for GGT, tumor size, ascites, cirrhosis, and TNM stage. OS: Overall survival; CI: Confidence interval.

that the G allele had a stronger binding affinity than the A allele.

DISCUSSION

The multidrug resistance protein family transports a wide range of physiological substrates and diverse therapeutic agents. In the past decade, much effort has been focused on MRP1-mediated drug resistance[18,19]; and emerging evidence indicates that SNPs within the *MRP1* gene have prognostic value in predicting the response to chemotherapy in different cancers[11,20]. Notably, MRP1 takes part in the transport of aflatoxin B1, a well-know human liver carcinogen that can induce a characteristic mutation in *p53* at codon 249[21,22]; and previous studies have shown that *p53* mutations are significantly associated with a poor prognosis for patients with HCC[15,23]. In addition, MRP1 also plays important roles in cellular antioxidant defense and immune cell function[4,5]. These observations and the upregulated expression level of *MRP1* gene in several liver diseases, including HCC, suggest the possibility that this protein is involved in tumorigenesis and progression[8,9]. Our present study examined the role of the *MRP1* G-1666A polymorphism as a prognostic factor in patients with HCC who were treated only with curative surgery, and proved that the GG genotype was an independent predictor of poor survival, especially in men with HCC. Furthermore, specific binding of nuclear proteins to G allele was found, which suggested a difference in transcription activity between different genotypes.

In the present study, there were 147 men (90.7%) and 15 women with HCC (9.3%), with a male-to-female ratio

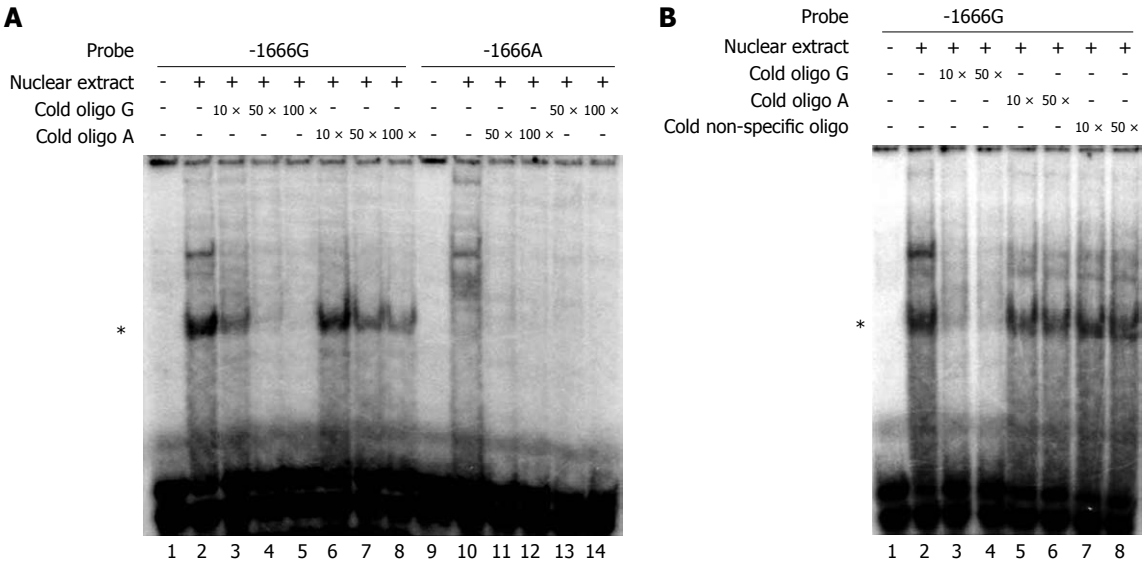

Figure 3 Electrophoretic mobility shift assay of the *multidrug resistance related protein-1* promoter region that contained the G-1666A site. A: Analysis was performed in the presence (+) or absence (-) of Hep3B nuclear extract. Each binding reaction contained γ-^{32}P-labeled -1666G (lanes 2-8) or -1666A (lanes 10-14) probes. A 10-, 50-, or 100-fold (as indicated) excess of unlabeled (cold) -1666A or G oligonucleotides (lanes 6-8, 11, and 12 or 3-5, 13, and 14) were included in the binding reactions as specific competitors. Labeled oligonucleotides incubated without the nuclear extracts were included as negative controls (lanes 1 and 9); B: In the present of Hep3B nuclear extract, 10- or 50-fold more excess of unlabeled -1666G oligonucleotides (lanes 3 and 4) or -1666A oligonucleotides (lanes 5 and 6) or non-specific oligonucleotides (lanes 7 and 8) were used as competitors. Lane 1 was the negative control. Lane 2 indicated the labeled -1666G oligonucleotides incubated with the nuclear extracts only. The asterisks indicated the DNA-protein complex.

of 9.8:1. We observed a remarkably significant association of the *MRP1* G-1666A polymorphism with 4-year OS in men with HCC, but not in the entire cohort. This phenomenon might result from the interaction between the polymorphism and sexual hormones during carcinogenesis, which has been demonstrated in the example of *MDM2* SNP309[24]. Therefore, the correlation between the *MRP1* G-1666A polymorphism and the survival of women with HCC requires further investigation to generate a definite conclusion.

SNPs in the promoter region of a gene can potentially alter the affinity of interactions between DNA and nuclear proteins and, in turn, affect the efficiency of transcription. We found that the G allele of the *MRP1* G-1666A polymorphism had a stronger binding affinity for nuclear proteins in hepatoma cells than the A allele had. This finding accords with our presumption that the G-1666A polymorphism might dominate the pumping ability of MRP1 by affecting the expression of the protein. Although a G-1666A polymorphism located 1.5 kb upstream of the core promoter of *MRP1*, and two major regulatory domains had already been found in tandem upstream of the core promoter[25], recent studies have revealed that distal regions (enhancer or suppressor) can influence gene transcription through physical association with the transcription start site[26]. Furthermore, allele G of the G-260C polymorphism could lead to lower activity of the *MRP1* promoter in cell lines, which suggests that nucleotide variants in the *MRP1* gene account, in part, for inter-individual variations and population differences in cellular efflux[27]. These data suggest that the G-1666A polymorphism functions as a distal element through the folding of the DNA strand.

The three transcription factors Sp1, NF-1 and CTF were predicted to bind to the promoter region, including the G-1666A site, by Alibaba2.1 software, but only the Sp1 consensus motif could partially disrupt the DNA-protein binding in a competition assay, whereas the other two did not show significant influence on the binding (data not shown). Sp1 antibody failed to reveal any super-shift band when added to the EMSA reaction complex (data not shown), which suggested that Sp1 binding to the cis-element, including the G-1666A site, required an interaction between Sp1 and other nuclear proteins. Future work will be required to identify such nuclear proteins.

In summary, our present study shows that the *MRP1* G-1666A polymorphism is an independent prognostic factor for patients with HCC, which implies a role for MRP1 in tumor progression. Clearly, much more work remains to be done to confirm our findings and overcome the limitations in our work before this SNP can be used as a marker for poor outcome in HCC.

ACKNOWLEDGMENTS

The authors are grateful to Professor Shi-Mei Zhuang for her advice and guidance on this paper.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer death, and long-term survival remains poor because of high rates of tumor recurrence or progression. Therefore, markers that can be used for improving therapeutic regimens and survival prediction are urgently needed.

Research frontiers

The finding that human multidrug resistance protein-1 (MRP1) is expressed

in unusually large amounts in HCC suggests it has a role in the growth and progression of this cancer. Expression of MRP1 is affected by the genetic sequence in its promoter region, therefore, the authors of this study examined the potential of different sequences (polymorphisms) in the *MRP1* promoter to serve as indicators of prognosis and outcome in patients with HCC.

Innovations and breakthroughs

Recent studies have demonstrated that mutations within the *MRP1* gene have value in predicting the response to chemotherapy in different cancers, but the clinical significance of such mutations in the *MRP1* promoter for patients with HCC is unknown. This is believed to be the first study to identify a polymorphism in the promoter of *MRP1* that is an independent prognostic factor for 4-year overall survival in men with HCC. The correlation between the *MRP1* polymorphism and the survival of women with HCC requires further investigation. The authors also demonstrated that the polymorphism altered the affinity of nuclear proteins for the DNA in the HCC cells, which might explain the mechanism by which the expression of MRP1 was reducing.

Applications

The genetic sequence identified in this study can be used to test tissue samples from patients with HCC to help predict their outcome after therapy. This information can be used to guide treatment decisions and improve therapeutic regimens for individual patients.

Terminology

MRP1 is one of a family of proteins found on the surface of cells. These proteins transport a wide variety of substances and can contribute to resistance to chemotherapy by transporting anticancer drugs out of cancer cells. The promoter region of a gene is a sequence of nucleotides that regulates whether and how much of a protein is synthesized from that gene.

Peer review

This is a study of an important area of cancer genomics. The authors found that a single nucleotide polymorphism of *MRP1* promoter region is an independent prognostic factor for HCC patients. The study design was well-organized and they reached a conclusion by making full use of the data.

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S- Editor Sun H **L- Editor** Kerr C **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6111

World J Gastroenterol 2010 December 28; 16(48): 6111-6118
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Impaired PI3K/Akt signal pathway and hepatocellular injury in high-fat fed rats

Ji-Wu Han, Xiao-Rong Zhan, Xin-Yu Li, Bing Xia, Yue-Ying Wang, Jing Zhang, Bao-Xin Li

Ji-Wu Han, Department of Gastroenterology, The 4th Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Xiao-Rong Zhan, Xin-Yu Li, Bing Xia, Yue-Ying Wang, Jing Zhang, Department of Endocrinology, First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Bao-Xin Li, Department of Pharmacology, State Key Laboratory of Biomedicine and Pharmacology, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Author contributions: Zhan XR and Li BX designed the research and contributed equally to this work; Han JW, Li XY, Xia B and Wang YY performed the research; Han JW and Zhang J analyzed the data; Han JW, Li XY and Zhan XR wrote the paper.
Supported by The Natural Science Foundation of Heilongjiang Province, No. 2005-13
Correspondence to: Xiao-Rong Zhan, PhD, Professor, Department of Endocrinology, First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China. xiaorongzhan@sina.com
Telephone: +86-451-53643849 Fax: +86-451-55607786
Received: July 28, 2010 Revised: September 13, 2010
Accepted: September 20, 2010
Published online: December 28, 2010

Abstract

AIM: To determine whether mitochondrial dysfunction resulting from high-fat diet is related to impairment of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway.

METHODS: Rat models of nonalcoholic fatty liver were established by high-fat diet feeding. The expression of total and phosphorylated P13K and Akt proteins in hepatocytes was determined by Western blotting. Degree of fat accumulation in liver was measured by hepatic triglyceride. Mitochondrial number and size were determined using quantitative morphometric analysis under transmission electron microscope. The permeability of the outer mitochondrial membrane was assessed by determining the potential gradient across this membrane.

RESULTS: After Wistar rats were fed with high-fat diet for 16 wk, their hepatocytes displayed an accumulation of fat (103.1 ± 12.6 *vs* 421.5 ± 19.7, $P < 0.01$), deformed mitochondria (9.0% ± 4.3% *vs* 83.0% ± 10.9%, $P < 0.05$), and a reduction in the mitochondrial membrane potential (389.385% ± 18.612% *vs* 249.121% ± 13.526%, $P < 0.05$). In addition, the expression of the phosphorylated P13K and Akt proteins in hepatocytes was reduced, as was the expression of the anti-apoptotic protein Bcl-2, while expression of the pro-apoptotic protein caspase-3 was increased. When animals were treated with pharmacological inhibitors of P13K or Akt, instead of high-fat diet, a similar pattern of hepatocellular fat accumulation, mitochondrial impairment, and change in the levels of PI3K, Akt, Bcl-2 was observed.

CONCLUSION: High-fat diet appears to inhibit the PI3K/Akt signaling pathway, which may lead to hepatocellular injury through activation of the mitochondrial membrane pathway of apoptosis.

Key words: Nonalcoholic fatty liver; Phosphatidylinositol 3-kinase/protein kinase B signaling pathway; Mitochondria; B-cell lymphoma gene 2; Caspase-3

Peer reviewer: Dr. Shawn David Safford, Department of Surgery, Duke University Medical Center, 994 West Ocean View Avenue, Norfolk, VA 23503, United States

Han JW, Zhan XR, Li XY, Xia B, Wang YY, Zhang J, Li BX. Impaired PI3K/Akt signal pathway and hepatocellular injury in high-fat fed rats. *World J Gastroenterol* 2010; 16(48): 6111-6118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6111.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6111

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is caused by

triglyceride (TG) accumulation within the liver and can either be a benign self-limiting state or a condition associated with steatohepatitis (NASH), which may develop to fibrosis, cirrhosis and liver failure[1,2]. Triacylglycerol formation in the hepatocytes may also be cytotoxic to hepatocytes[3,4]. Multiple lines of evidence support the role of intrahepatic fat in causing hepatic insulin resistance. Hepatic insulin resistance is considered to be the fundamental mechanism in the prevalence and progression of the disease. It is also a critical component in the development of NAFLD, which is characterized by a marked reduction in the activity of the insulin signaling pathway[5].

In the presence of insulin, the insulin receptor normally phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the activation of several signaling pathways, including the metabolic phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway. Phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types[6].

Hepatocyte apoptosis is a key histologic feature of NAFLD, and correlates with progressive inflammation and fibrosis[7]. The molecular pathways leading to hepatocyte apoptosis are not fully defined; however, mitochondrial dysfunction is an important element in the pathogenesis of NAFLD[8]. Recent evidence showed that mitochondria participate in the regulation of both cell proliferation and death, including apoptosis[9], and are thus potential mediators of the PI3K/Akt signaling pathway. Although multiple lines of evidence have suggested a close linkage between insulin-induced signaling and mitochondrial functions[10], the potential relationship between the PI3K/Akt signaling pathway and the mitochondrial abnormalities that underlie NAFLD remain unclear.

The present study was undertaken to investigate the relationship between mitochondria impairment and the activity of the PI3K/Akt signaling pathway during the development of NAFLD.

MATERIALS AND METHODS

Animal groups and diet

Male Wistar rats, 12 wk old were obtained from Harbin Medical University.

Laboratories (stock No. 002207). All experiments and animal care complied with the guidelines for the humane treatment of animals set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences, Harbin Medical University. At 6 wk of age, the 60 mice were randomly divided into four groups: (1) Normal control (NC) group; (2) NC plus the PI3K inhibitor LY294002 (NC + LY, 15 μg/kg daily injected *via* the tail CA 440206, Calbiochem); (3) NC plus the AKT inhibitor 1-L-6-hydroxymethyl-chiro-inositol2-(R)-2-O-methyl-3-O-octadecylcarbonate (NC + AI, 20 μg/kg daily *via* tail injection CA124005, Calbiochem); and (4) High-fat diet (HFD). The normal control rats were fed a commercial rat diet (7%-10% fat, 68%-70% carbohydrates, 18%-20% protein, 1%-2% vitamins and minerals; 210 kcal/100 g per day) for 16 wk, while rats in the treatment group (HFD group) were fed a high-fat diet (40% fat, 38%-40% carbohydrates, 18%-20% protein, 1%-2% vitamins and minerals; 210 kcal/100 g per day) for the same period of time.

Calculation of metabolic index and resistance index

Blood samples from the retro-orbital sinus were collected before and after the treatment. Rats were fasted overnight before the collection of the blood samples. Plasma insulin was determined using ELISA. Insulin resistance was evaluated using a homeostasis model assessment of insulin resistance (HOMA). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) levels were measured using spectrophotometric assay kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Insulin resistance was assessed by computing insulin resistant index (HOMA-IR). The formula used was as follows: HOMA-IR = Insulin (μg/L) × glucose (mmol/L)/22.5.

Measurement of hepatic TG

The liver (100 mg wet tissue) was homogenized in an ice-cold 0.05% butylhydroxytoluene solution. After lipids were extracted from the liver according to the method of Folch *et al*[11], TG content in each sample was measured with a commercial assay kit (Wako Pure Chemical Industries, Osaka, Japan CA 290-63701).

Isolation of hepatocytes

Hepatocytes were isolated from the liver (20-25 mg) of each mouse by the collagenase perfusion method. Each liver was pre-perfused at 37℃ with buffer containing 100 mmol/L HEPES (pH 7.4), 143 mmol/L NaCl, and 7 mmol/L KCl, and then perfused with buffer containing 0.05% collagenase and 5 mmol/L $CaCl_2$. Following digestion, the liver was dispersed in the perfusion solution and incubated in the perfusion buffer at 37℃ for an additional 5 min. The dispersed cell suspension was then filtered through a nylon mesh and centrifuged at 100 × *g* for 3 min at 25℃. The resulting cell pellets were resuspended in the hepatocyte medium, and cell viability was then determined using a trypan-blue-exclusion test.

Measurement of mitochondrial membrane potential of hepatocytes

The integrity of the inner mitochondrial membrane was assessed by determining the potential gradient across this membrane. Rhodamine 123 (Rh123) powder was dissolved in methanol and stored at -20℃ as a 1 g/L solution, which was diluted to 5 mg/L with phosphate buffered solution (PBS) before each experiment. Hepatocytes (1×10^6) were washed three times with PBS that had been preheated to 4℃. They were then resuspended in 300 mL PBS, incubating with Rh123 (final concentration 2.5 mg/L) for 1 h at 37℃, and then filtered through a 200-mesh screen. Approximately 10000 cells were measured using a FACS Calibur flow cy-

tometer (BD Biosciences, San Diego, CA, USA) using Cell Quest software (a maximum absorbing wave length 590 nm, an excitation wave length 488 nm) (BD Biosciences). Rh123 and tetramethylrhodamineethylester (TMRE) were purchased from Invitrogen (Karlsruhe, Germany).

Electron microscopy

For transmission electron microscopy, small liver fragments were fixed in 4% glutaraldehyde and then processed using standard methods. Sections were viewed under microscope by a pathologist (Dr. Chang H, Department of Pathology, Harbin Medical University). Mitochondrial number and size were determined using quantitative morphometric analysis under transmission electron microscope (Model HB601UX, Vacuum Generators, Hastings, United Kingdom).

Western blotting

Ten μg protein was subjected to SDS-PAGE (10% acrylamide gel) and then transferred to a PVDF membrane for 2 h (120 V) using a Bio-Rad Mini Trans Blot electrophoretic transfer unit (Bio-Rad, Marnes-la-Coquette, France). The membranes were blocked for nonspecific binding with 5% nonfat dry milk and then probed with the specific primary antibodies (Abcam, CA ab74136, ab63566, ab79360, ab8805, 1:1000 dilution) at 4℃ overnight. After 3 washes with TBS-T, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA SC2030). Separated proteins were visualized by an ECL kit (GE Healthcare Life Sciences, CA RPN2135) and light emission was captured on X-ray film (GE Healthcare). Intensities of the respective bands were examined by densitometric analysis (Scion Image Analyst program).

Statistical analysis

Results were presented as mean ± SE and were analyzed using one-way analysis of variance followed by the Bonferroni multiple comparisons test. All tests were 2-sided, and $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using INSTAT version 3 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Comparison of body and liver weight, biochemical parameters, insulin resistant index among four groups

Compared with normal control groups, the mean serum transaminase, hepatic TG content, body and liver weight, insulin resistant index were all increased in PI3K inhibitor, Akt inhibitor and high-fat groups ($P < 0.05$, Table 1). Hepatocytes could be damaged after PI3K/Akt pathway signal transduction was blocked, but blocked PI3K/Akt pathway signal transduction could lead to TG accumulation in liver to liver weight gain and insulin resistance.

Table 1 Weights of the liver and serum levels of biochemical parameters in high-fat fed rats

	NC	NC + LY	NC + AI	HFD
Tissue weight				
Liver (g)	1.55 ± 0.14	1.99 ± 0.32[a]	1.89 ± 0.43[a]	3.98 ± 0.64[a]
Serum				
TG (mg/dL)	183.8 ± 70.4	396.7 ± 72.3	376.7 ± 68.4	589 ± 98.4[b]
ALT (U/L)	34 ± 3.1	86 ± 5.58[a]	89 ± 5.2[a]	207 ± 35.5[b]
AST (U/L)	36 ± 3.4	88 ± 6.1	99 ± 5.2[a]	187 ± 35.5[b]
GGT (U/L)	52 ± 6.4	48 ± 3.9	62 ± 4.4	232 ± 67.8[b]
Glucose (mmol/L)	5.1 ± 0.4	6.1 ± 0.5	5.7 ± 0.4	7.1 ± 71.3[a]
Insulin (mIu)	4.7 ± 0.7	14.9 ± 2.0[b]	15.0 ± 2.9[b]	21.0 ± 3.8[b]
Liver				
IRI (HOMA-IR)	1.06	4.03[a]	3.8[a]	6.6[b]
TG (mg/g)	103. 1 ± 12.6	324.6 ± 13.4	2336.8 ± 11.6	421.5 ± 19.7[b]

Rats were fed and subjected to each measurement. Data are presented as the mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* rats fed with normal diets. NC: Normal control; NC + LY: NC plus the PI3K blocker LY294002; NC + AI: NC plus the AKT blocker; HFD: High-fat diets for 16 wk ($n = 10^{-12}$); ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. GGT: γ-glutamyltransferase; TG: Triglycerides; IRI (HOMA-IR): Insulin resistant index.

Expression of PI3K and Akt protein in hepatocytes in high-fat fed mice

To further investigate whether the PI3K/Akt pathway mediates injury of hepatocytes, we measured the protein expression levels of total and phosphorylated PI3K and Akt in the four groups. The protein expression levels of total PI3K and Akt showed no significant difference among the four groups (Figure 1). But compared with the normal control groups, the expression levels of pPI3K and pAkt markedly decreased in the high-fat group. Moreover, in PI3K inhibitor groups, neither pPI3K nor pAkt was expressed. In Akt inhibitor groups, pAkt showed no expression, and pPI3K had no significant difference compared with the normal control group (Figure 1). These results suggested that fat mass accumulation in the liver may lead to decreased expression of pPI3K and pAkt in fatty liver induced by high fat.

Fat accumulation in the liver and PI3K/Akt pathway

To investigate further whether the fat accumulation in the liver was involved in PI3K/Akt pathway, TG content in each sample was measured in the four groups. The results showed that TG content of liver was elevated in high-fat diet, PI3K and Akt inhibitor groups.

Ultrastructure of hepatocellular mitochondria and PI3K/Akt pathway

To determine whether the PI3K pathway has an anti-apoptotic effect in liver cells, we compared the mitochondrial morphology in the four groups (Figure 2A). Electron microscopy revealed that the hepatocytes of the control group rats were rich in mitochondria, the shape and size of mitochondria were normal and had few lipid droplets. In contrast, many mitochondria from rats in the high-fat group were enlarged and showed morphological changes, including a rarefied matrix and large lipid droplet. In the high-fat group, 83.0% ± 10.9% of mitochondria had abnormal morphology, compared to 9.0% ± 4.3% in the control group ($P < 0.05$) (Figure 2B). The changes

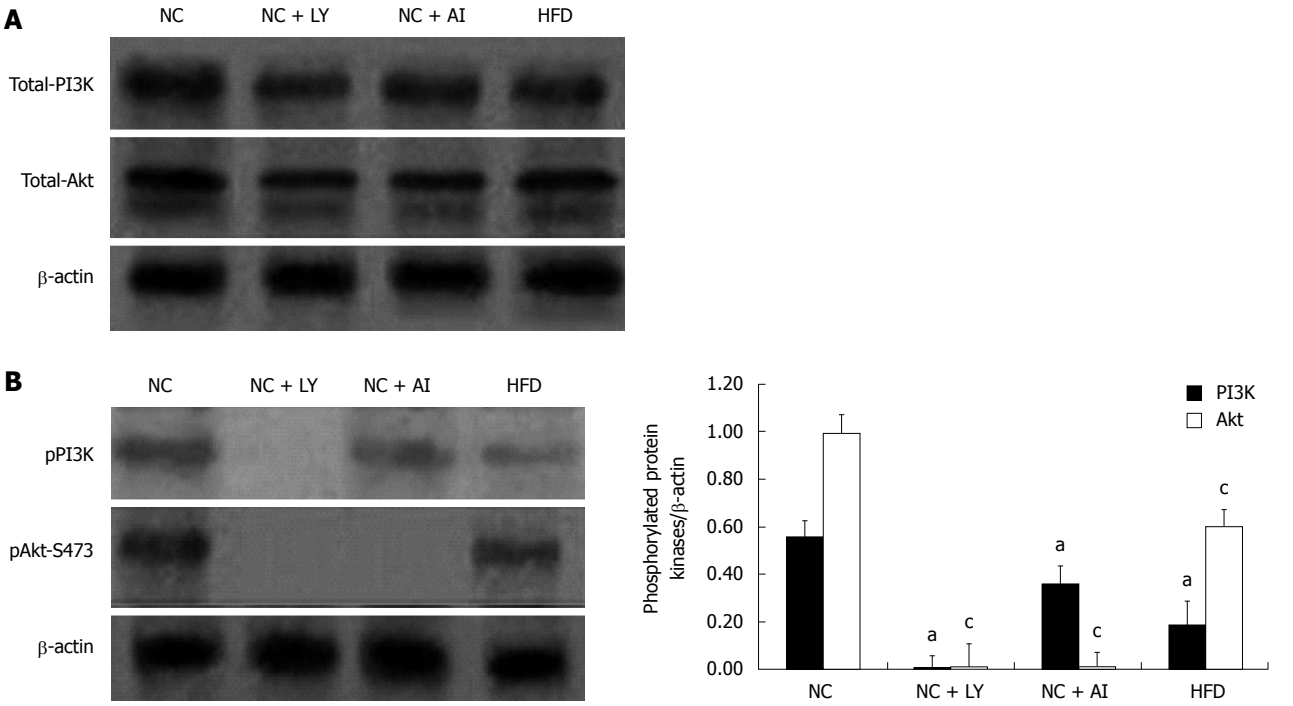

Figure 1 Western blotting analysis of phosphatidylinositol 3-kinase and protein kinase B in hepatocytes. A: Western blotting analysis of total phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt). The protein expression levels of total PI3K and Akt showed no significant difference among the four groups; B: Western blotting analysis of phosphorylated PI3K and Akt. Left: In high-fat fed groups (HFD), the protein expression levels of both phosphorylated PI3K and Akt decreased compared with the normal control (NC) group. In PI3K blocker groups (NC + LY), pPI3K and pAkt showed no expression. In AKT blocker groups (NC + AI), pPI3K was normally expressed and pAkt showed no expression. Right: Band density values are compared with those of the β-actin loading control. $^{a,c}P < 0.05$ *vs* NC group.

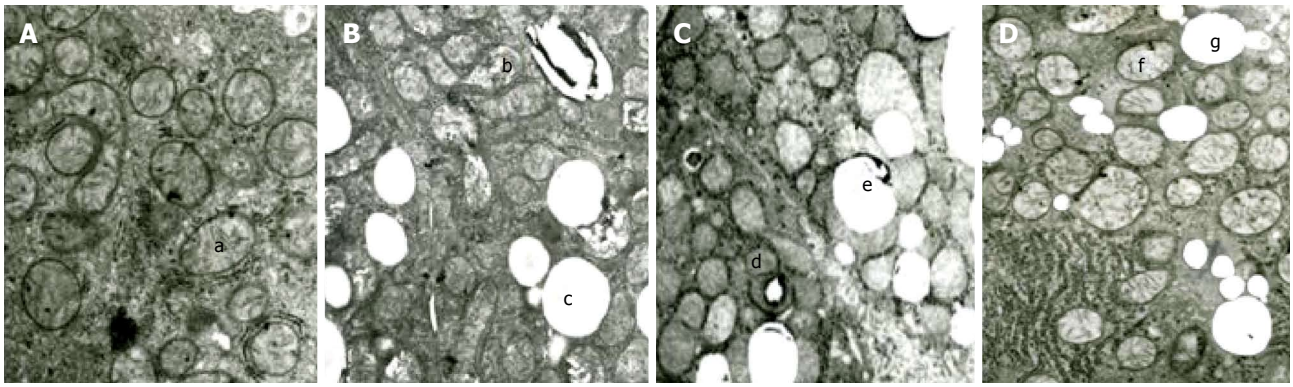

Figure 2 Ultrastructural changes in hepatocellular mitochondria. A: Mitochondria (a) in normal diet group; B: Enlarged mitochondria (b) and lipid droplet (c) in high-fat diet group; C and D: Animals treated with either phosphatidylinositol 3-kinase blocker (C) or protein kinase B blocker (D) showed similar changes in mitochondrial shape, size (d, f) and lipid droplet (e, g) to those of the high-fat group. Magnification × 18 000.

of mitochondria in the PI3K-inhibitor and Akt- inhibitor groups were similar to those of the high- fat group, although they differed significantly from the high-fat group in the PI3K and Akt inhibitor groups (Figure 2C and D). This indicated that PI3K/Akt pathway blocking could affect ultrastructural changes of hepatocellular mitochondria. The change was similar to that resulting from a high-fat diet.

Hepatocellular mitochondria function and PI3K/Akt pathway

To investigate whether PI3K/Akt signaling is associated with an increase in the permeability of the outer mitochondrial membrane, we measured the mean fluorescence intensity (MFI) of Rh123 on the mitochondrial membrane in animals of the four groups. The MFI was 249.121% ± 13.526% in the high-fat group, and 389.385% ± 18.612% in the control group, indicating that there was a significant decrease in the mitochondrial membrane potential in the high-fat group. The PI3K and Akt inhibitor groups showed similar decreases in the mitochondrial membrane potential (211.326% ± 12.114% and 214.326% ± 13.321%, respectively) compared with the control group (Figure 3). These results suggested that mitochondrial function was impaired to the same degree in the high-fat and both inhibitor groups, strongly sug-

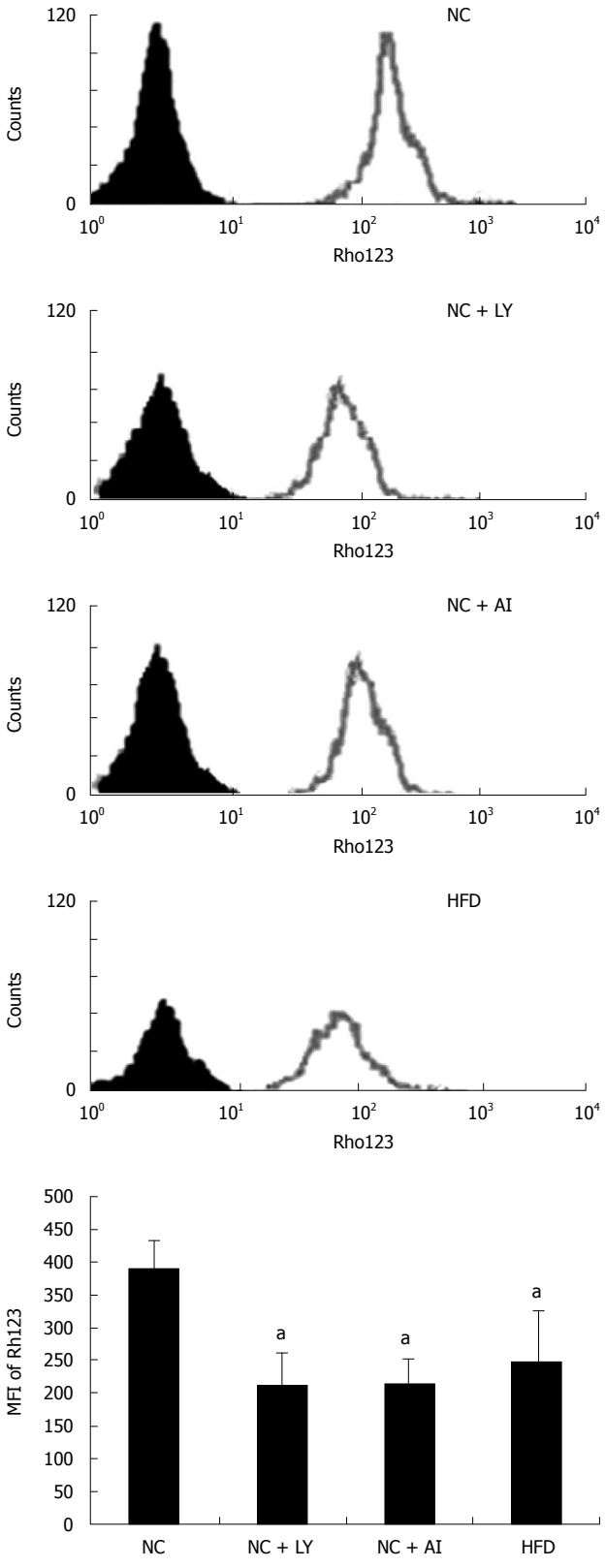

Figure 3 Changes in the membrane potential of hepatocyte mitochondria. Fluorescence intensity (MFI) of Rh123 was 389.385% ± 18.612% in the normal control (NC) group, but decreased to 268.326% ± 13.526% in the high-fat group (HFD); and in PI3K and Akt blocker groups, MFI had a very similar change (211.326% ± 12.114%, 214.326% ± 13.321%, respectively), indicating a decrease in the mitochondrial membrane potential (Dym) in high-fat, PI3K and Akt blocker groups. $^{a}P < 0.05$ *vs* NC group.

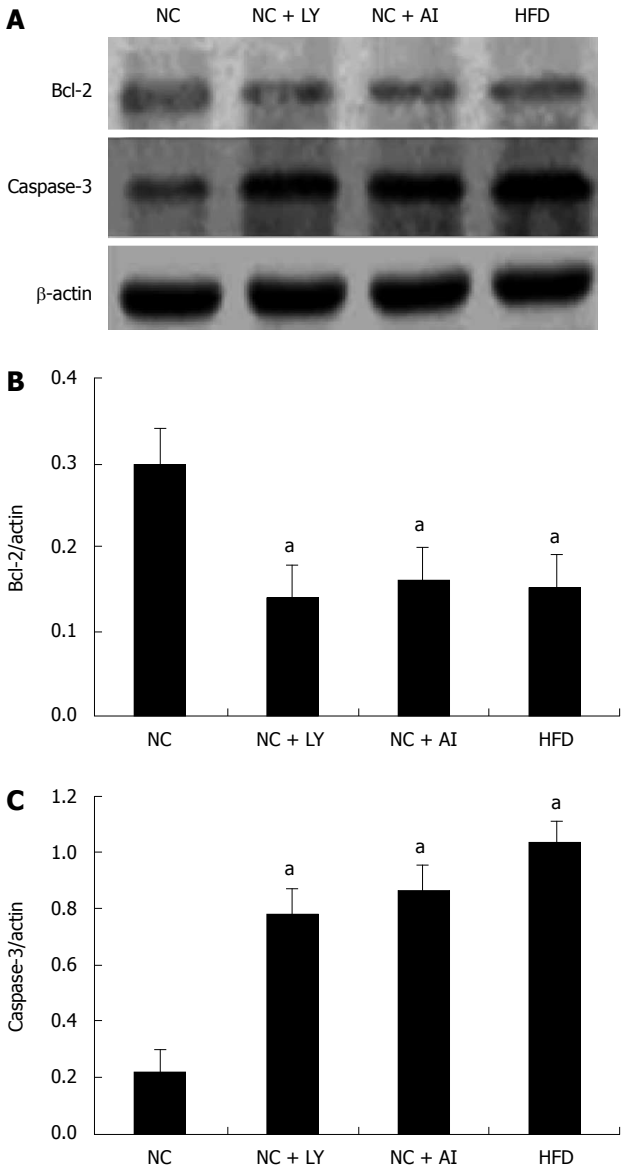

Figure 4 Western blotting analysis of caspase-3 and Bcl-2 in hepatocytes. A: Compared with control values [normal control (NC) group, lane 1], the expression levels of Bcl-2 protein decreased in the phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt)-blocker and high-fat groups (lanes 2-4). In contrast, caspase-3 expression was equally elevated in each of the three treatment groups compared with the control value; B, C: Band density values are compared with those of the β-actin loading control. $^{a}P < 0.05$ *vs* NC group.

gesting that the PI3K/Akt signal transduction pathway is associated with permeabilization of the outer mitochondrial membrane.

Apoptotic genes and PI3K/Akt pathway

One way in which changes in the PI3K/Akt signaling pathway might have induced mitochondrial apoptosis was through the effects of the apoptosis-related proteins Bcl-2 and caspase-3. To test this theory, we measured the expression level of these two proteins using Western blottings (Figure 4). We found that Bcl-2 expression showed a similar decrease in the high-fat, P13K-inhibitor, and

Akt-inhibitor groups (by 64%, 61% and 62%, respectively) compared to the control group, Conversely, the level of caspase-3 increased by a similar margin in the 3 experimental groups (42%, 31% and 29.5%, respectively) compared to the control group. This result demonstrated that there may be a link between the PI3K/Akt signaling pathway and the mitochondrial pathway of apoptosis in rats treated with high-fat, P13K inhibitor, or Akt inhibitor.

DISCUSSION

In this study, we attempted to investigate whether the PI3K/Akt pathway could mediate mitochondrial impairment during the development of NAFLD, and whether this might explain why hepatic insulin resistance is critical to the development of this disease. We found that hepatocytes of rats fed a high-fat diet accumulated fat and developed deformed mitochondria, a decreased mitochondrial membrane potential, and decreased expression of PI3K and Akt proteins. Unexpectedly, we found that blocking the PI3K/Akt pathway with either a PI3K or Akt inhibitor led to hepatocellular fat accumulation and mitochondrial impairment indistinguishable from that of high-fat fed rats. These findings suggest that signals transduced by the PI3K/Akt pathway are involved in the pathogenesis of NAFLD.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade is an important component of insulin signaling in normal tissues, where it mediates glucose uptake and homeostasis, as well as being an important regulator of cell survival in numerous cell types[12]. In this study, we found that both the insulin resistance index (HOMA-IR) and serum levels of hepatocellular enzymes were significantly increased in response to a high-fat diet, suggesting that insulin resistance and hepatocyte damage may coexist in our high-fat experimental model.

Mitochondrial dysfunction is known to play a central role in the hepatocyte damage of NAFLD[13]. Although our current experiment confirmed that mitochondrial dysfunction is associated with progression of liver pathological changes, the mechanisms initiating mitochondrial dysfunction in this disease are unknown. Specifically, it is not clear whether mitochondrial damage and changes in insulin signal transduction are directly related to the pathophysiology of nonalcoholic fatty liver induced by high fat. A number of studies have indicated that insulin can stimulate mitochondrial biogenesis[14] and alter mitochondrial morphology in obese, insulin-resistant and type 2 diabetic individuals[15]. In the current study, we found that a high-fat diet led to an increase in the proportion of morphologically abnormal mitochondria as well as an increase in the insulin resistance index. Similarly, less marked results were seen with treatment of the P13K- or Akt inhibitors, suggesting that changes in the PI3K/Akt signal transduction pathway may mediate these changes in mitochondria morphology.

The transmembrane potential (Dym) of mitochondria is known to play a crucial role in their normal function[16]. We found that the mitochondrial transmembrane potential

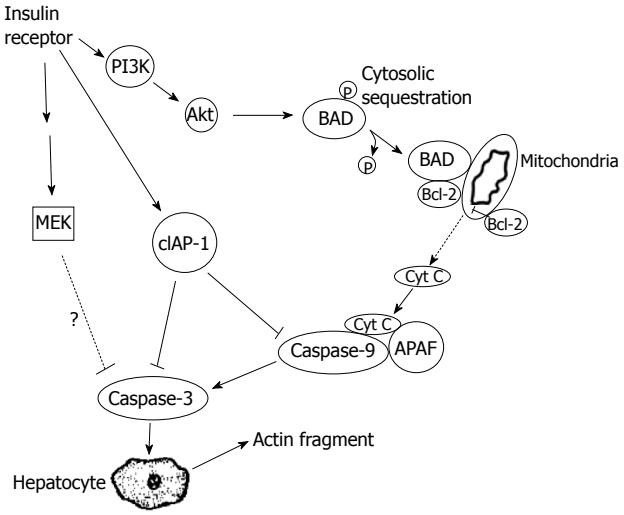

Figure 5 Simulation diagram of the relationship between insulin signaling pathway and hepatocyte apoptosis. PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B; BAD: Bcl2-associated agonist of cell death; MEK: Mitogen-activated protein kinase kinase; cIAP-1: Cell inhibitors of apoptosis protein-1; Cyt C: Cytochrome c; APAF: Apoptotic peptidase activating factor.

in the high-fat group, as well as those treated with either the PI3K- or AKT inhibitor, was significantly lower than control values, further implicating the P13K/Akt pathway in mitochondrial dysfunction.

Our results have confirmed some previous findings by Mehta *et al*[17], who reported that hepatic steatosis is frequently associated with obesity, type 2 diabetes, and hyperlipidemia, with insulin resistance being a key pathogenic factor in NAFLD and mitochondrial damage being characteristic of the disease. However, the current study is the first to implicate the PI3K/Akt signal transduction pathway in the morphological and functional changes in hepatocyte mitochondria, as well as insulin resistance induced by high fat.

Recent evidence suggests that hepatocellular injury in a number of liver diseases is accompanied by activation of the apoptotic pathways[18]. We found morphological (deformed mitochondria), and functional abnormalities (a reduction in the mitochondrial membrane potential) in hepatocyte mitochondria consistent with apoptosis in high-fat, PI3K and Akt inhibitor groups. We also examined the expression of two proteins in the Bcl-2 family of anti-apoptotic proteins. Proteins in this family are known to regulate apoptosis at peri-mitochondrial sites. The PI3K/Akt signaling pathway has been shown to have an anti-apoptotic effect by activating Bcl-2 to inhibit the apoptotic mediator caspase-3[19]. We found that, in rats fed a high-fat diet, expression of PI3K, phosphorylated Akt, and Bcl-2 decreased, but the expression of caspase-3 increased, suggesting a mechanism by which apoptosis may be triggered in NAFLD. To further support a role for the P13K pathway in mediating apoptosis in NAFLD, we found that the P13K- and Akt inhibitors led to a similar decrease in Bcl-2 expression and a significant increase in caspase-3 expression (Figure 5).

Apoptosis is a process of active cellular self-destruc-

tion that requires the expression of specific genes including those of the Bcl-2 gene family[20]. Of these, Bax, Bad and Bak promote cell death, whereas Bcl-2 and Bcl-xL inhibit apoptosis and promote cell survival[21]. The results of a recent study suggest that caspase-3 can cause permeabilization of cells, with the help of pro-apoptotic Bcl-2 proteins. Until recently, the prevailing view has been that caspase-3 activation represents the apex of the caspase cascade within the mitochondrial apoptotic pathway.

Some Bcl-2 family members located on the mitochondrial membrane have been shown to be able to alter the permeability of the mitochondrial membrane and trigger the activation of caspases[22]. Programmed cell death might thus be activated *via* a membrane bound pathway, in which the signal is initiated at the mitochondrion[23]. Pro-apoptotic compounds are normally sequestered in the intermembrane space[24]. When the permeability of the outer mitochondrial membrane increases, these proteins are released into the cytosol, forming the apoptosome and subsequently activating caspase-3[25]. The results of the current study indicate that not only high fat, but also blockage of the PI3K/Akt pathway signal, lead to an increase in the permeability of the hepatic mitochondrial membrane, implicating this pathway in the apoptotic mechanisms triggered by a high-fat diet. All these results lead us to propose a disease model in which the PI3K/Akt signal transduction pathway induces apoptosis *via* a Bcl-2/caspase-3 mitochondrial-dependent pathway, in which phosphorylation of Bad results in targeting of Bcl-xL to the mitochondrial membrane, where Bcl-2 interacts with and inactivates anti-apoptotic Bcl-2 proteins, thereby inducing apoptosis[26].

In conclusion, the present study suggests that fat accumulation in the liver may impair PI3K/Akt pathway signal transduction and thereby activate the mitochondrial membrane pathway of apoptosis, leading to hepatocyte damage.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is caused by triglyceride (TG) accumulation within the liver, which may progress to fibrosis, cirrhosis and liver failure. Mitochondrial dysfunction which caused hepatocyte apoptosis is an important element in the pathogenesis of NAFLD. Hepatic insulin resistance is also a critical component in the development of NAFLD, which is characterized by a marked reduction in the activity of the insulin signaling pathway, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway. But the potential relationship between the PI3K/Akt signaling pathway and the mitochondrial abnormalities that underlie NAFLD remain unclear.

Research frontiers

Recent reports have highlighted the insulin-induced signaling pathways in various cells. In the presence of insulin, the insulin receptor normally phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the activation of several signaling pathways, including the PI3K/Akt pathway. PI3K and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types. The mitochondrial impairment during the development of NAFLD is also an area of intense research. Mitochondrial dysfunction is an important element in the pathogenesis of NAFLD, mainly hepatocyte apoptosis. Recent evidence showed that mitochondria participates in the regulation of both cell proliferation and death, including apoptosis, and are thus potential mediators of the PI3K/Akt signaling pathway.

Innovations and breakthroughs

This is the first study to report the relationship between mitochondria impairment and the activity of the PI3K/Akt signaling pathway during the development of NAFLD, which suggests that fat accumulation in the liver may impair PI3K/Akt pathway signal transduction and thereby activate the mitochondrial membrane pathway of apoptosis, leading to hepatocyte damage.

Applications

The results of this study indicated that the PI3K/Akt pathway may mediate mitochondrial impairment during the development of NAFLD, and this might explain why hepatic insulin resistance is critical to the development of this disease.

Terminology

Insulin signaling pathways, including the metabolic PI3K/Akt pathway. PI3K and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types. Mitochondrial impairment, an important element in the pathogenesis of NAFLD. Mitochondria participates in the regulation of both cell proliferation and death, including apoptosis, and are thus potential mediators of the PI3K/Akt signaling pathway.

Peer review

This is a well written paper with well thought out, well-controlled data. The paper evaluates the effect of a high-fat diet and correlates it with similar findings seen with blocking the P13K or Akt inhibitors, and thereby implicated this pathway as the mechanism for high-fat diet hepatic injury.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6119

World J Gastroenterol 2010 December 28; 16(48): 6119-6122
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

High prevalence of nonalcoholic fatty liver in patients with idiopathic venous thromboembolism

Matteo Nicola Dario Di Minno, Antonella Tufano, Anna Russolillo, Giovanni Di Minno, Giovanni Tarantino

Matteo Nicola Dario Di Minno, Antonella Tufano, Anna Russolillo, Giovanni Di Minno, Giovanni Tarantino, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, 80131 Naples, Italy
Author contributions: Tarantino G and Di Minno MND conceived the study and wrote the manuscript; Tarantino G performed the statistical analysis; Di Minno G critically revised the paper; Tufano A and Russolillo A performed the clinical investigation.
Supported by An Institutional Grant of the Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, 80131 Naples, Italy
Correspondence to: Giovanni Tarantino, MD, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Via S. Pansini, 5, 80131 Naples, Italy. tarantin@unina.it
Telephone: +39-81-7462024 Fax: +39-81-5466152
Received: April 1, 2010 Revised: May 25, 2010
Accepted: June 1, 2010
Published online: December 28, 2010

Abstract

AIM: To assess the prevalence of nonalcoholic fatty liver disease (NAFLD) in patients with idiopathic venous thromboembolism (VTE).

METHODS: In a case-control study, after excluding subjects with well-consolidated risk factors for VTE, idiopathic VTE was documented in 138 consecutive patients who were referred to our department. Two hundred and seventy-six healthy sex/age/body-mass-index-matched subjects, without any clinical/instrumental evidence of VTE, served as controls. All underwent a clinical/laboratory/ultrasound assessment for the presence of metabolic syndrome and NAFLD.

RESULTS: NAFLD was detected in 112/138 cases (81%) and in 84/276 controls (30%) [risk ratio: 2.7, 95% confidence interval (CI): 2.2-3.2, $P < 0.0001$]. Metabolic syndrome and smoking habit were more prevalent in patients with idiopathic VTE. The high prevalence of NAFLD in VTE was also confirmed after adjustment for inherited thrombophilia. NAFLD was clearly predicted by VTE (odds ratio: 1.8, 95% CI: 1.2-2.7, $P < 0.0001$).

CONCLUSION: NAFLD was independently associated with idiopathic VTE.

Key words: Thromboembolism; Metabolic syndrome; Nonalcoholic fatty liver disease

Peer reviewer: Astrid van der Velde, PhD, Team Wetenschap, Netherlands Heart Foundation, PO Box 300, 2501 CH, The Hague, The Netherlands

Di Minno MND, Tufano A, Russolillo A, Di Minno G, Tarantino G. High prevalence of nonalcoholic fatty liver in patients with idiopathic venous thromboembolism. *World J Gastroenterol* 2010; 16(48): 6119-6122 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6119.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6119

INTRODUCTION

Venous thromboembolism (VTE) has an annual incidence of 1-2 events/1000 people in the general population, and is considered to be an emerging health problem[1,2]. Arterial and venous thromboses have been historically considered as distinct entities due to thrombus composition and different response to antiplatelet or anticoagulant drugs[3]. Metabolic syndrome (MS), which affects > 20% of the whole population[4,5], increases cardiovascular risk[6] by a blood hypercoagulability-related mechanism. This phenomenon is the result of abnormally high plasma levels of plasminogen activator inhibitor-1 (PAI-1), fibrinogen and factors Ⅶ, Ⅷ and von Willebrand, as well intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1[7-12]. Indeed, antiphospholipid syndrome and hyperhomocysteinemia predispose to venous and

cardiovascular events[13-15]. Ageno *et al*[16] have reported an association between idiopathic VTE and MS, which has been confirmed by Ay *et al*[17]. Arterial hypertension is a cardiovascular risk factor for VTE[18,19]. In addition, type 2 diabetes is associated with several coagulation and fibrinolysis alterations that lead to a procoagulant, thrombogenic predisposition, and is likely to have a significant impact on VTE occurrence[20]. Abdominal obesity is currently accepted as an independent risk factor for VTE[21].

Nonalcoholic fatty liver disease (NAFLD) has been strictly associated with MS[22]. Insulin resistance is reckoned to be the major mechanism. NAFLD refers to a wide spectrum of liver damage, which ranges from simple steatosis to nonalcoholic steatohepatitis, advanced fibrosis and cirrhosis.

In a series of patients with idiopathic VTE, we tried to assess the prevalence of NAFLD, further expression of MS, comparing the data with those achieved in control subjects.

MATERIALS AND METHODS

Inclusion criteria

One hundred and thirty-eight patients with recent (< 6 mo) objective diagnosis of idiopathic VTE were enrolled in the study. One hundred and twenty patients had deep vein thrombosis (DVT), of which, 21 were associated with superficial vein thrombosis, nine were suffering from isolated pulmonary embolism (PE), and 16 with PE plus DVT. DVT was confirmed by Doppler ultrasonography (DUS). PE was documented by computed tomography.

VTE was defined as idiopathic in the absence of pregnancy or puerperium, known active malignancies, recent (< 3 mo) surgery or trauma, fracture, immobilization, lack of prophylaxis, acute medical disease, use of oral contraceptives, long-distance travel, a history of VTE or repeated birth loss. In contrast, when at least one of the previous risk factors was present, VTE was defined as secondary and the patients were excluded from the study.

As many as 276 healthy sex/age/body mass index (BMI)-matched subjects served as controls. In all of them, exclusion of DVT was based on clinical examination, use of D-dimer testing, and clinical pretest probability and, in some uncertain cases, by two DUS examinations within 1 wk of each other.

Exclusion criteria

Cases and controls who presented with unstable medical conditions were excluded. Other exclusion criteria were a history of infectious chronic diseases including hepatitis B and C, autoimmune and storage diseases, drug-induced hepatic steatosis, and prior use of medication known to affect inflammation, glucose metabolism or blood lipids. Alcohol abuse was ruled out, according to the DSM-IV diagnostic criteria, by means of screening tests such as MAST (Michigan Alcohol Screening Test) and CAGE (Cut down, Annoyed, Guilty, and Eye opener), as well as random tests for blood alcohol concentration and the use of a surrogate marker, e.g. mean corpuscular volume. Patients on antihypertensive therapy maintained a balanced medical regimen throughout the study.

Clinical, laboratory and imaging data

Sex, age, BMI, waist circumference, history of symptomatic atherosclerosis (i.e. ischemic stroke, transient ischemic attack, acute myocardial infarction, angina, intermittent claudication), arterial hypertension or use of antihypertensive drugs, diabetes mellitus or use of antidiabetic drugs, hyperlipidemia or use of statins or clofibrate, smoking habit (daily consumption of $\geqslant$ 1 cigarette), current use of heparin, oral anticoagulant or antiplatelet drugs were recorded. Subsequently, all patients underwent liver ultrasound (US), measurement of blood pressure, fasting glucose, transaminases and γ-glutamyl transferase activity, high-density lipoprotein (HDL) cholesterol and triglyceride levels. MS was diagnosed by the presence of at least three criteria (National Cholesterol Education Adult Treatment Panel Ⅲ) on the basis of abdominal obesity (waist circumference > 102 cm for men and > 88 cm for women), triglycerides $\geqslant$ 150 mg/dL, HDL-cholesterol < 40 mg/dL for men and < 50 mg/dL for women, blood pressure $\geqslant$ 130 mmHg and/or $\geqslant$ 85 mmHg, and fasting glucose $\geqslant$ 100 mg/dL. Obesity was recognized as a BMI $\geqslant$ 30.

The classification of "bright liver" or hepatic steatosis grade was based on the following scale of hyperechogenity at US: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney[23]. Diagnostic criteria for DVT were observation of an intraluminal venous thrombus, loss of compressibility, and lack of flow at DUS.

Statistical analysis

We observed how many times the event of interest, i.e. NAFLD occurred in the experimental group or cases (VTE) and in controls. Statistical confidence was increased by taking two controls per case. The RR and 95% CI was the ratio of the proportions of cases with a positive outcome in the two groups. Patients' clinical characteristics were compared using Student's *t* test (continuous variables) and the χ^2 test (dichotomous variables). A logistic regression (stepwise model) was adopted, in which NAFLD was the dependent variable and sex, anthropometric parameters (BMI, waist circumference), metabolic features (serum HDL-cholesterol, triglycerides and glucose), systolic blood pressure, diastolic blood pressure, smoking habit and finally VTE were employed as independent variables. MS as entity was not considered in prediction, to avoid multicollinearity. The same tool (enter method) was carried out to predict VTE presence the by US grade of steatosis. Statistical analysis was performed with MedCalc® 11.2.

RESULTS

The mean age in the cases and controls was 41.8 ± 13.0 and 43.4 ± 15.7 years, and the mean BMI in the two groups was 30.4 ± 4.1 and 29.6 ± 3.9 (P = 0.79 and P

Table 1 Prevalence of clinical and laboratory findings and smoking in the whole population

	Cases (138) yes/not	Controls (276) yes/not	RR (95% CI)	*P*
Smoking habit	81/57	108/168	1.5 (1.2-1.8)	0.000
Fasting glucose ⩾ 110 mg/dL	84/54	110/166	1.5 (1.25-1.9)	0.000
Abdominal obesity	98/40	107/169	1.8 (1.5-2.2)	< 0.0001
Hypertriglyceridemia	72/66	131/145	1.1 (0.8-1.3)	0.35
Low HDL-cholesterolemia	91/47	146/130	1.2 (1.1-1.5)	0.008
Hypertension	94/44	111/165	1.7 (1.4-2.0)	< 0.0001

RR: Risk ratio; CI: Confidence interval; HDL: High-density lipoprotein.

= 0.81, respectively). Sex distribution between the two groups did not show significant differences (χ^2, $P = 0.95$).

Among the 138 VTE patients, 112 (81%) had concomitant NAFLD, whereas 84 out of 276 (30%) controls suffered from NAFLD (RR = 2.7, 95% CI: 2.2-3.2, $P < 0.0001$). The RRs of smoking habit and single components of MS are shown in Table 1.

Factor V G1691A and/or prothrombin G20210A polymorphisms (major determinants) were not detected in 80 out of 138 cases (58%) and in 188 out of 276 controls (68.1%). A higher prevalence of NAFLD in patients with VTE but without inherited thrombophilia versus controls was also confirmed on the basis of this further selection; 62 patients in cases and 61 in controls (RR = 2.4, 95% CI: 1.9-3, $P < 0.0001$). When predicting NAFLD, VTE played an important role, which confirmed the aforementioned findings, but also smoking habit and some MS components gave a good prediction (Table 2). In contrast, age, BMI and sex did not enter the model, because their significance was > 0.1. The presence of VTE was well predicted by grade of steatosis, as revealed by US (OR = 1.9, 95% CI: 1.05-3.8, $P < 0.0001$).

DISCUSSION

Our main finding was a significantly higher prevalence of NAFLD in idiopathic VTE patients than in controls, which was confirmed after adjusting for inherited thrombophilia. Although these results were partially expected, they were highlighted for the first time in the present study. What is more, this report extends previous data[16] on MS. In a recent report[24], rather than considering "all-or-nothing" definitions for MS, the additive effect of having more than one of the MS features has been considered. This is a controversial point. However, what if physicians use an indirect parameter of MS presence, e.g. NAFLD? The present study supports a significant correlation between every single component of MS and VTE, even though the strictest association was demonstrated between NAFLD, a further expression of MS as a whole[22], and VTE, which by-passes the restrictive criteria of MS.

These data lend credence to the possibility that VTE is an early clinical event in a generalized vascular disease

Table 2 Prediction of nonalcoholic fatty liver disease

	OR (95% CI)	*P*
Venous thromboembolism	1.8 (1.2-2.7)	< 0.0001
Fasting glucose ⩾ 110 mg/dL	1.0 (1.0-1.02)	0.04
Abdominal obesity	1.7 (1.14-2.5)	0.0001
Hypertension	1.02 (1.0-1.04)	0.03
Smoking habit	1.6 (0.8-2.3)	0.0002

Method: Stepwise; variable entered if $P < 0.05$, and removed if $P > 0.1$. OR: Odds ratio; CI: Confidence interval.

that involves venous and arterial circulation. Our results support the need for further studies to evaluate the risk of subsequent cardiovascular events in VTE patients without MS, but with NAFLD.

In trying to establish the complex interaction between VTE and NAFLD, we stress that they share common mechanisms. First of all, we should pinpoint the role of PAI-1. In fact, abdominal fat, liver steatosis and serum triglycerides levels have been shown to be significant and independent determinants of PAI-1 plasma level in an unselected sample of male adults upon adjustment for age and therapy[25]. Additionally, the pro-angiogenic factor, vascular endothelial growth factor, which is generally thought to be the main factor that determines VTE in patients with cancer[26], plays a key role in NAFLD[27]. Recent evidence has substantiated that NAFLD is associated with elevated circulating levels of ICAM-1, which throws further light on inflammation-related liver damage[28]. Another intriguing link is represented by smoking, which is a plain risk factor for the development of VTE. Indeed, this relationship could be justified by the presence of NAFLD. In fact, Yuan *et al*[29] have provided novel evidence to demonstrate that tobacco smoke exposure can accelerate the development of experimental NAFLD.

The limitations of the present study are as follows. Our control group comprised individuals who were referred for signs or symptoms initially suggestive of VTE, and it may not adequately represent a general healthy population. However, the prevalence of MS in our control group was comparable to that reported in the general Italian population[5], which suggests that, with a differently selected control group, our findings could have been comparable to those reported in the previous study. Although patients with cancer were excluded from our study, some VTE patients might have had occult cancer at the time of investigation. The impact of occult cancer on the components of MS is unknown; however, its impact on the results of our analysis was likely to have been low. With regard to the definition of idiopathic VTE, we have defined it as VTE that occurs in patients without the most common known risk factors. Based on our definition, other risk factors might have been missed, but this is unlikely to have significantly influenced our results. Another potential point of criticism relates to the size of the study, which was not very large.

In conclusion, an eventual association between VTE and NAFLD should be always pursued.

COMMENTS

Background

Venous thromboembolism (VTE) with an annual incidence of 1-2 events/1000 people in the general population is considered to be an emerging health problem.

Research frontiers

Metabolic syndrome affects > 20% of the whole population, and increases the cardiovascular risk by a blood hypercoagulability-related mechanism.

Innovations and breakthroughs

This is believed to be the first evidence to show a strict link between idiopathic VTE and nonalcoholic fatty liver disease (NAFLD). Smoking could increase the risk of VTE by worsening NAFLD.

Applications

Patients suffering from MS should be warned against their increased risk of VTE.

Peer review

In this paper, research on the prevalence of NAFLD in idiopathic VTE is presented, which is an interesting topic.

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S- Editor Wang JL **L- Editor** Kerr C **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6123

World J Gastroenterol 2010 December 28; 16(48): 6123-6127
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Extrahepatic portal vein thrombosis in children and adolescents: Influence of genetic thrombophilic disorders

Andrea Pietrobattista, Matteo Luciani, Juan G Abraldes, Manila Candusso, Simona Pancotti, Massimo Soldati, Lidia Monti, Giuliano Torre, Valerio Nobili

Andrea Pietrobattista, Manila Candusso, Giuliano Torre, Valerio Nobili, Liver Unit, Research Institute, Bambino Gesù Children's Hospital, 00165 Rome, Italy
Matteo Luciani, Department of Hematology, Bambino Gesù Children's Hospital, 00165 Rome, Italy
Juan G Abraldes, Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clínic, IDIBAPS and CIBERhed, University of Barcelona, 080036 Barcelona, Spain
Simona Pancotti, Massimo Soldati, Department of Laboratory Pediatrics, Bambino Gesù Children's Hospital, 00165 Rome, Italy
Lidia Monti, Radiology Department, Bambino Gesù Children's Hospital, 00165 Rome, Italy
Author contributions: Pietrobattista A and Luciani M designed the study and contributed equally to this work; Abraldes JG, Candusso M, Pancotti S, Soldati M and Monti L contributed to the collection and analysis of data; Torre G and Nobili V contributed to the manuscript preparation and revisions.
Supported by Grant from Bambino Gesù Children's Hospital
Correspondence to: Valerio Nobili, MD, Liver Unit, Research Institute, Bambino Gesù Children's Hospital, Piazza S. Onofrio 4, 00165 Rome, Italy. nobili66@yahoo.it
Telephone: +39-6-68592243 Fax: +39-6-68592192
Received: January 26, 2010 Revised: March 10, 2010
Accepted: March 17, 2010
Published online: December 28, 2010

Abstract

AIM: To explore the prevalence of local and genetic thrombophilic disorders as risk factors for portal vein thrombosis (PVT) in our series, the largest ever published in pediatric literature.

METHODS: We conducted a case-control study enrolling 31 children with PVT and 26 age-matched controls. All were screened for thrombophilia, including genetic disorders, protein C, protein S and homocysteine deficiencies. All coagulation parameters were studied at least 3 mo after the diagnosis of portal vein obstruction.

RESULTS: In our study we showed that most pediatric patients with PVT have local prothrombotic risk factors, which are probably the most important factors leading to PVT. However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal umbilical vein catheterization or sepsis.

CONCLUSION: Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor.

Key words: Portal vein thrombosis; Children; Thrombophilic disorders; Protein C; Protein S

Peer reviewers: Wallace F Berman, MD, Professor, Division of Pediatric GI/Nutrition, Department of Pediatrics, Duke University Medical Center, Duke University School of Medicine, Durham, Box 3009, NC 27710, United States; Dr. Marco Senzolo, Liver Transplantation and Hepatobiliary Unit, Royal Free Hospital, London, United Kingdom

Pietrobattista A, Luciani M, Abraldes JG, Candusso M, Pancotti S, Soldati M, Monti L, Torre G, Nobili V. Extrahepatic portal vein thrombosis in children and adolescents: Influence of genetic thrombophilic disorders. *World J Gastroenterol* 2010; 16(48): 6123-6127 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6123.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6123

INTRODUCTION

Portal vein thrombosis (PVT) is a common cause of portal hypertension. To date, the pathogenesis of PVT in children still remains unexplained, yet it is the major cause of portal hypertension in children and adolescents.

Variceal bleeding due to PVT is a recognized cause of upper gastrointestinal bleeding in children in developing countries[1,2]. Hereditary thrombophilias that are known to predispose to venous thrombosis and PVT include certain mutations of the prothrombin (*PTHR*), factor V Leiden (*FVL*) or methylenetetrahydrofolate reductase (*MTHFR*) genes or deficiency of one of the natural anticoagulant proteins C and S[3-6].

Some neonatal events such as abdominal surgery, sepsis or umbilical vein catheterization (UVC) are typically identified in patients with PVT[7], with an incidence of thrombosis complicating UVC reported in the literature as high as 44%[8-10]. Moreover, other factors, such as dehydration, also have been suggested to play a part in PVT development[11].

Finally, despite all efforts, the cause of the blocked portal vein remains obscure in 50% to 90% of children[12]. Unlike in adults, studies of thrombophilic disorders in children are scant, and to date only a few studies have evaluated the prevalence of hereditary thrombophilic disorders in children and adolescents with PVT[1,2,13,14].

The aim of our study was to explore the prevalence of local and genetic thrombophilic disorders as risk factors in PVT in our series, the larger ever published.

MATERIALS AND METHODS

A 2-year prospective case-control study (December 2006 to December 2008) was carried out at Bambino Gesù Children's Hospital in Rome, Italy. The study was conducted according to the principles of the Declaration of Helsinki, informed consent was obtained, and the authors' institutional review board approved the study.

We enrolled two groups of subjects for the study. Group 1 included 31 (20 male) Caucasian patients with PVT; mean age, 7 yr 8 mo (range: 11 mo - 18 yr 2 mo). Group 2 comprised 26 children (15 male) free of liver disease and thrombotic events, age matched with group 1, who were inpatient in our hospital during the study. Upper endoscopy was performed in all patients of Group 1 and showed signs of portal hypertension but not always the presence of varices.

PVT was diagnosed by Doppler ultrasound scan or angiography [14 patients underwent both these procedures and we found a very high concordance (98%)]; both procedures were performed by the same radiologist. Normal liver function tests or no other sign of liver disease was an inclusion criteria, as well as the absence of histological abnormalities on liver biopsy examination when performed.

All patients were screened for thrombophilia including genetic disorders (*MTHFR C677T, FVL, PTHR G20210A*) protein C (PC), protein S (PS) and homocysteine. All coagulation parameters were studied at least 3 mo after the diagnosis of portal vein obstruction to avoid falsely low levels related to active thrombosis. Abnormal values of coagulation factors might be observed in patients with PVT due to impaired liver synthesis. Due to the lack of standards to define PC or PS deficiency in this setting, we used a ratio of these levels to prothrombin rate lower than 0.7 as a working definition for these deficits. This definition should exclude those patients with low PC and PS levels related to impaired liver synthesis, which would also affect prothrombin rate.

None of the patients were on anticoagulant or antiplatelet therapy at the time of the study. Detailed history was obtained with special emphasis on history of umbilical catheterization (50%), umbilical sepsis (6%), admission to neonatal intensive care unit (72%), severe gastroenteritis and dehydration (6%), history of thromboembolism in the patients and their family members (3%), and history of parental consanguinity (1%).

Genetic and specific analysis

Genomic DNA was isolated from white blood cells by standard procedures. A 222 bp fragment of the Factor V gene, a 165 bp fragment of the Factor Ⅱ gene and a fragment of the MTHFR gene are amplified from human genomic DNA using specific primers and the amplicon is detected by fluorescence using a specific pair of probes consisting of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of a PCR cycle. The same specific probes are also used to determine the genotype by performing a melting curve analysis.

PC and PS activity was measured by coagulometric assay. PC activity was measured using a specific activator extracted from southern copperhead snake venom (*Agkistrodon c. contortrix;* STA protein C, Roche). PS activity was determined based on the principle of activated factor V inhibition (STA protein S, Roche). Protein activity was expressed as a percentage of a reference plasma pool.

Statistical analysis

Continuous variables were compared with unpaired *t*-test. Categorical variables were compared with the Fisher exact test. The association between the presence of an abnormality and PVT was assessed with odds ratios and their 95% confidence intervals (CI). All analyses were performed with SPSS version 15 (Chicago, IL, USA).

RESULTS

The characteristics of the patients and controls are summarized in Table 1. In patients, the first manifestation of portal vein thrombosis occurred at a mean age of 4 year 9 mo (range: 6 mo to 16 year 2 mo). This was upper gastrointestinal (GI) bleeding in 87% of patients followed by splenomegaly in 13%. Eighty-one percent of the patients had varices at presentation, while 74% had splenomegaly. Sixty-eight percent of the patients had a history of a local prothrombotic factor (neonatal sepsis or umbilical vein catheterization). Only 1 patient with a local prothrombotic factor was present in the controls.

Congenital thrombophilic disorders

FVL mutation was found in 2 (7%) patients and heterozygous *G20210A* mutation was found in 3 (10%), while

Table 1 Characteristics of the patients (mean ± SD)

	Controls	Patients
Age	6 yr 9 mo	7 yr 8 mo
Sex	15 males	20 males
INR (%)	1.05 ± 0.09	1.35 ± 0.21
ALT (UI/L)	31.15 ± 13.83	25.45 ± 14.71
AST (UI/L)	37.23 ± 11.86	31.42 ± 11.90
GGT (UI/L)	16.46 ± 16.89	17.68 ± 18.40
Albumin (g/dL)	4.16 ± 0.48	3.88 ± 0.37
Bil/tot (mg/dL)	0.78 ± 0.13	0.81 ± 0.27
Bil/dir (mg/dL)	0.12 ± 0.11	0.18 ± 0.14
ALP (UI/L)	404.09 ± 154.90	515.97 ± 180.44

INR: International normalized ratio; ALT: Aspartate aminotransferase; AST: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; Bil/tot: Bilirubin/total; Bil/dir: Bilirubin/direct; ALP: Alkaline phosphatase.

homozygosity for these two mutations was not found in any patient. No control patient had mutations in these two genes.

MTHFR-C667T mutation was found in 16 (68%) patients, and 4 (13%) were homozygous for this mutation. The corresponding figures in the control group were 6 (23%) and 0 patients, respectively (Table 2). Therefore, the odds ratio for having at least an allele with the *MTHFR-C677T* polymorphism in patients with portal vein thrombosis was 7.00 (95% CI: 2.15-22.85), suggesting that this polymorphism could increase the risk of PVT. However, intriguingly enough, levels of homocysteine in controls were similar to that found in cases ($P = 0.28$), and all subjects had normal values.

Coagulation inhibitor protein deficiency

Four patients presented PC levels compatible with prot C deficiency and 4 had PS levels compatible with prot S deficiency. We also investigated their parents to show any prot C alteration. Moreover, none of the controls had prot C deficiency while one had values consistent with prot S deficiency.

The overall frequency of inherited thrombophilic abnormalities (excluding mutations in *MTHFR*) in cases was 32% (8 patients had only one factor, one patient had two factors and one patient had three factors). This prevalence was significantly lower in controls (1/26, 4%) (Table 3). Thus, the OR of having any inherited prothrombotic disorder (excluding *MTHFR* polymorphism) in patients with portal vein thrombosis as compared to controls was 11.91 (95% CI: 1.41-100.77).

Eight patients (26%) had neither a thrombophilic nor a local factor (idiopathic portal vein thrombosis). This figure is in keeping with previously published series of adult portal vein thrombosis. Thirteen patients (42%) had only local factors, eight (26%) had both local and thrombophilic factors and only 2 patients (6%) had isolated inherited thrombophilic factors with no history of a local factor.

There were no associations between the presence of an inherited prothombotic disorder and the manner of initial presentation of PVT (splenomegaly or GI bleeding). However, patients with inherited thrombophilic factors were less likely to have varices at the time of presentation (6 out of 10 patients) as compared with patients without (19 out of 21; $P = 0.045$).

No recurrent thrombotic events were recorded in a 24 mo long follow-up, both in patients with and without prothrombotic disorders.

Table 2 Distribution of type and prevalence of mutations in study subjects *n* (%)

Type of mutations	Prevalence of mutations		
	Control (26)	Case (31)	Total (57)
Normal	20 (76.9)	10 (32.3)	30 (52.6)
Abnormal *MTHFRC677T*			
Heterozygous	6 (23.1)	17 (54.8)	23 (40.4)
Homozygous	0 (0)	4 (12.9)	4 (7.0)

$P = 0.001$. *MTHFR*: Methylenetetrahydrofolate reductase.

Table 3 Frequency of inherited thrombophilic abnormalities *n* (%)

Any thrombophilic factor	Control	Case	Total
No	25 (96.2)	21 (67.7)	46 (80.7)
Yes	1 (3.8)	10 (32.3)	11 (19.3)
Total	26 (100)	31 (100)	57 (100)

$P = 0.008$ (Fisher's exact test).

DISCUSSION

In this study we show that most patients with pediatric portal vein thrombosis have a history of a local prothrombotic factor, such as sepsis or umbilical vein catheterization, a figure much higher than that reported for adult portal vein thrombosis (around 30%)[15]. Therefore, and distinctly from adult PVT, local factors seem clearly to be the major players implicated in the development of PVT in children. However, a major finding of this study is that inherited disorders of coagulation are also frequently found in these patients (38%, as compared with 4% in controls), though most times in association with a local factor. This suggests, on one hand, that inherited thrombophilic disorders might facilitate the development of PVT thrombosis after a "local" event. On the other hand, since the presence of a thrombophilic disorder might have an impact on the management and follow-up of these patients, our data support the notion that children with PVT should be thoroughly investigated for the presence of a thrombophilic factor, even if an obvious history of a local factor is present.

The most frequent thrombophilic disorder was a deficit in naturally occurring anticoagulants (proteins C or S). It is possible, however, that the prevalence of coagulation inhibitor protein deficiency might be overestimated, since these factors might decrease due to altered liver synthesis and decreased hepatic blood flow secondary to the thrombosis, as already suggested in literature[6,16]. Notably, some

Table 4 Frequency of thrombophilic disorders in children and adolescents with portal vein thrombosis *n* (%)

Study	Coagulation inhibitor protein deficit		Gene mutations		
	PC	PS	*FVL*	*PTHR*	*MTHFR*
Dubuisson *et al*[13] (*n* = 20)	9 (45)	13 (65)	NP	NP	NP
Uttenreuther-Fischer *et al*[2] (*n* = 23)	NP	NP	2 (9)	NP	NP
Heller *et al*[1] (*n* = 24)	NP	NP	4 (17)	0	1 (4)
Pinto *et al*[12] (*n* = 14)	6 (43)	3 (21)	0	1 (7)	3 (21)
Current study (*n* = 31)	4 (13)	4 (13)	2 (6.5)	3 (9.7)	16 (67.7)

PC: Protein C; PS: Protein S; *FVL*: Factor V Leiden; *PTHR*: Prothrombin; *MTHFR*: Methylenetetrahydrofolate reductase; NP: Not provided.

studies have already shown a rise in the concentration of coagulation inhibitor proteins after a surgical correction directly bypassing the venous obstruction[17,18], confirming this hypothesis. However, even with our restrictive working definition of prot C and S deficit (less than 0.7 × prothrombin rate), we have found a high prevalence of these disorders, suggesting that their role is more relevant in pediatric than in adult PVT. Larger numbers with detailed family history (difficult to recruit in this setting) would be required to gain further insight into this finding.

In our population the *MTHFR-C677T* polymorphism was much more frequent in patients than in controls, suggesting that it behaves as a risk factor for PVT. However, in our cohort no difference in the levels of homocysteine between controls and patients was found. Thus, this polymorphism is not always associated with high plasma levels of homocysteine[19,20], even in patients with documented thrombotic events and no other risk factor for thrombophilia. This raises the question of whether the *MTHFR* gene polymorphism, without hyperhomocysteinemia, may itself contribute to thrombophilia. On the other hand, intermittent hyperhomocysteinemia may occur, which is not easily detectable even if clinically significant. In addition, the interpretation of homocysteine levels in these patients is problematic. Dietary imbalances, such as an inadequate intake of folate and vitamin B12 which are needed to break down excess homocysteine or methionine overabundance from dietary protein, may play a critical role in homocysteine metabolism[21,22].

Although the association between the *MTHFR* mutation and thrombosis has not yet been fully clarified[6,23,24], anticoagulation may be indicated in patients with *MTHFR* mutation (either homozygous or heterozygous, with or without hyperhomocysteinemia)[25] with previous thrombotic events and other thrombotic risk factors (pregnancy, oral contraceptives, surgery, sepsis and immobilization). Therefore, our data suggest that the presence of *MTHFR* mutations should be investigated in all pediatric patients with PVT. In addition, if hyperhomocysteinemia is present, therapy with folic acid and B6 and B12 vitamins should be instituted.

Another finding of this study was the lower prevalence of GI varices in patients with prothrombotic disorders. This might be an association by chance, or could reflect that those patients with a thrombophilic factor were more likely to be diagnosed in a phase of "recent thrombosis", when varices still have not developed. At any rate, no differences were observed in the clinical evolution of PVT between patients with genetic anomaly and those without.

In summary, most pediatric patients with PVT have local prothrombotic factors, which are probably the most important factors leading to PVT (Table 4). However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal UVC or sepsis. Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor. Future trials should evaluate the role of prophylactic low molecular weight heparins in children requiring UVC, especially in those with a family history of thrombotic events or other thrombotic risk factors.

COMMENTS

Background

Portal vein thrombosis (PVT) is a common cause of portal hypertension. To date, the pathogenesis of PVT in children still remains unexplained despite the fact that it is the major cause of portal hypertension in children and adolescents. Unlike in adults, studies of thrombophilic disorders in children are scant, and to date only a few studies have evaluated the prevalence of hereditary thrombophilic disorders in children and adolescents with PVT.

Research frontiers

To date, many pediatric patients with PVT have local prothrombotic factors, which are probably the most important factors leading to PVT. However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal umbilical vein catheterization (UVC) or sepsis. Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor. Future studies should evaluate the role of prophylactic low molecular weight heparins in children requiring UVC.

Innovations and breakthroughs

This series is the larger ever published so far. In this study the authors show that most patients with pediatric PVT have a history of a local prothrombotic factor, a figure much higher than that reported for adult portal vein thrombosis (around 30%). The authors suggest extending the thrombophilic screening of three different genetic mutations to better analyze this population.

Applications

Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor.

Peer review

This is a well written and important contribution to the pediatric literature. The paper describes the wider case series of patients with these conditions and few published series are present at the moment, the data could be of interest for the readers.

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S- Editor Wang JL **L- Editor** Logan S **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6128

World J Gastroenterol 2010 December 28; 16(48): 6128-6134
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Tissue factor in predicted severe acute pancreatitis

Ellen Andersson, Jakob Axelsson, Gunilla Eckerwall, Daniel Ansari, Roland Andersson

Ellen Andersson, Jakob Axelsson, Gunilla Eckerwall, Daniel Ansari, Roland Andersson, Department of Surgery, Clinical Sciences Lund, Lund University and Lund University Hospital, SE-221 85 Lund, Sweden
Author contributions: Andersson E designed the experiment, calculated data, wrote the paper; Axelsson J set up and performed the ELISA analyses; Eckerwall G included patients and performed ELISA analyses; Ansari D contributed in writing the paper and analysing data; Andersson R took part in the design of the experiment.

Supported by The Skane County Council Research and Development Foundation, No. REGSKANE-61401; and the Erik and Angelica Sparre Foundation, No. 081230
Correspondence to: Ellen Andersson, MD, Department of Surgery, Clinical Sciences Lund, Lund University and Lund University Hospital, SE-221 85 Lund,
Sweden. ellen.andersson@med.lu.se
Telephone: +44-121-4142972 Fax: +44-121-6272384
Received: June 18, 2010 Revised: August 30, 2010
Accepted: September 7, 2010
Published online: December 28, 2010

Abstract

AIM: To study tissue factor (TF) in acute pancreatitis and evaluate the role of TF as a predictive marker of severity.

METHODS: Forty-nine consecutive patients admitted to Lund University Hospital, fulfilling the criteria of predicted severe acute pancreatitis (AP), were recruited prospectively between 2002 and 2004. Blood samples for TF analyses were drawn at inclusion in the study and 12 h, 1 d and 3 d later.

RESULTS: Twenty-seven patients developed mild AP, and 22 patients severe AP. At inclusion in the study, the groups were comparable with respect to gender, aetiology, Acute Physiology and Chronic Health Evaluation Ⅱ score, and duration of pain. At inclusion in the study and at 12 h, TF was higher in the severe AP group (P = 0.035 and P = 0.049, respectively). After 1 and 3 d, no differences in TF levels were noted. Interleukin (IL)-6 was significantly higher in the severe AP group at all of the studied time points. C-reactive protein (CRP) was significantly higher in the AP group at 1 and 3 d. In receiver operating characteristic-curves, the area under the curve (AUC) for TF was 0.679 (P = 0.035) at inclusion in the study, and a cut off level for TF of 40 pg/mL showed a sensitivity of 71% and a specificity of 67%, whereas corresponding AUC for IL-6 was 0.775, P = 0.001, and for CRP was 0.653. IL-6 showed better AUC-values than TF at all time points studied.

CONCLUSION: TF-levels are raised early in severe AP. TF as an early predictive marker of severe AP is superior to CRP, but inferior to IL-6.

Key words: Acute pancreatitis; Coagulation; Prediction of severity; Tissue factor

Peer reviewer: Natalia A Osna, MD, PhD, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha, NE 68105, United States

Andersson E, Axelsson J, Eckerwall G, Ansari D, Andersson R. Tissue factor in predicted severe acute pancreatitis. *World J Gastroenterol* 2010; 16(48): 6128-6134 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6128.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6128

INTRODUCTION

Severe acute pancreatitis (AP) is one example of critical illness where both the inflammatory system and the coagulation system are to be considered as ticking bombs, where the most extreme scenarios result in multiple organ dysfunction and disseminated intravascular coagulation. Microcirculatory disturbances with micro vascular thromboses appear to play an important role both in the inflamed pancreas itself and in remote organ failure[1,2]. Clinical evidence is still sparse[3-5], but several experimental studies have suggested an important role of the coagulation system in the pathophysiology of AP[6-8]. One key to the cross-talk

between inflammation and coagulation are proteases, with enzymatic capacity to activate both inflammation and coagulation. Coagulation factors, such as factor Ⅶ (FⅦ) and tissue factor (TF), as well as thrombin, can bind to protease activated receptors (PARs) on various cells and elicit intracellular signalling, resulting in modulation of inflammatory response[9]. The PAR family has at least four members (PAR 1-4) where TF-FⅦ has been shown to be able to act through PAR-2, while TF-FⅦ-FX also activates PAR-1. PAR-2 is the only PAR not activated by thrombin[10].

Tissue factor is a trans-membrane glycoprotein, initiating the most important pathway of coagulation[11,12]. Tissue factor is expressed in the vascular adventitia, but may also be expressed in micro-particles which can be shed from leukocytes, endothelial cells, vascular smooth muscle cells and platelets[13]. In the normal setting TF is not in contact with circulating blood. When vessels are injured or when TF-expressing cells are stimulated by circulating pro-inflammatory cytokines or lipopolysaccharide (LPS), TF is exposed to the bloodstream. TF then binds and activates factor Ⅶ. Factor Ⅶ is a vitamin K-dependent trypsin-like serine protease, produced in the liver. It circulates in an inactive form, and requires the action of its allosteric regulator, TF, to convert it to the active enzyme (FⅦa). The TF-factor Ⅶ complex initiates coagulation by activating FX, eventually resulting in conversion of pro-thrombin to thrombin. Thrombin cleaves fibrinogen, resulting in abundant fibrin production and the formation of a clot. The activity of TF is counterbalanced by circulating tissue factor pathway inhibitor (TFPI). In addition to its well-established role in coagulation, TF, and to a lesser extent FⅦ, have also been associated with various other physiological processes of gene transcription, apoptosis and cytoskeleton reformation, such as in inflammation, sepsis, metastasis, angiogenesis and atherosclerosis, where the TF-FⅦa complex acts as a signalling receptor[14-17]. The role of TF/FⅦa signalling in inflammatory conditions is confirmed by TF/FⅦa regulated expression of the pro-inflammatory cytokine interleukin (IL)-8 in keratinocytes[18], and a role in the regulation of both IL-6 and IL-8 expression in monocytes/macrophages[19]. Confirming the effect of FⅦa on expression of interleukins, recombinant FⅦa administered to healthy humans caused a three- to four-fold increase in plasma levels of IL-6 and IL-8[20]. A role of TF/FⅦa signalling in the regulation of inflammatory genes has been demonstrated in LPS-stimulated macrophages, where TF-FⅦa signalling activated genes coding for tumor necrosis factor-α, IL-6, and IL-8[21].

Recent clinical studies have suggested a potential role of coagulation variables, such as TF, TFPI and D-dimer, in predicting risk of developing organ failure and severe AP[22-24]. However, the evidence supporting their use as predictors of severity of AP is still weak, compared to C-reactive protein (CRP) and IL-6, which to date are the most well-documented laboratory parameters to predict severe AP[25-28].

The present study aimed to investigate plasma levels of TF in the initial phase of predicted severe AP, and to assess the ability of this biochemical marker to predict severe AP.

MATERIALS AND METHODS

Consecutive patients admitted to Lund University Hospital with the clinical diagnosis of acute pancreatitis, were recruited prospectively between June 2002 and December 2004. Inclusion and exclusion criteria are listed in Table 1.

Written informed consent was obtained and the study was approved by the local research ethics committee. This study was part of a prospective single-centre study on early enteral nutrition *vs* total parenteral nutrition in AP, where parts of the data on IL-6 and CRP have been published[29]. Venous blood was taken for measurement of plasma levels of TF, FⅦ, fibrinogen, IL-6 and CRP. Not all markers were measured at all time points in the study. TF and IL-6 were measured at inclusion, after 12 h, and after 1 and 3 d. CRP was measured at inclusion, and after 1 and 3 d. Fibrinogen and FⅦ were only measured at inclusion in the study.

Descriptive data were recorded including age, gender, aetiology, time from onset of pain to inclusion in the study, Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score on day 1 and 3, organ failure, and mortality. The severity of pancreatitis was assessed according to the Atlanta classification[30].

Blood samples and assays

Peripheral blood samples were taken from each patient on study inclusion, at 12 h, and after 1 and 3 d. Admission plasma levels of FⅦ were analysed, and to detect the prevalence of fibrinolysis and fibrinogen consumption at admission, plasma fibrinogen was analysed. Fibrinogen is an acute phase protein, affected by pathologic proteolysis such as in disseminated intravascular coagulation, where low levels of fibrinogen are to be expected. TF, IL-6 and CRP were analysed at repeated time points during three days after inclusion in the study.

Tissue factor and fibrinogen were collected using citrate tubes, and ethylenediaminetetraacetic acid tubes were used for IL-6 and CRP. All samples were centrifuged at 2200 *g* for 10 min (3200 r/min, rotor diameter 19.1 cm). The plasma was decanted and stored at -70℃ until further analysis.

TF and FⅦ were assessed by enzyme-linked immunosorbent assay (ELISA)-kits according to the manufacturer's instructions (Assaypro St. Charles, MO, USA). The TF-ELISA recognizes TF-apo, TF and TF-Ⅶ complexes. The FⅦ-ELISA detects free FⅦ and FⅦa, as well as complexes with TF, TF/factor Ⅶ and TF/FⅦa.

Fibrinogen was analysed by Sysmex CA-7000 (Sysmex Corporation, Kobe, Japan) according to the operator's manual. The procedure involves mixing citrate plasma with buffer. After incubation, coagulation was initiated by adding an excess of thrombin. The time between addition of thrombin and coagulation was registered photo-optically and is inversely proportional to the concentration of fibrinogen.

IL-6 was measured by an ELISA-kit according to the manufacturer's instructions (Quantikine, R6D systems Europe, Abingdon, UK). CRP was measured by Cobas

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
> 18 yr	Acute pancreatitis due to surgery
Abdominal pain	Trauma
Amylase > 3 times upper normal limit	Cancer
Onset of abdominal pain within 48 h	Inflammatory bowel disease
APACHE Ⅱ score > 8 and/or	Stoma
CRP > 150 and/or	Short bowel
Peripancreatic fluid collection on CT	Chronic pancreatitis

APACHE: Acute Physiology and Chronic Health Evaluation; CRP: C-reactive protein; CT: Computed tomography.

6000 (Roche Corporation, Basel, Switzerland) according to the operator's manual. The complex binding between CRP and CRP monoclonal antibodies attached to latex particles was registered as an increase in absorbance, measured photo-optically, and the increase in absorbance was related to the concentration of CRP.

Statistical methods

Data are presented as median and interquartile range, when applicable. Outliers are not shown in the box-plots, but are included in all calculations. Comparisons between groups were performed with the χ^2 test for binary data or Fisher's exact test for small samples. Continuous variables were compared with the Mann-Whitney *U*-test. To evaluate TF as a predictor of severe AP, receiver operating characteristics (ROC) curves were plotted and positive likelihood ratios (PLR) and negative likelihood ratios (NLR) were calculated to detect optimal cut-off levels. As a comparison, figures calculated from levels of CRP and IL-6 were used, as they are known to be good predictors of severity, IL-6 already at admission[27] while CRP peaks about 48 h later.

In a ROC curve, the true positive rate (sensitivity) is plotted in function of the false positive rate (100 - specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC plot that passes through the upper left corner (100% sensitivity, 100% specificity). The closer the ROC plot is to the upper left corner, and the greater the area under the curve, the higher the overall accuracy of the test is[31]. The Likelihood Ratio (LR) is the likelihood that a given test result would be expected in a patient with the target disorder, compared to the likelihood that the same result would be expected in a patient without the target disorder.

Statistical analyses were performed with SPSS version PASW Statistics 18 (SPSS Inc, Chicago, IL, USA).

RESULTS

Patient characteristics

According to the Atlanta classification, 22 patients (45%) fulfilled the criteria of severe AP, and 27 patients (55%) were classified as having mild AP. One patient in the severe AP group died, rendering an overall mortality rate of 2.0%. At inclusion in the study, the groups with mild and severe pancreatitis were comparable with respect to gender, aetiology, APACHE Ⅱ score, and duration of pain prior to inclusion. Age was lower in the severe AP group, compared to the mild AP group. Patient characteristics and laboratory variables at time of inclusion are presented in Table 2.

Table 2 Patient characteristics and laboratory variables at time of inclusion

	MAP (n = 27)	SAP (n = 22)	P
Age (yr)[1]	76 (63-85)	63 (56-77)	0.042
Sex (M:F)	14:13	10:12	0.664
APACHE Ⅱ[1]	9 (8-11)	9 (7-13)	0.860
Aetiology			
Biliary	15	16	0.248
Alcohol	4	3	1.000
ERCP	3	1	0.617
Unknown	5	2	0.436
Duration of pain (h)[1]	34 (21-43)	25 (22-29)	0.160
Amylase[1]	8.2 (2.7-13.7)	9.8 (4.3-15.3)	0.690
IL-6 (pg/mL)[1]	100 (55-210)	275 (158-315)	0.001
CRP (mg/mL)[1]	106 (69-167)	173 (104-209)	0.071
Tissue factor (pg/mL)[1]	35 (23-50)	49 (36-101)	0.035
Fibrinogen (g/L)[1]	4.8 (4.4-6.2)	4.0 (3.8-7.2)	0.047
FⅦ (ng/mL)[1]	155 (46-294)	136 (88-296)	0.608

[1]Values are expressed as median and inter-quartile range. MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis; APACHE: Acute Physiology and Chronic Health Evaluation; ERCP: Endoscopic retrograde cholangiopancreatography; IL-6: Interleukin-6; CRP: C-reactive protein; FⅦ: Factor Ⅶ.

Markers

Because some blood samples were not taken properly, there are different numbers of patients at the different time points. At inclusion in the study, TF was higher in the severe AP group, whereas fibrinogen was lower in the severe AP group compared to the group with mild AP [Figure 1, tissue factor (pg/mL)].

There was no difference in FⅦ-levels between the groups (P = 0.608). A large variation in inter-individual levels of FⅦ was noted [Figure 2, scattergram of FⅦ plasma levels at admission (ng/mL)]. IL-6 was higher in the severe AP group (P = 0.001), and CRP showed a tendency towards higher levels in the severe AP group (P = 0.071) at time of inclusion in the study (Table 2).

When looking at changes over time, TF was slightly higher in the severe AP group at 12 h (P = 0.049). After 1 and 3 d no differences in TF levels were noted between the mild and the severe AP group [Figure 1, tissue factor (pg/mL)].

IL-6 peaked at 12 h and was significantly higher in the severe AP group at all of the studied time points (at inclusion P = 0.001, 12 h P < 0.001, 1 d P < 0.001 and 3 d P = 0.000, respectively). CRP peaked at day 3, and was significantly higher in the AP group at 1 and 3 d (P = 0.001 and P < 0.001, respectively).

Prediction of severity

To evaluate the utility of TF as an early predictor of se-

Table 3 Area under the curve-values, possible cut-off levels, sensitivity, specificity, positive and negative likelihood ratio for tissue factor

Time point[1]	AUC	*P*	Cut-off TF (pg/mL)	Sensitivity	Specificity	PLR	NLR
0	0.679	0.035	32	86	48	1.65	0.30
			40	71	67	2.14	0.43
			46	62	74	2.39	0.51
0.5	0.681	0.049	33	90	43	1.57	0.26
			41	78	56	1.79	0.39
			47	72	70	2.37	0.40
1	0.652	0.078					
3	0.621	0.151					

[1]Time points: 0 = inclusion in study, 0.5 = 12 h, 1 = 24 h, 3 = 3 d. AUC: Area under the curve; TF: Tissue factor; PLR: Positive likelihood ratios; NLR: Negative likelihood ratios.

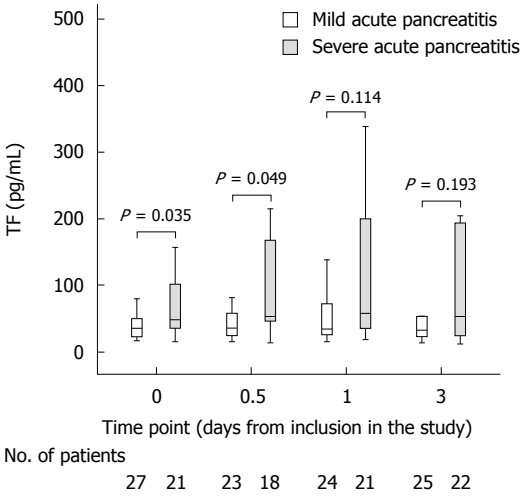

Figure 1 Tissue factor. Time points: 0 = inclusion in study, 0.5 = 12 h, 1 = 24 h, 3 = 3 d. TF: Tissue factor.

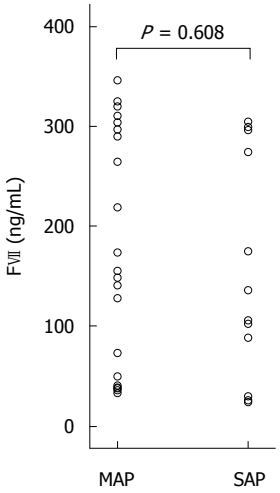

Figure 2 Scattergram of factor Ⅶ plasma levels at inclusion (ng/mL). FⅦ: Factor Ⅶ; MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

vere AP, ROC-curves were plotted for the time of inclusion (Figure 3A, ROC curves of TF, IL-6 and CRP at time of inclusion in the study), and for 12 h (Figure 3B, ROC curve of TF and IL-6 at 12 h after inclusion in the study), 1 d (results not shown) and 3 d after inclusion in the study (Figure 3C, ROC curves of TF, CRP and IL-6 at 3 d after inclusion). As a comparison, ROC-curves were plotted for CRP and IL-6. Area under the curve (AUC) values at the different time points were studied. Based on these results, possible cut-off levels for TF are suggested at inclusion and after 12 h, based on sensitivity, specificity, PLR and NLR. Table 3 shows AUC-values, P-values, possible cut-off levels, sensitivity, specificity, PLR and NLR for TF (pg/mL).

DISCUSSION

Several previous studies on coagulation factors in AP have been published. In a study on 36 patients with AP, elevated levels of TF were detected at admission. In that study only 5 patients were classified as having moderate AP, while 31 had severe AP according to the Japanese Severity Score[22]. A correlation between higher levels of TF and development of organ failure was demonstrated, but in contrast to the results from the present study no correlation with overall severity was detected. In the present study, TF was higher in severe AP compared to mild AP at inclusion in the study, i.e. close to admission, and after 12 h.

The levels of fibrinogen in both mild and severe AP were in the higher span or above the reference for normal human plasma levels, consistent with fibrinogen being an acute phase protein. A slightly lower level of fibrinogen was noted in the group with severe AP at inclusion in the study. The results are, however, hard to interpret as fibrinogen is an acute phase protein and the level of fibrinogen in the severe AP group was just above normal level. It should be stressed that the result for fibrinogen was of weak significance, and further studies of other parameters of fibrinolysis, such as D-dimer and fibrin degradation products should be conducted in order to tell whether early fibrinolysis is the explanation for the lower levels of fibrinogen in the severe AP group.

In a study on 91 patients with AP, D-dimer, prothrombin time and fibrinogen were different when comparing patients developing organ failure and patients not developing organ failure, both at admission and 24 h later. D-dimer was the best predictive marker of organ failure (sensitivity 90%, specificity 89%)[23]. In a study on 139 patients with AP, the levels of antithrombin Ⅲ (AT-Ⅲ),

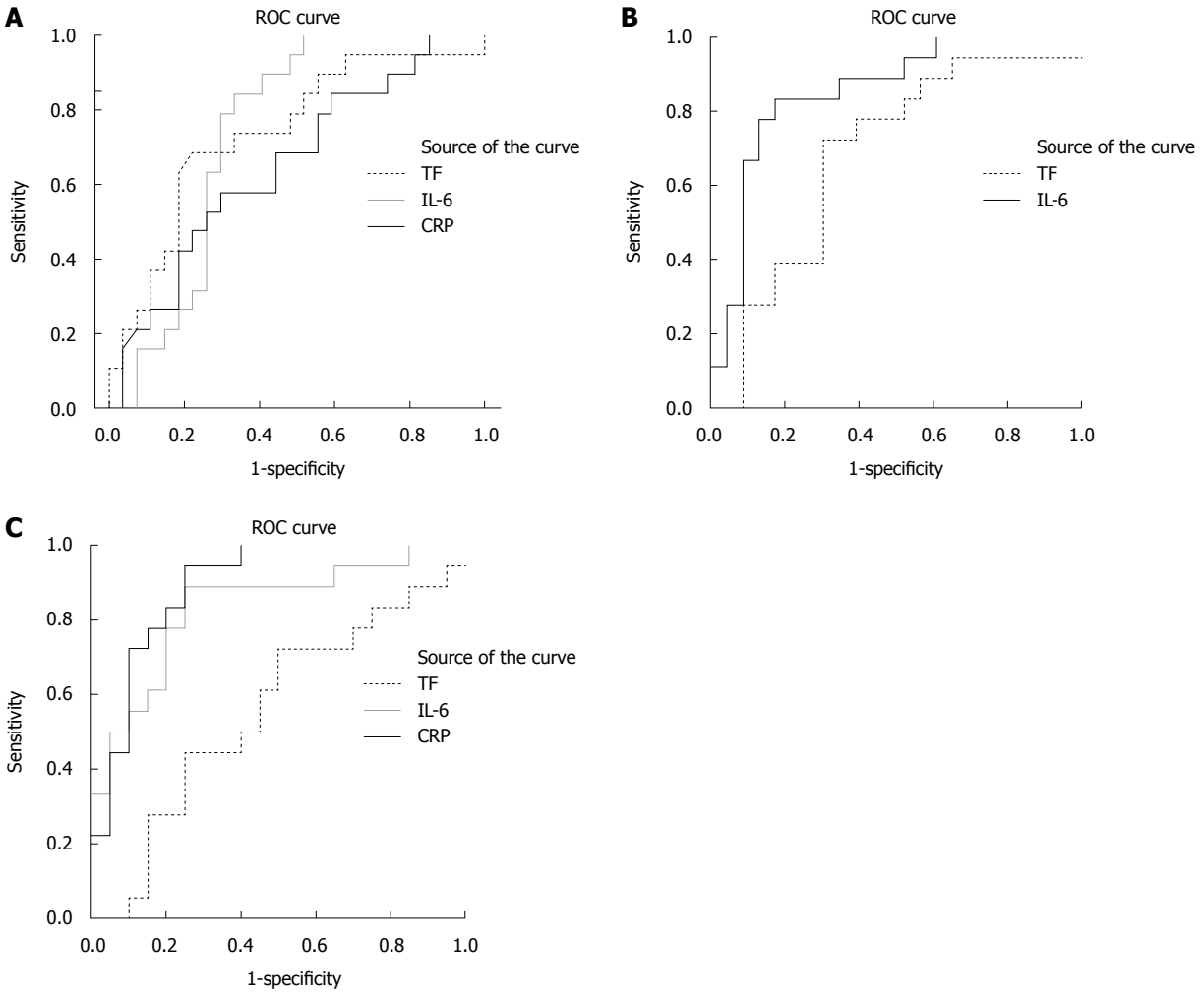

Figure 3 Receiver operating characteristic curves. A: Receiver operating characteristic (ROC) curves of tissue factor (TF), interleukin-6 (IL-6) and C-reactive protein (CRP) at time of inclusion in the study; B: ROC curves of TF and IL-6 at 12 h after inclusion in the study; C: ROC curves of TF, IL-6 and CRP at 3 d after inclusion.

fibrin/fibrinogen degradation products, platelet count, D-dimer, and antithrombin-AT-Ⅲ complex at admission were associated with severity and prognosis of AP. AT-Ⅲ, fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-Ⅲ complex at admission showed better area under the ROC curve values compared to CRP. AT-Ⅲ was the best predictor of fatal outcome (sensitivity 81%, specificity 86%)[32].

In experimental studies, deficiency of FⅦ has been shown to reduce inflammation[33,34] and high levels of FⅦ have been suggested to be associated with ischemic heart disease and inflammation[35,36]. In the present study, concentrations of FⅦ did not differ between the mild AP group and the severe AP group, and hence levels of FⅦ do not seem to be affected in the early course of the disease. The large variation in levels of FⅦ is consistent with reported findings on a strong contribution of the FⅦ genotype to levels of FⅦ. Different FⅦ genotypes can result in up to several-fold differences in mean FⅦ levels[37].

Early recognition of patients at risk of developing severe AP with multiple organ failure and high risk of mortality remains a challenge, despite the use of multifactor scoring systems such as APACHE-Ⅱ and Ranson's score[38]. Obesity, age, alcohol consumption and use of tobacco are known to predispose to a severe disease course[39,40]. The most widely used laboratory parameter to predict severity of AP and development of complications is CRP. A meta-analysis on the ability of IL-6 to predict severe AP concludes that these cytokines perform at an acceptable level in predicting severe AP[26]. The pooled IL-6 sensitivities ranged between 81.0% and 83.6% and specificities between 75.6% and 85.3% with PLRs of 3.43, 4.90 and 4.40 for days 1, 2 and 3, respectively. The IL-6 AUCs were 0.75, 0.88 and 0.85 for days 1, 2 and 3, which are in accordance with the AUCs for IL-6 in the present study.

Data concerning the role of coagulation variables as predictors of severe AP are scarce. In a study of 44 patients with AP, TFPI measured at admission was shown to be related to severity[24]. Among the three variables in the present study, fibrinogen, FⅦ and TF, TF was significantly raised at admission, when comparing the severe and the mild AP group. With this result in mind, TF was explored as a marker of severity at four different time points, by area under ROC-curves. At admission and after 12 h, the AUC for TF was 0.68, and when evaluating different cut-off points the best PLR was 2.4, with a sensitivity of 62%

and a specificity of 72%, which implies a quite low impact on the likelihood of severe disease, much less impact than IL-6 at corresponding time points.

We conclude that levels of TF, but not FVII, are higher in "true severe" AP than in those patients with predicted severe AP who turn out to develop mild AP. Our results stress the need of more reliable predictors of severity, as only 45% of the patients in our study with predicted severe disease actually developed severe AP. The value of TF as a predictive marker of severe AP early in the course of the disease is not as good as IL-6, but superior to CRP. The results do not indicate a role for TF as a valuable predictive marker of severity on its own. The higher levels of TF in the early course of severe AP suggest, however, a potential role of TF in the development of severe disease, and may reflect a window for therapeutic inhibition of TF in AP.

COMMENTS

Background

Acute pancreatitis affects about 20-40/100 000 inhabitants each year. One fifth of these patients will develop a severe form of AP with multiple organ failure and a high risk of death. There is no reliable marker to early predict which patients will develop the severe form. In severe disease, such as AP, a close interplay between coagulation and inflammation is known to exist, and take part in the development of the disease. In this paper, tissue factor, which is a key player in the crosstalk between inflammation and coagulation, is measured in the plasma of patients with predicted severe pancreatitis.

Research frontiers

Data concerning the role of coagulation variables as predictors of severe acute pancreatitis (AP) are still sparse. The results from one study on patients with AP, showed that levels of tissue factor (TF) were related to the development of pancreatic necrosis in alcoholic severe AP, but no association with overall severity was demonstrated (Sawa *et al* 2006). In another study of AP, the coagulation parameters D-dimer, pro-thrombin time and fibrinogen were different in the group of AP patients developing organ failure compared to the patients who did not develop organ failure, both at admission and 24 h later. D-dimer was the best predictive marker of organ failure (Radenkovic *et al* 2009). In yet another study on AP, the levels of the coagulation parameters antithrombin Ⅲ (AT-Ⅲ), fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-Ⅲ complex at admission were associated with severity and prognosis of AP. AT-Ⅲ was the best predictor of fatal outcome (Maeda *et al* 2006).

Innovations and breakthroughs

The authors show that levels of TF measured early in the course of the disease are higher in patients who develop severe AP. These results are consistent with the possible role of coagulation variables in the development of AP.

Applications

The role of TF as an early predictor of severe AP is inferior to interleukin-6, which has been shown to be of value in various previous studies, however, TF is superior to the most frequently used laboratory parameter, C-reactive protein. The results indicate a role for TF in the development of severe AP, and the effect of tissue factor pathway-inhibitors in AP should be studied.

Terminology

Acute pancreatitis is an acute inflammation of the pancreatic gland, most often elicited by alcohol ingestion or gall stone disease. Tissue factor is located in the membrane of various cells surrounding the blood vessels throughout the body, and is exposed to circulating blood when vessels are ruptured or may be expressed by white blood cells or cells on the inside of blood vessels in inflammatory conditions, such as acute pancreatitis. When tissue factor binds to factor Ⅶ, circulating in the blood, the coagulation cascade is initiated, but tissue factor - factor Ⅶ may also modulate the inflammatory response.

Peer review

This clinically relevant study of the predictors of pancreatitis severity looks fine.

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S- Editor Sun H **L- Editor** Webster JR **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6135

World J Gastroenterol 2010 December 28; 16(48): 6135-6138
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Role of serotonin in development of esophageal and gastric fundal varices

Jelena S Rudić, Đorđe M Ćulafić, Duško S Mirković, Rada S Ješić, Miodrag N Krstić

Jelena S Rudić, Đorđe M Ćulafić, Rada S Ješić, Miodrag N Krstić, Clinic of Gastroenterology, Clinical Center of Serbia, Faculty of Medicine, University of Belgrade, Dr. Koste Todorovića Street, No 2, 11000 Belgrade, Republic of Serbia
Duško S Mirković, Centre of Medical Biochemistry, Clinical Center of Serbia, School of Pharmacy, University of Belgrade, Pasterova No 2, 11000 Belgrade, Republic of Serbia
Author contributions: Rudić JS and Ćulafić ĐM contributed equally to this work; Rudić JS, Ćulafić ĐM, Mirković DS, Ješić RS and Krstić MN designed the research; Rudić JS, Ćulafić ĐM and Mirković DS performed the research; Rudić JS, Ćulafić ĐM and Mirković DS contributed the new reagents/analytic tools; Rudić JS, Ćulafić ĐM, Mirković DS, Ješić RS and Krstić MN analyzed the data; Rudić JS and Ćulafić ĐM wrote the paper.
Supported by The Ministry for Science, Technology and Development of Republic of Serbia, No. 14501B
Correspondence to: Jelena S Rudić, MD, Clinic of Gastroenterology, Clinical Center of Serbia, Faculty of Medicine, University of Belgrade, Dr. Koste Todorovića Street, No 2, 11000 Belgrade, Republic of Serbia. jelena_rudic@yahoo.com
Telephone: +381-11-3663733 Fax: +381-11-3615587
Received: April 9, 2010 Revised: September 7, 2010
Accepted: September 14, 2010
Published online: December 28, 2010

Abstract

AIM: To determine the effect of free serotonin concentrations in plasma on development of esophageal and gastric fundal varices.

METHODS: This prospective study included 33 patients with liver cirrhosis and 24 healthy controls. Ultrasonography and measurement of serotonin concentration in plasma were carried out in both groups of subjects. The upper fiber panendoscopy was performed only in patients with liver cirrhosis.

RESULTS: The mean plasma free serotonin levels were much higher in liver cirrhosis patients than in healthy controls (219.0 ± 24.2 nmol/L *vs* 65.4 ± 18.7 nmol/L, $P < 0.0001$). There was no significant correlation between serotonin concentration in plasma and the size of the esophageal varices according to Spearman coefficient of correlation ($r_s = -0.217$, $P > 0.05$). However, the correlation of plasma serotonin concentration and gastric fundal varices was highly significant ($r_s = -0.601$, $P < 0.01$).

CONCLUSION: Free serotonin is significant in pathogenesis of portal hypertension especially in development of fundal varices, indicating the clinical value of serotonergic receptor blockers in these patients.

Key words: Serotonin; Portal hypertension; Esophageal varices; Fundal varices; Platelets

Peer reviewer: Hiroshi Yoshida, MD, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Rudić JS, Ćulafić ĐM, Mirković DS, Ješić RS, Krstić MN. Role of serotonin in development of esophageal and gastric fundal varices. *World J Gastroenterol* 2010; 16(48): 6135-6138 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6135.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6135

INTRODUCTION

Portal hypertension has been increasingly regarded as a multi-organ disease with complex blood flow changes in the systemic and splanchnic vascular network.

The hepatic stellate cell (HSC) has a significant position in the sinusoid for regulation of portal flow. During liver damage, HSCs are "activated" which leads to HSC transformation into myofibroblast-like cells with a resulting increased collagen production[1,2]. Several mitogens are included in the triggering of HSC proliferation: platelet-derived growth factor (PDGF), insulin-like growth factor 1 and connective-tissue growth factor. During activation,

HSCs acquire the ability to express PDGF receptors on the cell membrane surface[3]. The HSC membrane contains numerous receptors whose expression is increased with the extent of liver damage, to which different vasoconstrictors are bound: catecholamines, endothelin, angiotensin I and Ⅱ, leukotrienes and serotonin [5-hydroxytriptamin (5-HT)][4,5].

Serotonin, at the level of hepatic sinusoids, causes endothelial fenestrae contractions of liver sinusoids through 5-HT_1 receptors mediated by a Ca^{2+}-dependent process. Due to different proinflammatory mediators releasing from the damaged liver, it comes to platelet adherence to sinusoidal endothelium, translocation into Disse's space and serotonin release. Thereafter, serotonin binds to receptors (5-HT_{2A}, 5-HT_{1B}, 5-HT_{1F} and 5-HT_7) which are expressed on HSC and hepatocytes, which additionally interferes with HSC proliferation[6,7].

The aim of our study was to determine to what extent a free serotonin concentration in plasma has an effect on development of esophageal and gastric fundal varices.

MATERIALS AND METHODS

The study included 33 patients with liver cirrhosis who were examined and treated at the Clinic of Gastroenterology, Clinical Center of Serbia, and 24 healthy subjects who made up the control group. The study was prospective and conducted during the period 2008-2009.

Ultrasonography was carried out by Toshiba Core Vision, with 3.5 MHz duplex Doppler convex tube in a standard procedure. Ultrasonography examined the liver size, echo structure of the hepatic parenchyma and possible focal changes with a view to rule out the patients with primary and secondary liver tumors from the study. To determine the spleen size, standard parameters were used, according to which in physiological conditions the spleen diameter measured in the X intercostal space exceeded no more than 12.0 cm and anteroposterior diameter was not over 5.0 cm.

The upper endoscopy was performed by endo-video system Olympus GIF-Q 165. To measure the esophageal varices size, Paquet's classification was used: I degree-lesser snake-like mucosal protrusions, Ⅱ degree-varices were predominating up to a half of the esophageal lumen radius, Ⅲ degree-varices were in contact at some points, and Ⅳ degree-heralds of the imminent rupture (cherry red spots)[8]. Endoscopic examination showed portal hypertensive gastropathy (snake skin mucosa) and varices of the gastric fundus.

Platelet poor plasma (PPP) was obtained from the venous blood which was collected in 3 mL original Vacutainer "BD" tubes, with 75 g/L K_3EDTA 0.072 mL. Blood samples were taken between 8 and 9 a.m. Platelet rich plasma (PRP) was obtained by low speed centrifugation (200 *g*, 10 min) on "Heraeus Digifuga GL". Exactly 1 mL of PRP was centrifuged at 1000 *g*, 10 min. The obtained PPP was separated and stored at -20℃ for no longer than 20 d[9].

The number of PPP serotonin samples was estimated in one series. One hundred μL plasma samples were spiked with 10 μL of original N-methyl serotonin solution (Recipe, Munchen), which was an internal standard. After that, PPP samples were deproteinized with 100 μL original deproteinating reagent (Recipe, München), and centrifuged at 10 000 *g*[10]. The obtained 20 μL supernatants were analyzed on reverse phase HPLC column (Recipe, München), with original mobile phase for serotonin (Recipe, München). Original "Recipe" external standard solution was used for calibration. The HPLC system consisted of "Bio-Rad AS 100" HRLC automatic sampling system with "Rheodine 7125 valve", "Bio-Rad 1350" HPLC pump and "Bio-Rad 1640" electrochemical detection. Chromatographic data were calculated using the "Chrome Line V 4.20" HPLC software. Amperometric detection was done at 0.6 V. The duration of chromatographic separation was 10 min.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS®, version 17.0). Basic descriptive statistics included means, standard deviations, ranges and percentages. Differences between groups were compared with parametric *t*-test because data had a Gaussian distribution. Correlation analysis was processed *via* the Spearman method. Values at the $P \leqslant 0.05$ level were considered statistically significant.

RESULTS

The study included 11 (33.3%) female and 22 (66.7%) male patients, mean age of 52.32 (SD ± 11.55) years. The most common cause of liver cirrhosis was alcohol-in 15 (45.4%) cases. The incidence of posthepatitic cirrhosis was lower; HCV-8 (24.2%), HBV-5 (15.1%), while autoimmune diseases were quite rare-in 5 (15.1%) patients.

Splenomegaly was detected in 28 (84.8%) patients with liver cirrhosis. An average longitudinal splenic diameter was 17.5 ± 3.57 cm, and transversal diameter was 6.8 ± 1.77 cm, which was significantly different in relation to the controls, in whom an average longitudinal diameter was 10.21 ± 1.65 cm and transversal diameter was 3.03 ± 0.87 cm (*t*-test, $P < 0.05$).

There was a highly significant difference between the platelet count in the studied groups of patients ($t = -9.779$, $P < 0.01$).

The mean plasma free serotonin level was much higher in liver cirrhosis patients than in healthy controls (219.0 ± 24.2 nmol/L *vs* 65.4 ± 18.7 nmol/L; *t*-test, $P < 0.0001$).

There was no significant correlation between serotonin concentration in plasma and the platelet count according to Spearman coefficient of correlation ($r_s = 0.158$, $P > 0.05$).

Esophageal varices were not detected in 5 (15.1%) patients, grade Ⅰ-Ⅱ varices were detected in 9 (27.2%), grade Ⅱ-Ⅲ in 15 (45.4%) and the remaining 4 patients (12.1%) were grade Ⅳ. Gastric fundal varices were found in 7 (21.2%) patients, out of whom 2 had Ⅰ-Ⅱ, 4 had grade Ⅱ-Ⅲ esophageal varices, and one patient had Ⅳ degree varices.

Spearman's rank correlation verified a statistically significant correlation between the platelet count and varices size ($r_s = -0.479$, $P < 0.05$).

Spearman's rank correlation verified no significant difference between the serotonin concentration in plasma in relation to the size of esophageal varices (r_s = -0.217, P > 0.05). However, the mean plasma free serotonin level was higher in patients with esophageal varices than in patients without varices (t = -2.301, P < 0.05). Furthermore, the correlation of plasma serotonin concentration and fundal varices was highly significant (r_s = -0.601, P < 0.01). Also, we proved that the mean plasma free serotonin level was much higher in patients who had esophageal and gastric fundal varices than in patients who had only esophageal varices (t = -5.862, P < 0.01).

DISCUSSION

Different factors may affect the concentrations of circulating serotonin in liver cirrhosis, such as: impaired serotonin catabolism due to higher activity of the monoamino oxidases; impaired metabolism of tryptophane as a serotonin precursor; platelet sequestration in the spleen and/or platelet activation[11]. In addition, 5-HT as well as other vasoactive substances synthesized in the gastrointestinal tract *via* porto-systemic collaterals bypass the liver and directly enter the systemic circulation[12].

In the study of Beaudry *et al*[13], in 1994, the whole-blood serotonin levels were significantly lower in 30 patients with cirrhosis than in the age-matched controls, and no correlation was found between these levels and the severity of cirrhosis. However, in the same study the unconjugated plasma serotonin levels, an indication of the active form of serotonin, were significantly higher in patents with cirrhosis than in the controls.

In our previous study, free or unconjugated serotonin levels were investigated. The levels of free serotonin were higher in patients with liver cirrhosis than in healthy subjects[14].

In the study of Vorobioff *et al*[15], in 1989, it was confirmed that the application of ketanserin and ritanserin (serotonergic receptor inhibitors) caused the lowering of portal hypertension in patients with liver cirrhosis. The authors reported that the spleen congestion in liver cirrhosis brought about the platelet breakdown. Free serotonin, released in the sinusoidal spaces of the spleen, induced by S-2 receptor produced an intense vasoconstricting response in portal circulation which led to maintenance and elevation of the portal pressure. Moreover, it was documented that the reaction of the isolated mesenteric vein in rats with portal hypertension to 5-HT was hypersensitive, which was additional evidence of the role of this substance in pathogenesis of portal hypertension.

In our study, the correlation of unconjugated serotonin concentration (active form of serotonin) in plasma and varices of the gastric fundus was highly significant while the plasma unconjugated serotonin concentration did not correlate with the size of the esophageal varices. Moreover, mean longitudinal and transversal diameters of the spleen in patients was significantly higher as compared to controls.

The spleen has a crucial role in pathogenesis and maintenance of portal hypertension[16,17]. In portal hypertension, the anatomic changes of the spleen (pulp hyperplasia, congestion and fibrosis) and specific vascularization affect the hemodynamics of the splenic circulation[18].

Our finding may be accounted for different porto-systemic collateral pathways in esophageal and fundal varices as well as valuable flow changes in the left part of the portal venous system. Perisic *et al*[19] reported, in 2005, that the splenic vein flow in patients with liver cirrhosis was slower in comparison with healthy controls. In addition, in healthy controls, the splenic vein flow was significantly slower than in the portal vein. However, in patients with liver cirrhosis splenic vein flow was significantly faster than in the portal vein, probably because of the splenic venous congestion and compensatory hemodynamic mechanisms of the spleen.

Gastric varices are drained through the short gastric veins into the splenic vein. Serotonin released by platelet sequestration in the enlarged spleen reaches the lienal vein where the blood flow is faster than in the portal vein, and directly, *via* short gastric veins, it enters the fundal gastric veins, leading to vasoconstriction.

Our conclusion is that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices which may have clinical value in use of serotonin receptor blockers in these patients.

COMMENTS

Background

In acute and chronic hepatic insufficiency, the serotonin system changes lead to development of hepatic encephalopathy, portal hypertension and hyperdynamic circulation. Portal hypertension has been increasingly regarded as a multi-organ disease with complex blood flow changes in systemic and splanchnic vascular network. The hepatic stellate cell (HSC) has a significant position in sinusoid for regulation of portal flow and during liver damage serotonin binds to receptors [5-hydroxytriptamin (5-HT)$_{2A}$, 5-HT$_{1B}$, 5-HT$_{1F}$ and 5-HT$_7$] which are expressed on HSC and hepatocytes, which additionally interferes with HSC proliferation.

Research frontiers

Free serotonin, released in the sinusoidal spaces of the spleen, induced by S-2 receptor, produces an intense vasoconstricting response in the portal circulation, which leads to maintenance and elevation of portal pressure. The highlight of our study was to determine to what extent a free serotonin concentration in plasma has an effect on development of esophageal and gastric fundal varices.

Innovations and breakthroughs

In the study of Vorobioff *et al*, in 1989, it was confirmed that the application of ketanserin and ritanserin (serotonergic receptor inhibitors) caused the lowering of portal hypertension in patients with liver cirrhosis. Moreover, it was documented that the reaction of the isolated mesenteric vein in rats with portal hypertension to 5-HT was hypersensitive, which was additional evidence of the role of this substance in pathogenesis of portal hypertension. In this study, the correlation of unconjugated serotonin concentration (active form of serotonin) in plasma and varices of the gastric fundus was highly significant while the plasma unconjugated serotonin concentration did not correlate with the size of the esophageal varices. The authors' finding may be accounted for by different porto-systemic collateral pathways in the esophageal and fundal varices as well as valuable flow changes in the left part of the portal venous system.

Applications

The conclusion is that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices. This may have clinical value in use of serotonin receptor blockers in these patients.

Terminology

Portal hypertension: Portal hypertension (> 10 mmHg) most commonly results from increased resistance to portal blood flow. Cirrhosis is the most common

cause of portal hypertension. One of the major clinical manifestations of portal hypertension includes life-threatening hemorrhage from gastrointestinal varices. Serotonin: Serotonin is a vasoactive substance, synthesized by the intestinal enterochromaffin cells, which is actively incorporated into platelets and stored in platelet dense-storage granules.

Peer review

The manuscript reports that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices which may have clinical value in use of serotonin receptor blockers in these patients.

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S- Editor Wang YR **L- Editor** O'Neill M **E- Editor** Ma WH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6139

World J Gastroenterol 2010 December 28; 16(48): 6139-6144
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Value of duplex doppler ultrasonography in non-invasive assessment of children with chronic liver disease

Mortada HF El-Shabrawi, Maissa El-Raziky, Maha Sheiba, Hanaa M El-Karaksy, Mona El-Raziky, Fetouh Hassanin, Abeer Ramadan

Mortada HF El-Shabrawi, Maha Sheiba, Hanaa M El-Karaksy, Mona El-Raziky, Abeer Ramadan, Department of Pediatrics, Cairo University, Cairo 11559, Egypt
Maissa El-Raziky, Department of Tropical Medicine, Cairo University, Cairo 11559, Egypt
Fetouh Hassanin, Department of Clinical Pharmacy, Misr International University, Cairo 11559, Egypt
Author contributions: El-Shabrawi MHF, El-Raziky M, Sheiba M, El-Karaksy HM, El-Raziky M, Hassanin F and Ramadan A contributed equally to this work; El-Shabrawi MHF and El-Karaksy HM designed the research; Ramadan A performed the clinical examination; El-Raziky M, Sheiba M and El-Raziky M performed the ultrasound examination; Hassanin F analyzed the data statistically; El-Shabrawi MHF and Hassanin F wrote the paper.
Supported by Cairo University, as six of the researchers are employees of that University
Correspondence to: Mortada HF El-Shabrawi, MD, Professor of Pediatrics and Chief of Pediatric Hepatology, Department of Pediatrics, Cairo University, Cairo 11559, Egypt. melshabrawi@medicine.cu.edu.eg
Telephone: +20-2-35721790 Fax: +20-2-37619012
Received: August 1, 2010 Revised: September 13, 2010
Accepted: September 20, 2010
Published online: December 28, 2010

Abstract

AIM: To investigate the value of duplex Doppler ultrasonography (US) in the assessment of the hemodynamics of the portal and hepatic veins in a cohort of children with chronic liver disease (CLD) and to detect any relationship between the US changes, etiology and severity (or stage) of CLD.

METHODS: We prospectively enrolled 25 children with biopsy-proven CLD. Thirteen had cirrhosis (aged 8.9 ± 2.0 years) and 12 had chronic hepatitis (aged 9.3 ± 2.3 years). Gray scale and color-coded duplex Doppler US were performed for all, as well as 30 healthy age and sex-matched controls. Findings were correlated with clinical, laboratory and histopathological characteristics.

RESULTS: Prominent caudate lobe was detected in 100% of cirrhotics, but none of the chronic hepatitis or controls. Thickened lesser omentum and loss of the triphasic waveform of the hepatic vein were present in 69.2% and 53.8% of cirrhotics *vs* 33.3% and 8.3% of chronic hepatitis respectively. Portal vein flow velocity was significantly lower ($P < 0.0001$) and the congestion index was significantly higher ($P < 0.005$) in both patient groups compared to controls. Child-Pugh's staging showed a positive correlation with both abnormal hepatic vein waveform and direction of portal blood flow; and a negative correlation with both hepatic and portal vein flow velocities. No correlation with the etiology of CLD could be detected.

CONCLUSION: Duplex Doppler added to grayscale US can detect significant morphologic and portal hemodynamic changes that correlate with the severity (stage) of CLD, but not with etiology.

Key words: Chronic hepatitis; Chronic liver disease; Cirrhosis; Doppler; Grayscale; Pediatrics; Ultrasound

Peer reviewer: Dr. Herwig R Cerwenka, Professor, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

El-Shabrawi MHF, El-Raziky M, Sheiba M, El-Karaksy HM, El-Raziky M, Hassanin F, Ramadan A. Value of duplex doppler ultrasonography in non-invasive assessment of children with chronic liver disease. *World J Gastroenterol* 2010; 16(48): 6139-6144 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6139.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6139

INTRODUCTION

The search for a non-invasive biochemical or imaging marker for the severity (stage) and/or etiology of chronic liver disease (CLD) in adults, as well as children, is in extremely active nowadays. Real time ultrasonography (US) has become an integral part of the non-invasive evaluation of the liver in many clinical settings in adults. Color-coded duplex Doppler information regarding the presence or absence of flow and the direction and velocity of that flow can be obtained non-invasively, rapidly and relatively inexpensively[1]. In spite of being evaluated since 1983[2]; the accuracy, sensitivity and specificity of duplex Doppler imaging as a non-invasive diagnostic and prognostic modality for liver cirrhosis, and its correlation to the histopathologic findings as well as the degree of functional impairment of the liver, remains controversial and is still debated by many investigators[3]. Adding duplex Doppler evaluation in numerous studies has clarified the role of this modality in the evaluation of various CLD in adults and children, including liver cirrhosis[4-7]; portal hypertension[8-12]; presence or absence of esophageal varices[13], noninvasive diagnosis of the degree of hepatic fibrosis[14,15], assessment of the portal venous blood flow in cystic fibrosis[16], perioperative monitoring in orthotropic liver transplantation (LTx)[17], prediction of the severity of veno-occlusive disease and assessing its prognosis[18], assessment of the functional hepatic flow and total hepatic flow[19] and investigating the effect of fatty infiltration of the liver on the Doppler waveform pattern in the hepatic veins of obese children[3,20]. Other studies evaluated Doppler US measurement of the blood flow in the hepatic artery, hepatic veins and portal vein as a noninvasive indicator of disease severity in children who had undergone Kasai portoenterostomy for extrahepatic biliary atresia[21] and those who had CLD of unknown etiology[22]. However in the setting of LTx, serial intra- and post-operative Doppler US has largely been accepted as a useful technique for making an early diagnosis of abnormal hemodynamics of the graft circulation. Furthermore, intraoperative Doppler US is used to assess the reconstructed vessels objectively in order to reduce the incidence of vascular complications following LTx[6] and it is gradually replacing the more invasive angiographic techniques[23].

The aim of this study was to investigate the value of abdominal color-coded duplex Doppler US when added to the conventional grayscale scanning in the non-invasive assessment of the splanchnic morphology, as well as hemodynamics of the portal and hepatic veins in a cohort of Egyptian children suffering from CLD. We aimed also to detect any relationship between the US changes, etiology and severity (or stage) of the CLD.

MATERIALS AND METHODS

Materials and methods

We prospectively enrolled 25 children with CLD from the Pediatric Hepatology Unit at Cairo University Children Hospital, Cairo, Egypt. Thirteen patients (group 1) were diagnosed with established cirrhosis (7 girls and 6 boys) with a mean age of 8.9 ± 2.0 years, and 12 (group 2) were diagnosed with chronic hepatitis without cirrhosis (6 girls and 6 boys) with a mean age of 9.3 ± 2.3 years. Thirty healthy child relatives of the patients (13 girls and 17 boys) with a mean age of 8.1 ± 2.2 years were included as a control group. All patients were subjected to: (1) Careful interrogation and thorough physical examination for signs of CLD (jaundice, palmar erythema, bleeding diathesis, hand tremors, hepatomegaly, splenomegaly, ascites and edema); (2) biochemical tests of liver functions; (3) serological markers of viral and autoimmune hepatitis; (4) testing for inborn errors of metabolism when indicated; and (5) percutaneous liver biopsy using the Menghini aspiration technique for histopathological diagnosis, grading and staging of the CLD.

Patients, as well as controls, underwent conventional grayscale US using Toshiba® Sonolayer 2000 apparatus (Toshiba Corporation, Tokyo, Japan) equipped with 3.5 and 5 MHz convex linear transducers. Examination included liver size and echo pattern, portal vein diameter, splenic size and echo pattern, thickness of the lesser omentum in comparison to the aorta as well as detection of ascites. Both patients and controls also underwent color-coded duplex Doppler examination using Toshiba® Sonolayer SSH-60A apparatus (Toshiba Corporation, Tokyo, Japan) with a low frequency (3.5 MHz) transducer, in order to optimize the return of Doppler signals from deeper-lying tissues.

After being fasted overnight for a minimum of 8 h, all children were examined in the supine position. A low pulse repetition frequency was used initially, with manual adjustment when aliasing occurred. Approach for the vein of interest was selected to keep the beam vessel (angle 0) always less than 60°. Doppler recording of the hepatic veins was initially examined using a transverse sub-xiphoid approach, with the probe slightly cephalad. The right intercostal approach was used to obtain a longitudinal view of the middle hepatic vein. Every child was asked to stop breathing for a few seconds, in deep inspiration, during examination to avoid motion artifacts. Measurements were obtained from the hepatic veins at least 2 cm from the confluence with the inferior vena cava, to reduce the possible influence of the changes of flow pattern in the inferior vena cava on hepatic veins hemodynamics. We classified the hepatic vein Doppler waveforms according to Gorka *et al*[24] into: normal triphasic, abnormal biphasic or monophasic, and those with loss of the reverse-flow. Mean flow velocity of the middle hepatic vein was also measured in cm/s in all patients and controls.

The portal vein was examined from an anterior abdominal subcostal and/or right intercostal approach and scanned longitudinally throughout its entire length. Measurements were obtained in the middle segment between the splenoportal junction and the intrahepatic bifurcation (1-2 cm before the bifurcation). It was examined at a standard point for the diameter, patency, presence or absence

Table 1 Patients demographic and clinical data *n* (%)

Diagnosis	Group 1 (cirrhosis)	Group 2 (chronic hepatitis)
Symptoms		
Hematemesis	2 (15.4)	3 (25)
Jaundice	4 (38.5)	3 (25)
Dark urine	5 (38.5)	4 (33.3)
Abdominal distension	8 (61.7)	8 (66.7)
General physical signs		
Pallor	5 (38.5)	3 (25)
Jaundice	5 (38.5)	4 (33.3)
Lower limb edema	6 (46.2)	4 (33.3)
Abdominal signs		
Hepatomegaly	7 (53.8)	9 (75)
Splenomegaly	10 (76.9)	4 (33.3)
Ascites	5 (38.5)	3 (25)

of intraluminal echogenic material, direction of flow within the vein and blood flow velocity in cm/s.

The congestion index (CI) is determined by duplex Doppler US. It is the ratio between the cross-sectional area of the portal vein in cm^2 and the blood flow velocity in that vein in cm/s, and calculated using the following formula: CI = cross sectional area/blood flow velocity[25,26].

The cross sectional area was calculated using the following formula: Cross sectional area = $\pi \times (d^2/4)$; d = diameter of portal vein in cm; and $\pi = 3.14$[27].

Statistical analysis

All data were statistically analyzed using independent samples test, χ^2 test, post-Hock test and Armitage[28]. Probability (*P* value) was considered significant if $P < 0.05$.

RESULTS

Group 1 included 13 children with established cirrhosis (5 of metabolic etiology and 8 viral hepatitis C or B) and group 2 included 12 with chronic hepatitis and no cirrhosis (5 viral hepatitis C or B and 7 autoimmune). Table 1 shows the demographic and clinical data of the 2 groups. Biochemical tests of liver function showed no significant difference between either group (similar letters meant no significant difference, while dissimilar letters meant a significant difference, the *P* value is significant).

A prominent caudate lobe (as an important US sign) was found in 100% of patients with cirrhosis and none of patients with chronic hepatitis or controls. Thickened lesser omentum (i.e. lesser omentum: aortic diameter ratio > 1:1.7 in children[29]) was present in 69.21% of cirrhotic patients in comparison to 33.3% of patients with chronic hepatitis. Loss of the normal triphasic oscillation of the hepatic vein waveform was detected in 53.8% of group 1 in comparison to 8.3% of group 2 and none of the controls. Abnormal direction of portal blood flow was detected in 46.2% of group 1 and 25% of group 2, and none of the controls (Figures 1 and 2).

Hepatic vein flow velocity showed non-significant negative correlation with liver size (r = -0.125) and weak

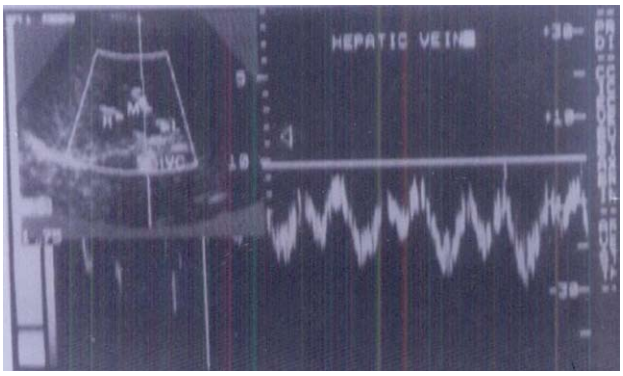

Figure 1 Triphasic waveform pattern of normal hepatic vein.

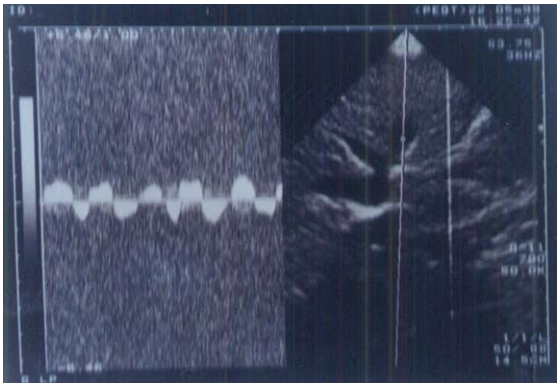

Figure 2 Abnormal waveform pattern of hepatic veins in a cirrhotic liver.

but significant correlation with both splenic size (r = -0.374) and portal vein diameter (r = -0.304) (Table 2).

Portal vein flow velocity had weak significant negative correlation with liver size (r = -0.431) and powerful significant negative correlation with both splenic size (r = -0.699) and portal vein diameter (r = -0.743, Figure 3).

CI had a weak significant negative correlation with liver size (r = -0.431) and powerful significant negative correlation with both splenic size (r = -0.699) and portal vein diameter (r = -0.743).

According to Child-Pugh's[30] classification, 4 (31%) of our cirrhotic patients were Class A; 4 (31%) Class B and 5 (38%) Class C. Those classes showed a powerful significant positive correlation with both abnormal hepatic waveform and abnormal direction of portal blood flow. Also there was a powerful significant negative correlation with both hepatic vein and portal vein flow velocity (r = -0.785 and -0.688, respectively, Figure 4) and weak significant positive correlation with CI (r = -0.595).

Analysis of the US findings according to the etiological categories (metabolic, viral or autoimmune CLD) did not reveal any significant correlations.

DISCUSSION

In the present study we tried to correlate the splanchnic morphological and hemodynamic parameters of the portal and hepatic veins with the severity of hepatic affection, as

Table 2 Grayscale and Doppler ultrasonography measurements in the studied groups (mean ± SD)

	Controls	Group 1	Group 2	*P* value
Liver size in cm	8.55 ± 0.69	10.19 ± 2.33[b]	12.2 ± 2.02[b,d]	< 0.01
Splenic size in cm	8.43 ± 0.92	11.82 ± 1.30[b]	10.10 ± 1.50[b,d]	< 0.01
Portal vein diameter (cm)	0.61 ± 0.13	1.16 ± 0.23[b]	0.95 ± 0.22[b,d]	< 0.01
Hepatic vein flow velocity (cm/s)	23.1 ± 2.09	21.84 ± 1.90	22.41 ± 2.02	> 0.05
Portal vein flow velocity (cm/s)	31.1 ± 3.92	21.23 ± 3.30[b]	22.22 ± 1.23[b]	< 0.01
Congestion index	4.61 ± 5.75[d]	9.50 ± 1.60	4.97 ± 1.14[d]	< 0.01

[b]$P < 0.01$ *vs* controls; [d]$P < 0.01$ *vs* group 1.

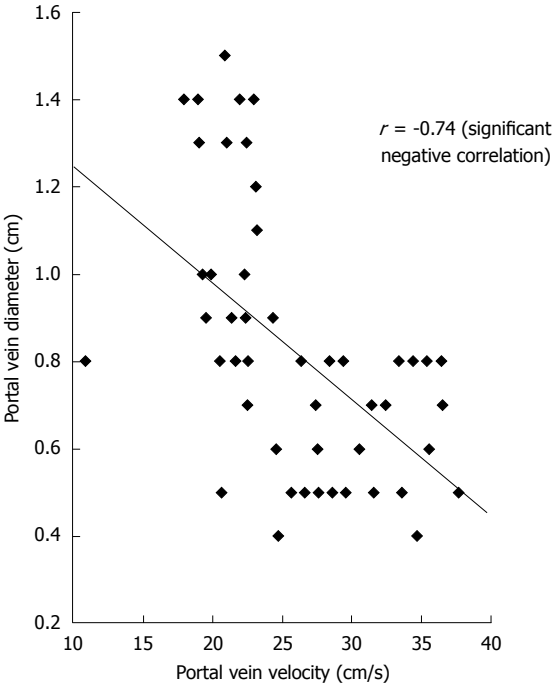

Figure 3 Correlation between portal vein flow velocity and portal vein diameter.

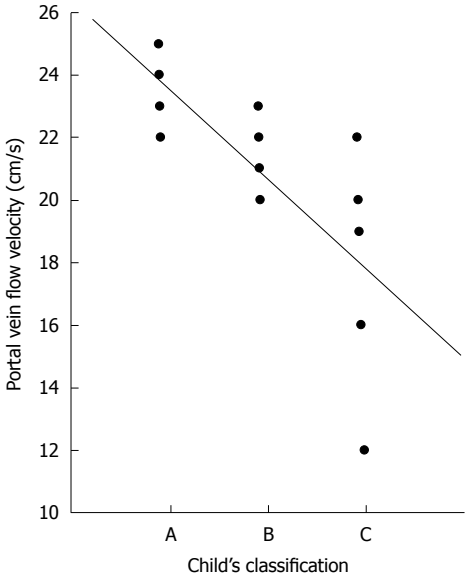

Figure 4 Correlation between Child-Pugh's classification and portal vein velocity.

evaluated by liver histopathology and Child-Pugh's classification of cirrhosis[30]. Prominent caudate lobe was a constant finding in all our patients with cirrhosis and not found in chronic hepatitis. The prominence of the caudate lobe in cirrhosis results from marked hyperplasic changes in the regenerative nodules with no cellular or structural atypia. The density of the regenerative hepatocytes becomes much higher, the quantity of bound water larger and free water smaller, increasing the US signals reflected. The reason why only the caudate lobe shows such huge hyperplasia in cirrhosis remains unclear[31].

Thickened lesser omentum (i.e. lesser omentum: aortic diameter more than 1:1.7[29]) is highly-suggestive of portal hypertension and the presence of esophageal varices in children, and allows the detection of portal hypertension earlier than detection of collaterals by Doppler and even earlier than clinical signs[32]. In our study there was a significant increase of lesser omentum thickness in cirrhotic patients than chronic hepatitis.

Koda *et al*[8], in 1996 described the decrease in portal vein flow velocity with the progress of chronic hepatitis as a sensitive indicator and a useful test with close correlation with the histological degree of liver fibrosis. Schneider *et al*[33], combined portal vein velocity with hepatic artery pulsatility index as a reliable, non-invasive evaluation of patients with liver cirrhosis. On the other hand Dinç *et al*[34], reported that portal vein flow velocity and portal vein flow volume alone are not useful parameters for discriminating cirrhotic patients from healthy subjects. In our study we did not measure the hepatic artery pulsatility index; however values of portal vein flow velocity were significantly lower in patients compared to controls and significantly lower in the group with liver cirrhosis than the chronic hepatitis. A significant correlation was also detected between both splenic size and Child-Pugh's class and the portal flow velocity; the lower the velocity, the larger the splenic size and the worse the Child-Pugh's class. Therefore in children with cirrhosis, portal flow velocity might be correlated with the severity of portal hypertension and the severity of liver parenchymal dysfunction as worsening Child-Pugh's class was associated with lower portal vein flow velocity conforming to reports in adults[35].

In the present study CI was significantly higher in the group with liver cirrhosis than both the chronic hepatitis group and the controls. CI showed no significant differ-

ence between chronic hepatitis group and controls. CI showed a positive correlation with Child-Pugh's class of cirrhotics. CI was reported to be a significant parameter in the evaluation of the risk of bleeding varices and prognosis of patients with liver cirrhosis, while in chronic hepatitis patients it was found to be similar to healthy controls and was not related to the grade of hepatic inflammation[36].

Loss of the normal triphasic oscillation of the hepatic vein waveform was detected in 53.8% of our cirrhotics, in comparison to 8.3% of chronic hepatitis and none of the controls. Bolondi *et al*[37], Ohta *et al*[38] and Arda *et al*[39], reported the loss of triphasic oscillation even in early-stage chronic parenchymal liver disease (Child-Pugh's class A).

In conclusion, grayscale and color-coded duplex Doppler US are very valuable, non-invasive diagnostic modalities in children with CLD. They could detect splanchnic morphological and portal hemodynamic changes that could be correlated to the degree of liver parenchymal affection but not to the etiology of the CLD. Therefore we recommend their wider application in the assessment of children with CLD.

COMMENTS

Background

The search for a non-invasive biochemical or imaging marker for the severity (stage) and/or etiology of chronic liver disease (CLD) in adults as well as children is in extremely active nowadays. Real time ultrasonography (US) has become an integral part of the non-invasive evaluation of the liver in many clinical settings in adults. Color-coded duplex Doppler information regarding the presence or absence of flow and the direction and velocity of that flow can be obtained non-invasively, rapidly and relatively inexpensively.

Research frontiers

The aim of the research is to investigate the value of duplex Doppler US in the assessment of the hemodynamics of the portal and hepatic veins in a cohort of children with CLD, and to detect any relationship between the US changes and etiology and severity (or stage) of CLD.

Innovations and breakthroughs

In the present study, the authors tried to correlate the splanchnic morphological and hemodynamic parameters of the portal and hepatic veins with the severity of hepatic affliction. In this study, the authors found that the values of portal vein flow velocity were significantly lower in patients compared to controls and significantly lower in the group with liver cirrhosis than the chronic hepatitis group. A significant correlation was also detected between both splenic size and Child-Pugh's class and the portal flow velocity; the lower the velocity, the larger the splenic size and the worse the Child-Pugh's class. Therefore in children with cirrhosis, portal flow velocity might be correlated with the severity of portal hypertension and the severity of liver parenchymal dysfunction.

Applications

In conclusion, grayscale and color-coded duplex Doppler US are very valuable, non-invasive diagnostic modalities in children with CLD. They could detect splanchnic morphological and portal hemodynamic changes that could be correlated to the degree of liver parenchymal affection but not to the etiology of the CLD. Therefore the authors recommend their wider application in the assessment of children with CLD.

Peer review

This article well documented that the grayscale and color-coded duplex Doppler US are very valuable for non-invasive diagnostic modalities in children with CLD, and this will interest the readers.

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S- Editor Sun H **L- Editor** Rutherford A **E- Editor** Zheng XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6145

World J Gastroenterol 2010 December 28; 16(48): 6145-6150
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Pegylated interferon α-2b up-regulates specific CD8+ T cells in patients with chronic hepatitis B

Ji Chen, Yan Wang, Xue-Jie Wu, Jun Li, Feng-Qin Hou, Gui-Qiang Wang

Ji Chen, Yan Wang, Xue-Jie Wu, Jun Li, Feng-Qin Hou, Gui-Qiang Wang, Department of Infectious Diseases, Center for Liver Diseases, Peking University First Hospital, Beijing 100034, China

Author contributions: Chen J and Wang Y contributed equally to this work; Chen J performed the laboratory work and wrote the paper; Wang Y collected the clinical samples and data, joined part in writing; Wu XJ performed the laboratory work; Li J and Hou FQ collected the samples and the data; Wang GQ designed the project and prepared the writing and supported all the work.

Supported by National Natural Science Foundation of China, No. 30771905; National Basic Research Program of China (973 Program), No. 2007CB512800; Mega-projects of Science Research, No. 008ZX10002-008; Beijing Municipal Science & Technology Commission, No. D08050700650803

Correspondence to: Gui-Qiang Wang, MD, Professor of Medicine, Chairman of Department of Infectious Diseases, Center for Liver Diseases, Peking University First Hospital, No. 8, Xishiku Street, Xicheng District, Beijing 10034, China. wanggq@hotmail.com
Telephone: +86-10-66551122 Fax: +86-10-66551680

Received: July 7, 2010 Revised: September 26, 2010
Accepted: October 3, 2010
Published online: December 28, 2010

Abstract

AIM: To investigate the effect of pegylated interferon (IFN) α-2b on specific CD8+ T lymphocytes in patients with chronic hepatitis B (CHB).

METHODS: Twenty-one patients with CHB were treated with pegylated IFN α-2b. Periphery blood mononuclear cells were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen) at weeks 0, 4, 8, 12, and 24, respectively. Frequency of circulating hepatitis B virus (HBV) epitope-specific CD8 T cells was detected by flow cytometry. Cytokines were detected by cytometric bead assay.

RESULTS: The frequency of circulating HBV core or env-specific CD8 T cells was higher ($P < 0.05$), the number of HBV core specific CD8 T cells was greater at week 24 ($P < 0.05$), the level of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor-α, and IFN-γ] was higher, while that of Th2-type cytokines (IL-4, IL-6, and IL-10) was lower in responders than in non-responders ($P < 0.05$) after pegylated IFN α-2b treatment. The IL-6 level was correlated with HBV DNA ($r = 0.597$, $P = 0.04$), while the inducible protein-10 (IP-10) level was correlated with serum alanine aminotransferase (ALT) ($r = 0.545$, $P = 0.005$). The IP-10 level at week 8 after pegylated IFN α-2b treatment could predict the normalization of ALT in CHB patients (positive predict value = 56%, negative predict value = 92%).

CONCLUSION: Pegylated IFN α-2b can enhance the immune response of CHB patients by increasing the frequency of HBV specific CD8+ T cells and regulating the Th1/Th2 cytokines.

Key words: Chronic hepatitis B; Pegylated interferon α-2b therapy; Immune response; Cytokine

Peer reviewer: Dr. Jeff Butterworth, MB, FRCP, Department of Gastroenterology, Shrewsbury and Telford Hospital NHS Trust, Mytton Oak Road, Shrewsbury, Shropshire, SY3 8XQ, United Kingdom

Chen J, Wang Y, Wu XJ, Li J, Hou FQ, Wang GQ. Pegylated interferon α-2b up-regulates specific CD8+ T cells in patients with chronic hepatitis B. *World J Gastroenterol* 2010; 16(48): 6145-6150 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6145.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6145

INTRODUCTION

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide[1,2]. Chronic hepatitis B (CHB) is a chronic inflammatory liver disease,

which can progress to end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma.

Adaptive immunity plays a central role in the pathogenesis of chronic HBV infection, and it is crucial to understanding the behavior of T cell response for the design of effective strategies for the control of HBV infection[3-5]. Different studies in chronic and early acute phases of HBV infection suggested that the functional impairment of HBV-specific cell-mediated immune response plays an important role in HBV persistence[6-14]. Moreover, recent studies showed that both positive and negative signals regulate the antigen-specific T cell function and are important for the better outcome of patients with HBV infections[15-17].

Pegylated interferon (IFN) α-2b can modulate and reduce antiviral function of CHB patients by enhancing their immune responses. However, the exact effect of pegylated IFN α-2b on the immune responses of patients with HBV infections remains unclear. The present study was designed to investigate the effect of pegylated IFN α-2b on HBV specific CD8+ T cells and secretion of cytokines in CHB patients.

MATERIALS AND METHODS

Patients and study design

Twenty-one consecutive CHB patients (17 males and 4 females) at the age of 20-39 years (mean 25 years), admitted to our hospital from January 2008 to May 2009 were included in this study. Diagnosis of HBV infection was established as previously described[18]. Clinical data and characteristics of the patients are summarized in Table 1. The patients were treated with pegylated IFN α-2b (PegIntron from Schering-Plough), at the dose of 0.5-1 μg/kg of body weight, once a week for 24 wk. Clinical and laboratory data about the patients were detected before treatment, or at weeks 4, 8, 12, and 24 after treatment. Patients co-infected with HBV and HCV or with detectable antibodies against hepatitis delta virus or against human immunodeficiency virus were excluded, as were those with other causes of liver disease, including alcohol abuse. No patient had decompensated liver disease (evidence or history of ascites, variceal bleeding, hepatic encephalopathy or jaundice).

Isolation of peripheral blood monouclear cells

Peripheral blood monouclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen). Blood was two-fold diluted with RPMI 1640 medium containing 300 μg/mL L-glutamin, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum, then added into the isovolumic Ficoll, centrifuged for 400 × *g* at 21℃ for 35 min. The cells were washed twice with phosphate buffered saline (PBS).

Human leukocyte antigen-A2 typing

One hundred microliters of fresh heparinized blood (100) was incubated with human leukocyte antigen-A2 primary antibody for 30 min. Erythrocytes were lysed with an erythrocyte lysate at 37℃, washed with PBS, and then incubated with secondary antibody, washed again and analyzed on Becton Dickinson FACS (Becton Dickinson, USA).

Analysis of HBV epitope-specific CD8+ T cells

Frequency of HBV epitope-specific CD8 T cells was detected by flow cytometry after incubated with HBV core18-27 tetramers (ProImmune, Oxford, UK) and HBV env 335-343 pentamers (ProImmune, Oxford, UK). Freshly isolated PBMC were incubated with PE-labeled tetramer or pentamer in PBS (10% FCS) for 15 min at 37℃, washed once with PBS (1% FCS) and then incubated on ice for 30 min with FITC-anti-CD8 (ProImmune, Oxford, UK), washed twice with PBS, adjusted to 1×10^6 cells/vial, and fixed in 2% paraformaldehyde for analysis. About 1×10^6 PBMC were harvested and analyzed within the CD8 gate on Becton Dickinson FACS using the CELLQuest™ software.

Secretion of cytokines

Serum levels of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and inducible protein-10 (IP-10) in CHB patients were measured by cytometric bead assay (BD, USA) according to its manufacturer's instructions.

Serological assessment

Fasting serum levels of liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase] were measured with a Hitachi-7180 automatic biochemistry analyzer (Hitachi Inc., Japan) following the standard laboratory methods. HBV DNA was detected by real time polymerase chain reaction (Amplicor, Roche).

Statistical analysis

All data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Descriptive baseline data were expressed as mean ± SD for continuous variables. Differences between groups were assessed using Kruskal-Wallis H for continuous variables. Spearman *P* test was performed for correlation analysis. The accuracy of serum factors for predicting virologic response was assessed using the receiver operating characteristic curve. $P < 0.05$ was considered statistically significant.

RESULTS

Frequency of circulating HBV epitope-specific CD8 T cells in CHB patients after pegylated IFN α-2b treatment

Circulating HBV epitope-specific CD8 T cells were detected 13 out of the 21 CHB patients (Table 1). The frequency of HBV core 18-27 tetramers+/CD8+ T cells at week 0 was 0.013 ± 0.002, which increased to 0.026 ± 0.015, 0.029 ± 0.019, 0.036 ± 0.025, and 0.045 ± 0.027, respectively, at weeks 4, 8, 12, and 24 after IFN α-2b treatment (Figure 1), with a significant difference between weeks 8 and 0, and between weeks 24 and 0 ($P < 0.05$). The frequency of HBV env 335-343 pentamers+/CD8+ T cells began to increase at week 8 with a significant difference between weeks 24 and 0 ($P < 0.05$). No significant difference was observed in frequency of HBV core and HBV env specific CD8 T cells.

Table 1 Clinical characteristics of chronic hepatitis B patients included in this study

Patient	Age (yr)/sex	HBV DNA (IU/L)	ALT (U/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	Platelets ($\times 10^9$/L)	HBeAg	HBeAb	HBsAg	HBsAb	Genetype
1	27/M	201 000 000	143	9.1	43.7	113	+	-	+	-	C
2	25/M	160 000 000	147	12.9	47.4	167	+	+	+	-	C
3	30/M	471 000 000	205	11.9	44.3	127	+	-	+	-	C
4	21/M	32 200 000	123	10.8	45.8	181	+	-	+	-	C
5	21/M	186 000 000	148	14.2	44.1	110	+	-	+	-	C
6	38/F	30 800 000	347	18.1	45.5	126	+	-	+	-	C
7	20/F	29 000 000	171	10.0	44.9	284	+	-	+	-	C
8	20/M	143 000 000	-	13.6	48.0	248	+	-	+	-	B
9	20/F	621 000 000	112	13.4	48.5	170	+	-	+	-	C
10	23/M	597 000 000	196	18.9	43.2	201	-	+	+	-	C
11	20/F	2 910 000	98	15.6	48.0	137	+	+	+	-	C
12	38/M	63 700 000	206	11.1	51.2	142	+	+	+	-	C
13	28/M	134 000 000	138	9.0	45.9	174	+	+	+	-	C
14	25/M	237 000 000	93	16.9	51.3	130	+	-	+	-	B
15	39/M	1 190 000 000	122	18.2	50.6	166	+	-	+	-	C
16	36/M	8 820 000	170	22.9	47.1	169	+	-	+	-	C
17	25/M	655 000 000	90	20.9	46.8	161	+	-	+	-	B
18	25/M	157 000 000	164	24.3	47.3	209	-	+	+	-	C
19	20/M	290 000 000	124	11.9	47.8	140	+	-	+	-	B
20	23/M	655 000 000		19.9	44.6	194	+	-	+	-	B
21	28/M	15 300 000	237	14.5	45.3	154	+	-	+	-	C

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody.

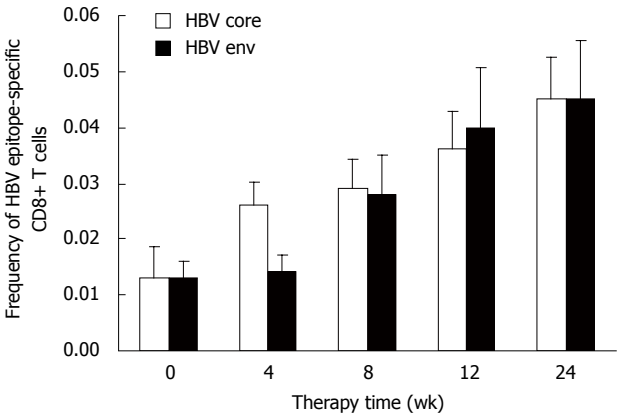

Figure 1 Frequency of hepatitis B virus epitope tetramer+/CD8+ T cell after pegylated interferon α-2b treatment. The frequency of hepatitis B virus (HBV) specific CD8+ T cells was increased connectively at weeks 4, 8, 12 and 24 after pegylated interferon α-2b treatment with no difference in frequency of HBV core specific CD8+ T cells and HBV env specific T cells.

To further analyze the effect of pegylated IFN α-2b on HBV-specific CD8 T cells, 13 patients were divided into responders (n = 7) and non-responders (n = 6). Responders were defined as their ALT returned to its normal level and their HBV DNA was decreased to over 2log, and/or their serum HBeAg was conversed. The frequency of HBV core18-27 tetramers+/CD8+ T cells was 0.014 ± 0.011, 0.029 ± 0.022, 0.029 ± 0.021, 0.067 ± 0.029, and 0.05 ± 0.025, respectively, in responders at weeks 0, 4, 8, 12 and 24 after treatment, which was higher than that in non-responders (0.012 ± 0.007, 0.018 ± 0.009, 0.028 ± 0.019, 0.025 ± 0.021 and 0.030 ± 0.01, respectively). No significant difference was found in frequency of HBV core specific CD8 T cells between responders and non-responders at baseline, even at weeks 4, 8, and 12 after treatment (Figure 2), with a significant difference observed at week 24 ($P < 0.05$, Figure 3). The frequency of HBV env specific CD8 T cells was higher in responders than in non-responders ($P < 0.05$, Figure 2).

Secretion of cytokines after pegylated IFN treatment and its correlation with virologic responses

The serum levels of IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFN-γ, IL-12, and IP-10 were measured at baseline, during the treatment and follow-up. The serum IL-2 level was very low in CHB patients, which was almost undetectable. The levels of Th1-type cytokines including IL-12, TNF-α and IFN-γ were increased while those of Th2-type cytokines including IL-4, IL-6 and IL-10 were decreased at week 48 after treatment (Figure 4). The baseline IP-10 level was increased from week 4 and decreased from week 48 after treatment.

The baseline IL-6 level was correlated with HBV DNA in responders (r = 0.597, P <0.05) but not with HBV DNA in non-responders. IL-10 was correlated with IL-6 (r = 0.762, P = 0.002), and IL-12 was correlated IFN-γ (r = 0.485, P = 0.026).

The IP-10 level was closely correlated with the serum ALT level not only in responders but also in non-responders (r = 0.545, P = 0.005, Figure 5), indicating that IP-10 level fluctuates with serum ALT level. The baseline IP-10 level was lower in patients with their ALT < 40 U/L than in those with their ALT > 40 U/L.

Predictability of IP-10

To determine whether IP-10 can predict the normalization of ALT (< 40 U/L) after peg-IFN α-2b treatment, receiver operating characteristic curve was plotted for IP-10. The

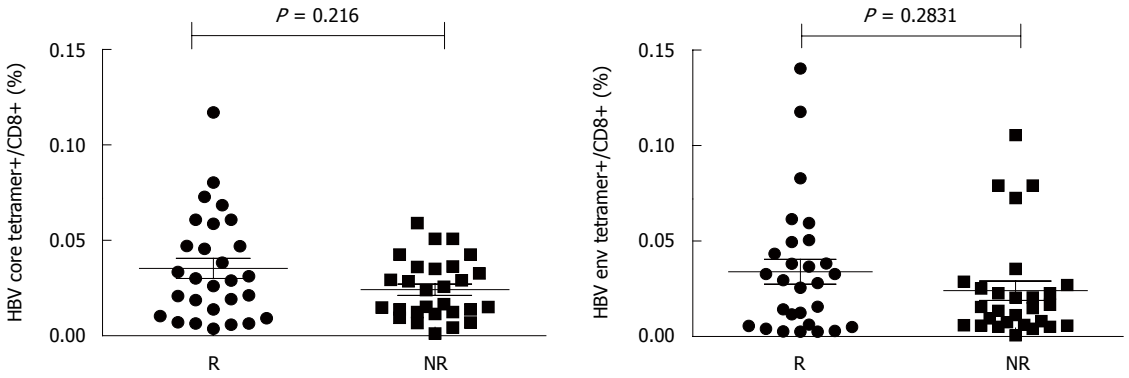

Figure 2 No correlation between increased hepatitis B virus epitope-specific CD8+ T cells and treatment outcome. The frequency of hepatitis B virus (HBV) core or env epitope-specific CD8+ T cells was higher in non-responders (NR) than in responders (R).

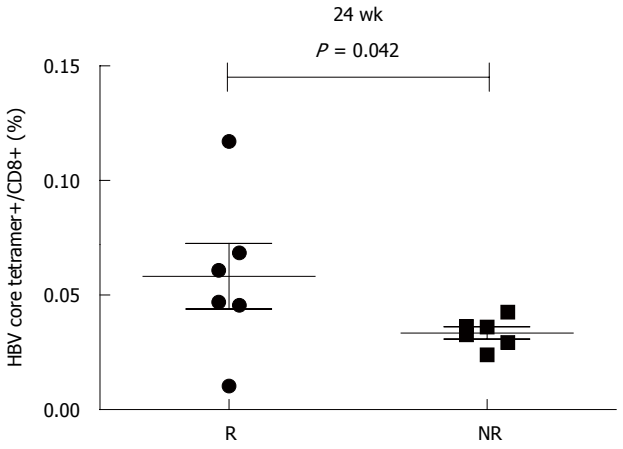

Figure 3 Correlation between increased hepatitis B virus specific T cells and treatment response 24 wk after therapy. The frequency of hepatitis B virus (HBV) core epitope-specific CD8+ T cells at week 24 was higher in responders (R) than in non-responders (NR).

IP-10 level at week 8 after treatment was predictable. The area under the curve was 0.741 ($P = 0.065$). A cutoff value of 437.78 was chosen. Correspondingly, the positive and negative predictive value was 56% and 92%, respectively (Table 2).

DISCUSSION

HBV has a high propensity to persist and several strategies have been developed for control of its evading from T cell responses, including the direct inhibitory effect of viral proteins on T cell responses and the emergence of escape mutations[19-21]. Moreover, HBV infection is more common in immune deficient individuals, such as infants, patients with cancer and those treated with steroid hormone, thereby can interfere with viral clearance by the innate immune system[22,23]. Inefficient innate responses and rapid spread of HBV may in turn delay and impair adaptive responses because of inefficient promotion of T cell priming by innate immunity and through T cell exhaustion induced by a rapidly increased viral load. However, the actual impact of exhaustion by persistent exposure to high antigen concentrations on virus persistence has only been partially defined.

Furthermore, two kinds of drugs (nucleoside analogs and IFN) are usually used in antiviral treatment of CHB

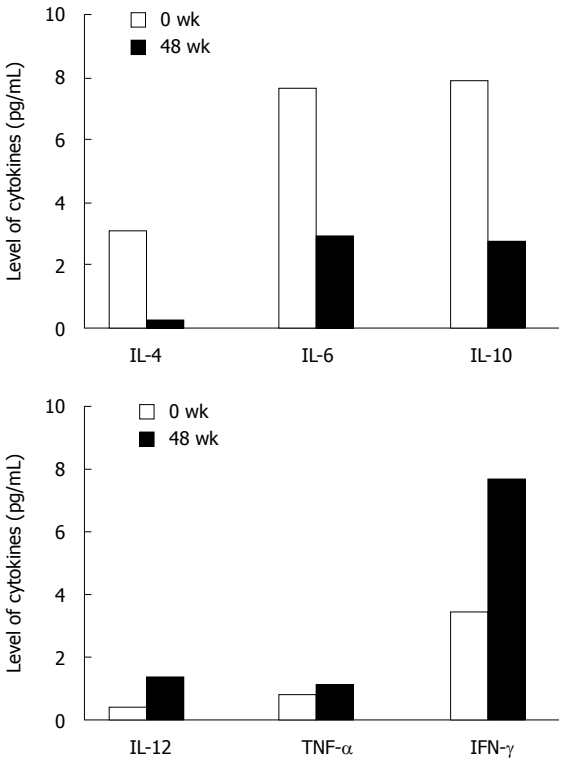

Figure 4 Level of cytokines after treatment. The levels of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ] were higher, while the levels of Th2-type cytokines (IL-4, IL-6 and IL-10) were lower at week 48 after treatment.

patients. IFN is involved in numerous immune interactions during viral infection, as an inducer, regulator, and effector of both innate and adaptive antiviral systems. IFN-α and beta are produced rapidly due to viral factors, such as envelope glycoprotein, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors[24]. In addition, IFN modulates both innate and adaptive immunity, ultimately resulting in an enhanced antiviral effector function.

In the present study, the frequency of HBV epitope-specific CD8+ T cells in peripheral blood was persistently increased at weeks 4, 8, 12 and 24 after peg-IFN α-2a treatment, while the number of HBV epitope-specific CD8 T cells in HBV core 18-27 tetramers and HBV env 335-343

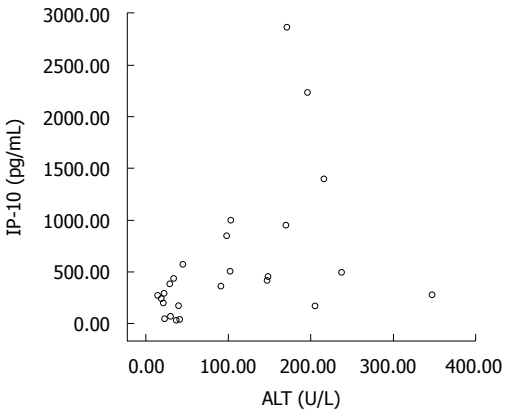

Figure 5 Positive correlation between alanine aminotransferase and inducible protein-10 levels ($r = 0.545$, $P = 0.005$). ALT: Alanine aminotransferase; IP-10: Inducible protein-10.

Table 2 Predictive value of serum inducible protein-10 levels 8 wk after treatment

IP-10 (pg/mL)	ALT < 40 U/L	ALT ≥ 40 U/L	Predictive value
< 437.78	5	4	PPV = 56%
≥ 437.78	1	11	NPV = 92%

IP-10: Inducible protein-10; ALT: Alanine aminotransferase; PPV: Positive predict value; NPV: Negative predict value.

pentamers was greater in responders than in non-responders after pegylated IFN α-2b treatment, suggesting that the therapeutic effect of pegylated IFN α-2b on HBV infection may be attributed to the elevated HBV-specific CD8 T cells, and that the immune response mediated by HBV-specific cells plays an important role in control of HBV. However, the frequency of HBV core 18-27 tetramers+/CD8+ T cells was higher than that of HBV env 335-343 pentamers+/CD8+ T cells after pegylated IFN treatment, suggesting that the HBV core epitope plays a more critical role in induction of a stronger immune response to HBV infection than to HBV env epitope. Pegylated IFN α-2b could enhance specific immune response of CHB patients. Further study should be performed with a large sample size.

Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. IL-10, a Th2-type cytokine secreted by T-cells, activated B cells and monocytes, is a powerful inhibitor of Th1 activation and suppresses cell-mediated immunity in mice and humans[25,26]. Of the detected cytokines, Th2-type cytokines such as IL-4, IL-6 and IL-10, were altered conspicuously. After treatment, the level of Th2-type cytokines (IL-4 and IL-10) was down-regulated, thus confirming the immune recover potential of pegylated IFN α-2b, the level of IL-12 which can promote the differentiation of Th1-type cytokines was low, and the production of Th1-type cytokines was increased, indicating that the immune function of pegylated IFN α-2b can be achieved by regulating the balance of Th1/Th2 cytokines.

IL-6 is a multifunctional cytokine with both differentiation and growth-promoting effects for a variety of target cells. IL-6 is generally considered an important cytokine in the network of cytokines that regulate immune reactions and acute phase responses[27]. It was reported that IL-6 is correlated with liver fibrosis/cirrhosis[28] and is a cell attachment site for HBV[29]. In the present study, the IL-6 level was correlated with HBV DNA plasma only in responders.

IP-10, a chemotactic CXC chemokine of 77 aa in its mature form[30,31], can be produced by a variety of cells, including hepatocytes[32,33]. The correlation between IP-10 levels and necroinflammatory activity, as well as the high and low IP-10 levels before and after pegylated IFN α-2b treatment, may imply that IP-10 plays a role in the natural pathogenesis of HBV-induced liver damage[34]. It was reported that the baseline IP-10 level can predictive the response of CHB patients to HCV treatment, and is correlated with liver inflammation and fibrosis[35,36]. In this study, the baseline IP-10 level in CHB patients could predict the normalization of ALT after pegylated IFN α-2b treatment.

In conclusion, given the importance of protective T cell responses in control of HBV, the correlation between immunomodulatory molecules and pegylated IFN α-2b treatment in restoration of the immune responses of antiviral T cells are highly desirable. Pegylated IFN α-2b therapy can enhance the immune response of CHB patients by influencing the production of cytokines. IP-10 can potentially predict the normalization of ALT, which is correlated with liver damage. Further study is needed with a large sample size.

ACKNOWLEDGMENTS

The authors thank Dr. Ming Yu and Hong-Li Xi for their technical support help in this study.

COMMENTS

Background

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide. Two kinds of drugs [nucleoside analogs and interferon (IFN)] are mainly used in treatment of chronic hepatitis B (CHB) patients. IFN is involved in numerous immune interactions as an inducer, regulator, and effector in treatment of viral infections. Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. Inducible protein 10 (IP-10) is a chemotactic CXC chemokine of 77 aa in its mature form.

Research frontiers

IFN-α and β are produced rapidly due to viral factors, such as envelope glycoproteins, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors. IP-10 can be produced by a variety of cells, including hepatocytes. The results of this study show that the baseline IP-10 level can predict the response of patients with HBV infection to its treatment with pegylated IFN α-2b.

Innovations and breakthroughs

The present study demonstrated the correlation between pegylated IFN α-2b treatment and HBV-specific T lymphocytes. In addition, the effect of pegylated IFN α-2b on HBV infection could be achieved by balancing the production of Th1/Th2 cytokines and IP-10 could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment.

Applications

In this study, pegylated IFN α-2b could up-regulate HBV epitope specific CD8+ T cells. The specific cellular immune response could control HBV. IP-10 serum

level could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment, thus providing a new index for the treatment of HBV infection. Pegylated IFN α-2b may be used as a novel strategy for the treatment of HBV infection by regulating the cytokines.

Terminology

Human leukocyte antigen (HLA) typing is a method to define the HLA+ and HLA+ blood for studied subjects. Flow cytometry is used to define the HBV epitope specific CD8+ T lymphocytes. Cytometric bead assay is a new technique for detecting serum concentration of cytokines.

Peer review

This is a very interesting study, showing that pegylated IFN α-2b therapy can increase the frequency of specific CD8+ T lymphocytes in CHB patients. This may contribute to the better control of HBV replication and to the recovery of CHB patients, thus having a promise for therapeutic interventions. The experiments support the claim of the authors.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6151

World J Gastroenterol 2010 December 28; 16(48): 6151-6154
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Short-segment Barrett's esophagus and cardia intestinal metaplasia: A comparative analysis

Ying Chang, Bin Liu, Gui-Sheng Liu, Tao Wang, Jun Gong

Ying Chang, Digestive Endoscopic Center, Shanghai Sixth Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
Bin Liu, Department of Emergence Surgery, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China
Gui-Sheng Liu, Department of Gastroenterology, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China
Tao Wang, Department of Gastroenterology, First Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Jun Gong, Department of Gastroenterology, Second Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Author contributions: Chang Y and Gong J designed the research; Chang Y, Liu B, Liu GS and Wang T performed the research; Chang Y, Liu GS and Wang T analyzed the data; Chang Y and Liu GS wrote the paper.
Supported by The Medical and Technology Cross Foundation of Shanghai Jiao Tong University, No. YG2010MS44
Correspondence to: Dr. Ying Chang, Digestive Endoscopic Center, Shanghai Sixth Hospital, Shanghai Jiao Tong University, Yishan Road 600, Shanghai 200233, China. mulan929@hotmail.com
Telephone: +86-21-64369181 Fax: +86-21-64369181
Received: August 30, 2010 Revised: September 26, 2010
Accepted: October 3, 2010
Published online: December 28, 2010

Abstract

AIM: To investigate the endoscopy and histology of short-segment Barrett's esophagus (SSBE) and cardia intestinal metaplasia (CIM), and their correlation with *Helicobacter pylori* (*H. pylori*) gastritis and gastroesophageal reflux disease (GERD).

METHODS: Biopsy specimens were taken from 32 SSBE patients and 41 CIM patients with normal appearance of the esophagogastric junction. Eight biopsy specimens from the lower esophagus, cardia, and gastric antrum were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff, Alcian blue/high iron diamine and Gimenez dye. Results were graded independently by one pathologist.

RESULTS: The SSBE patients were younger than the CIM patients ($P < 0.01$). The incidence of dysplasia and incomplete intestinal metaplasia subtype was higher in SSBE patients than in CIM patients ($P < 0.01$). *H. pylori* infection was correlated with antral intestinal metaplasia ($P < 0.05$), but not with reflux symptomatic, endoscopic, or histological markers of GERD in CIM patients. SSBE was correlated with reflux symptomatic and endoscopic esophagitis ($P < 0.01$), but not with *H. pylori* infection and antral intestinal metaplasia.

CONCLUSION: Dysplasia risk is significantly greater in SSBE patients than in CIM patients. CIM is a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, whereas SSBE may result from GERD.

Key words: Endoscopy; Barrett's esophagus; Cardia intestinal metaplasia; Esophagogastric junction; Gastroesophageal reflux disease

Peer reviewer: Bernabe Matias Quesada, MD, Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9°A, Buenos Aires 1013, Argentina

Chang Y, Liu B, Liu GS, Wang T, Gong J. Short-segment Barrett's esophagus and cardia intestinal metaplasia: A comparative analysis. *World J Gastroenterol* 2010; 16(48): 6151-6154 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6151.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6151

INTRODUCTION

The incidence of adenocarcinoma in esophagus and gastroesophageal junction (GEJ) has increased in recent years in North America, Europe, Japan and China[1-3]. Barrett's esophagus (BE) is thought to be a premalignant condition of esophageal adenocarcinoma, accounting for most cases of adenocarcinoma of the GEJ. The reported prevalence of Barrett's-associated adenocarcinoma varies widely, with an average of 10%[4-7]. A meta-analysis[8] of 4120 patients

in China reported that BE is found in 2.44% of patients undergoing endoscopy for various symptoms of upper gastrointestinal tract diseases.

It was reported that the frequency of short-segment Barrett's esophagus (SSBE), < 3 cm in length, is increased and implicated as a risk factor for adenocarcinoma of the cardia[9-11]. Endoscopic diagnosis of this entity is difficult and always requires histological demonstration of specialized columnar epithelium (SCE). Since most endoscopists do not perform biopsies unless the columnar epithelium is seen to extend from the proximity to the GEJ. Short segments are frequently unrecognized. Spechler *et al*[12] have recently described the presence of intestinal metaplasia in certain normal-appearing GEJ. The relation of this condition to SSBE has not yet been investigated.

In this study, SSBE and cardia intestinal metaplasia (CIM) were compared and their correlation with *Helicobacter pylori* (*H. pylori*) gastritis and gastroesophageal reflux disease (GERD) was studied, which may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

MATERIALS AND METHODS

Patients

Tissue specimens used in this study were provided by The Sixth Hospital of Shanghai Jiaotong University, with the approval of the hospital and patients. Endoscopy was performed in a standardized manner by experienced endoscopists. Appearance of the squamocolumnar junction was carefully studied in a prograde view after insufflation of air and retroversion in the stomach. Thirty-two consecutive patients with endoscopically apparent SSBE (< 3 cm in length) included in the study (group A) were selected from The Outpatient Clinic of our hospital over a two-year period. Two endoscopic features of the squamocolumnar transition were considered indicative of SSBE: a straight and regular Z line (< 3 cm) displaced upwards in relation to the GEJ (circumferential type), and an irregular Z line with eccentric tongues of red mucosa extending above the GEJ (digital type). The severity of SSBE was measured according to the Prague C&M classification[13]. The specimens were stained with Alcian blue (pH 2.5).

Group B was consisted of 41 adult ambulatory consecutive patients who underwent upper endoscopy in our endoscopy unit and were considered by the endoscopist to have a normal-appearing GEJ. Patients with a history of cancer or prior gastric/esophageal surgery were excluded, as were those who were unable to give their informed consent, or who had any contraindication to endoscopic biopsies. CIM was defined based on the presence of barrel-shaped goblet cells in normal-appearing GEJ.

All patients included in this study were questioned about symptoms of GERD (heartburn, regurgitation, and odynophagia). Endoscopic signs of esophagitis were recorded and graded according to the Los Angeles classification[14].

Endoscopy and biopsy protocol

Biopsy specimens were taken from 32 patients with SSBE and 41 CIM patients with normal-appearing GEJ. Eight biopsy specimens, taken from the lower esophagus, cardia, and gastric antrum, were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff (AB/PAS, pH 2.5), AB/high iron diamine (AB/HID) and Gimenez dye. Results were graded independently by one pathologist.

Table 1 Incidence of dysplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients

Patients	*n*	Dysplasia	%
CIM	41	1	2.4
SSBE	32	4	12.5[b]

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Biopsy specimens taken from 41 CIM patients and 32 short-segment Barrett's esophagus (SSBE) patients were stained with hematoxylin and eosin. The incidence of dysplasia was calculated. The incidence of dysplasia was significantly higher in SSBE patients than in CIM patients (12.5% *vs* 2.4%, $P < 0.01$).

Histology

Formalin-fixed, paraffin-embedded biopsy samples were stained with hematoxylin/eosin. PAS/AB (pH 2.5) was used to show the presence of acid mucins. BE was diagnosed based on the presence of SCE, which was defined by the unequivocal demonstration of intestinal-type goblet cells.

Statistical analysis

Statistical analysis was performed using the χ^2 test.

RESULTS

Incidence of dysplasia in SSBE and CIM patients

The SSBE patients were younger than the CIM patients ($P < 0.01$). The incidence of dysplasia was higher in SSBE patients than in CIM patients ($P < 0.01$) (Table 1).

Incidence of incomplete intestinal metaplasia in SSBE and CIM patients

The incidence of incomplete intestinal metaplasia (IM) was significantly different between the two types of epithelium ($P < 0.01$ *vs* CIM) (Table 2).

Prevalence of GERD in SSBE and CIM patients

The prevalence of GERD symptoms was higher in SSBE patients than in CIM patients ($P < 0.01$), as was endoscopic and histological evidence of esophagitis (Table 3).

Correlation between H. pylori and antral IM in SSBE and CIM patients

The correlation between *H. pylori* infection and antral IM in SSBE and CIM patients is shown in Table 4.

DISCUSSION

Over the past two decades, the incidence of adenocarcinoma of the esophagus and gastric cardia has increased rapidly. BE is recognized as a precancerous lesion of esophageal adenocarcinoma in most cases of adenocarcinoma

Table 2 Incidence of incomplete intestinal metaplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients

Patients	*n*	Incomplete IM	Complete IM	%
CIM	41	8	33	19.5
SSBE	32	21	11	65.6[b]
Total	73	29	44	39.7

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Eight biopsy specimens taken from the lower esophagus and cardia were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff (pH 2.5), AB/high iron diamine or Gimenez dyes. The prevalence of incomplete intestinal metaplasia (IM) was significantly higher in short-segment Barrett's esophagus (SSBE) patients than in CIM patients (65.6% *vs* 19.5%, $P < 0.01$).

Table 3 Incidence of reflux symptomatic, endoscopic, or histological markers of gastroesophageal reflux disease in short-segment Barrett's esophagus and cardia intestinal metaplasia patients *n* (%)

Patients	*n*	Reflux symptoms	Endoscopic esophagitis	Histological features of reflux esophagitis
CIM	41	12 (29.3)	5 (12.2)	12 (29.3)
SSBE	32	26 (81.2)[b]	30 (93.8)[b]	31 (96.9)[b]

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). All patients were questioned about symptoms of gastroesophageal reflux disease (GERD). Endoscopic signs of esophagitis were recorded and graded. All biopsy specimens were stained with hematoxylin and eosin. Alcian blue/periodic acid-Schiff (pH 2.5) was used to show the presence of acid mucins. The incidence of reflux symptomatic, endoscopic, or histological markers of GERD was higher in short-segment Barrett's esophagus (SSBE) patients than in CIM patients ($P < 0.01$).

Table 4 Relation between *Helicobacter pylori* infection and antral intestinal metaplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients *n* (%)

Patients	*n*	Cardia *H. pylori* infection	Antral IM	Antral *H. pylori* infection
CIM	41	18 (43.9)	21 (51.2)	20 (48.8)
SSBE	32	5 (15.6)[a]	4 (12.5)[b]	7 (21.9)[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Eight biopsy specimens taken from the lower esophagus, cardia, and gastric antrum were stained with Alcian blue/periodic acid-Schiff (pH 2.5) and Gimenez dyes, respectively. The incidence of *Helicobacter pylori* (*H. pylori*) infection and antral intestinal metaplasia (IM) was lower in short-segment Barrett's esophagus (SSBE) patients than in CIM patients ([a]$P < 0.05$, [b]$P < 0.01$).

of the GEJ. Progression from metaplasia to dysplasia and adenocarcinoma is well documented[7]. Traditionally, BE is arbitrarily defined as a circumferential segment of columnar-lined epithelium (2 or 3 cm in length) in the lower esophagus. However, this macroscopic definition has been recently questioned, because it excludes shorter segments and "tongues of columnar-lined epithelium", which are frequently found in the distal esophagus, and endoscopic measurements can be imprecise. It has therefore been proposed that the diagnosis of BE should be reserved for patients with IM detected in biopsy specimens from the distal esophagus[15,16]. Recently, the presence of CIM in certain normal-appearing GEJ has been described[17-19]. Detection of IM in the distal esophagus as well as within the gastric cardia has been reported with an increasing frequency[15,16]. It was reported that the prevalence of BE and CIM is 2%-12% and 5%-23%, respectively, in patients undergoing routine upper gastrointestinal endoscopy[20,21]. Detection of IM in BE patients potentially commits the patients to regular surveillance endoscopy with biopsy. The incidence of adenocarcinoma in patients with BE is estimated to be 30-50 times greater than that in general populations, and is on the increase[6,7]. However, the exact incidence of cancer in patients with BE is unknown, and the role of CIM as a premalignant lesion is still unclear. The relation of this condition to BE has not yet been investigated. Whether CIM and IM originating from the esophageal mucosa have a common pathogenesis and identically associated risk factors remains unknown.

In the present study, the dysplasia risk was significantly higher in SSBE patients than in CIM patients (12.5% *vs* 2.4%). Sharma *et al*[15] also compared the incidence of dysplasia in 177 SSBE patients and 76 CIM patients. As in our study, the risk of dysplasia differed significantly between the two groups. Dysplasia was detected in 11.3% (20/177) of SSBE patients and in 1.3% (1/76) of CIM patients, indicating that dysplasia is two potentially different clinical processes. Future studies should separate SSBE from CIM to improve our understanding of the pathophysiology and malignant potential of each entity.

Although a few authors reported that the areas adjacent to CIM show normal foveolar epithelium, whereas those adjacent to BE contain pre-goblet cells that can be positively stained with Alcian blue[15,16]. Since these characteristics cannot be found in all biopsy specimens, it is not reliable to distinguish SSBE from CIM histologically. HID/AB staining has also been used to distinguish SSBE from CIM[17-19]. It was reported that IM at the GEJ (or ultra-short-segment BE) is more frequently found to express sulfomucins, which is defined as type Ⅲ IM and involves the surface glandular epithelium[11,17]. Liu *et al*[10] also found that the area covered by incomplete IM is significantly greater and the level of sulfomucins is obviously higher in the esophagus than in the stomach. In our study, the prevalence of type Ⅲ IM was significantly higher in SSBE patients than in CIM patients (65.6% *vs* 19.5%, $P < 0.01$). HID/AB staining can be used to distinguish SSBE from CIM initially, based on the different expressions of neutral mucins, sialomucins, and sulfomucins.

The incidence of reflux symptomatic, endoscopic, or histological markers of GERD was higher while that of *H. pylori* infection and antral IM was lower in SSBE patients than in CIM patients ($P < 0.05$). Since CIM is a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, and SSBE can result from GERD, it is necessary to explore new and efficacious diagnostic methods to distinguish BE from CIM.

cDNA microarray methods have been used in the study of gene expression, DNA sequencing, novel genes and mutations, DNA polymorphism, drug screening, diagnosis of disease, and gene mapping, since they were reported by Schena *et al*[22] in 1995. We have previously

performed an analysis of three 4096 chips to investigate the difference in gene expression profiles between BE and CIM epithelium[23]. A total of 141 genes were screened that exhibited a differential expression in the three chips. A comparison between the two gene profiles showed that the gene expression patterns were different in BE and CIM epithelium, illustrating that detection of differences in gene expression between BE and CIM with gene chips is a new method for the diagnosis, treatment and prevention of BE. Future studies should separate SSBE from CIM to improve our understanding of the pathophysiology and malignant potential of such diseases.

COMMENTS

Background

The incidence of adenocarcinoma in the esophagus and gastroesophageal junction (GEJ) has increased in recent years. Barrett's esophagus (BE) is thought to be a premalignant condition. Recently, the presence of cardia intestinal metaplasia (CIM) in certain normal-appearing GEJ has been described. The relation between CIM and BE has not yet been investigated.

Research frontiers

Short-segment Barrett's esophagus (SSBE), < 3 cm in length, has been reported as a risk factor for adenocarcinoma of the cardia. Whether CIM and IM originating from the esophageal mucosa have a common pathogenesis still remains unknown. In this study, the authors demonstrated that CIM was a manifestation of *Helicobacter pylori* (*H. pylori*)-associated and multifocal atrophic gastritis, whereas SSBE could result from gastroesophageal reflux disease (GERD).

Applications

This study describing the he different characteristics of SSBE and CIM may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

Terminology

BE is thought to be a premalignant condition of esophageal adenocarcinoma, accounting for most cases of adenocarcinoma of the GEJ. BE is defined as IM detected in biopsy specimens from the distal esophagus. The extent of the Barrett segment is measured according to the Prague C&M classification.

Peer review

The authors examined the different characteristics of SSBE and CIM and revealed that CIM was a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, whereas SSBE could result from GERD. The results are interesting and may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

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S- Editor Sun H **L- Editor** Wang XL **E- Editor** Lin YP

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i48.6155

World J Gastroenterol 2010 December 28; 16(48): 6155-6162
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Increasing the frequency of CIK cells adoptive immunotherapy may decrease risk of death in gastric cancer patients

Jing-Ting Jiang, Yue-Ping Shen, Chang-Ping Wu, Yi-Bei Zhu, Wen-Xiang Wei, Lu-Jun Chen, Xiao Zheng, Jing Sun, Bin-Feng Lu, Xue-Guang Zhang

Jing-Ting Jiang, Chang-Ping Wu, Lu-Jun Chen, Xiao Zheng, Department of Tumor Biological Treatment, Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu Province, China
Jing-Ting Jiang, Yi-Bei Zhu, Jing Sun, Xue-Guang Zhang, Key Laboratory of Stem Cell of Jiangsu Province, Institute of Biotechnology, Key Laboratory of Clinical Immunology of Jiangsu Province, Soochow University, Suzhou 215123, Jiangsu Province, China
Yue-Ping Shen, Wen-Xiang Wei, Department of Medicine, Soochow University, Suzhou 215123, Jiangsu Province, China
Bin-Feng Lu, Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, United States
Author contributions: Jiang JT and Shen YP contributed equally to this work, designed the study and drafted the manuscript; Wu CP and Zhu YB participated in the experimental design; Wei WX and Chen LJ provided the clinical samples; Zheng X and Sun J analyzed the data and formatted the figures; Zhu YB performed all the experiments; Lu BF and Zhang XG drafted the manuscript and designed the study; all authors read and approved the final manuscript.
Supported by The National Natural Science Foundation of China, No. 30872176, 30950022 and 30972703; grants of Jiangsu Province and Soochow University Medical Development Foundation, No. EE126765
Correspondence to: Xue-Guang Zhang, MD, PhD, Key Laboratory of Stem Cell of Jiangsu Province, Institute of Biotechnology, Key Laboratory of Clinical Immunology of Jiangsu Province, Soochow University, Suzhou 215123, Jiangsu Province, China. xueguangzh@yahoo.com.cn
Telephone: +86-512-65125022 Fax: +86-512-65104908
Received: October 12, 2010 Revised: November 16, 2010
Accepted: November 23, 2010
Published online: December 28, 2010

Abstract

AIM: To analyze the correlation between cytokine-induced killer (CIK) cells adoptive immunotherapy and cancer-related death in gastric cancer patients.

METHODS: One hundred and fifty-six gastric cancer patients after operation at the Third Affiliated Hospital of Soochow University were enrolled in this study. Their clinical data including demographic characteristics, operation time, tumor size, pathological type and staging, tumor metastasis, outcome of chemotherapy or CIK cells adoptive immunotherapy, survival time or time of death were collected with a standard structured questionnaire. Kaplan-Meier method was used to estimate the median survival time, and the 2- and 5- year survival rates. Hazard risk (HR) and 95% confidence interval (95% CI) of CIK cells adoptive immunotherapy for gastric cancer were calculated using the two-stage time-dependent covariates Cox model.

RESULTS: The survival time of gastric cancer patients was longer after CIK cells adoptive immunotherapy than after chemotherapy (χ^2 = 10.907, P = 0.001). The median survival time of gastric cancer patients was also longer after CIK cells adoptive immunotherapy than after chemotherapy (49 mo *vs* 27 mo, P < 0.05). The 2- and 5-year survival rates of gastric cancer patients were significantly higher after CIK cells adoptive immunotherapy than after chemotherapy (73.5% *vs* 52.6%, 40.4% *vs* 23.9%, P < 0.05). A significant difference was observed in the survival curve for patients who received CIK cells adoptive immunotherapy (0, 1-10, 11-25, and over 25 frequencies) (χ^2 = 14.534, P = 0.002). The frequencies of CIK cells adoptive immunotherapy were significantly related with the decreasing risk of death in gastric cancer patients after adjustment for sex and age of the patients, tumor stage and relapse (HR = 0.54, 95% CI: 0.36-0.80) when the first stage Cox model was used to define the subjects who remained alive beyond 36 mo as survivors. However, no correlation was observed between the frequencies of death in CIK cells adoptive immunotherapy and the risk of gastric cancer patients (HR = 1.09, 95% CI: 0.63-0.89) when the second stage Cox model was used to define the subjects who survived for more than 36 mo as survivors.

CONCLUSION: The survival time of the gastric cancer

patients treated with chemotherapy combined with CIK cells adoptive immunotherapy is significantly longer than that of the patients treated with chemotherapy alone and increasing the frequency of CIK cells adoptive immunotherapy seems to benefit patients more.

Key words: Immunotherapy; Cytokine-induced killer cells; Gastric cancer; Survival analysis; Probability

Peer reviewer: Ming Li, Associate Professor, Tulane University Health Sciences Center, 1430 Tulane Ave Sl-83, New Orleans, LA 70112, United States

Jiang JT, Shen YP, Wu CP, Zhu YB, Wei WX, Chen LJ, Zheng X, Sun J, Lu BF, Zhang XG. Increasing the frequency of CIK cells adoptive immunotherapy may decrease risk of death in gastric cancer patients. *World J Gastroenterol* 2010; 16(48): 6155-6162 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i48/6155.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i48.6155

INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related death in China[1]. Its incidence in Jiangsu Province of China is particularly high, and its mortality rate is much higher than the national average[2]. The early clinical detection rate of gastric cancer is less than 15%, and about 85% cases of gastric cancer are at the advanced stage when their gastric cancer is diagnosed[3]. Surgery is the standard treatment procedure for localized and resectable gastric cancer[4]. However, surgery alone does not improve the 5-year survival rate of local advanced gastric cancer patients[3]. Although standardized surgical resection and adjuvant therapeutic modalities are available for gastric cancer, the survival rate of advanced gastric cancer patients remains very low after operation[5]. About 60% of gastric cancer patients usually experience local recurrence and metastasis to other organs[6]. It has been demonstrated that local recurrence and distant metastasis constitute a major problem in the failure of cancer therapies[7]. Therefore, considerable efforts are needed to improve the current therapeutic modalities and to explore new therapies. In recent years, immune therapy has become the fourth important treatment modality for malignant tumors following surgery, radiotherapy and chemotherapy[8-10].

A number of adoptive immunotherapy with killer cells have been reported, including lymphokine-activated killer cells[11], tumor infiltrating lymphocytes[12], or anti-CD3 monoclonal antibody-induced killer cells[13]. However, their therapeutic efficacy is limited due to their low anti-tumor activities[14]. At present, cytokine-induced killer (CIK) cells are a new type of anti-tumor effector cells, which can proliferate rapidly *in vitro*, with a stronger anti-tumor activity and a broader target tumor spectrum than the reported anti-tumor effector cells[10,15]. Moreover, CIK cells can regulate and enhance immune function[16]. Studies have reported the level of tumor markers, change in cellular immune functions, exploration of molecule targets and a short-term efficacy in gastric cancer patients after chemotherapy plus CIK cells immunotherapy despite some side effects[17-19]. However, the relation between the frequencies of CIK cells immunotherapy and its clinical efficacy has not been examined. In the present study, data obtained from 156 gastric cancer patients in fitting multivariate Cox model showed that more frequencies of CIK cells immunotherapy could improve the survival rate of gastric cancer patients.

MATERIALS AND METHODS

Patients

One hundred and fifty-six primary gastric cancer patients after operation at the Third Affiliated Hospital of Soochow University (Jiangsu Province, China) were enrolled in this study. Those who did not meet the inclusion criteria, or had other tumors were excluded.

A standard questionnaire was designed to collect the data from the patients, including demographic characteristics, operation time, tumor size and location, pathological type and staging, tumor metastasis, outcome of chemotherapy or CIK cells immunotherapy. Meanwhile, time of relapse and death of the patients was recorded. Patients received 6 cycles of chemotherapy before CIK cells immunotherapy. Some patients underwent CIK cells immunotherapy due to cancer recurrence during chemotherapy. Recurrence of gastric cancer was defined when local, peritoneal or distant metastasis was detected at any site during chemotherapy[20]. The study was conducted according to the principles of the Declaration of Helsinki. All patients gave their informed consent prior to inclusion in the study. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.

Eighty-one patients (62 males, 19 females) at the age of 59.9 ± 10.5 years with a median age of 60.5 years who underwent chemotherapy alone served as chemotherapy group (group Ⅰ), and those (60 males at the age of 62.4 ± 10.8 years with a median age 60.5 years, 15 females at the age of 51.0 ± 10.7 years with a median age of 50 years) who received chemotherapy plus CIK cells immunotherapy served as treatment group (group Ⅱ) (Table 1).

Preparation of CIK cells and treatment

Peripheral blood mononuclear cells (PBMC) were collected with a COBE spectra blood cell separator (Gambro BCT, Inc., Lakewood, USA). Viability of PBMC was assessed by trypan blue exclusion. PBMC (2.0×10^6/mL) were plated onto 6-well dishes (Nunc, Denmark) and cultured with medium I containing RPMI 1640 in the presence of 1.0×10^6 U/L human interferon-γ (IFN-γ, Shanghai Fosun Pharma Co., China), 5.0×10^5 U/L recombinant human interleukin-2 (IL-2, Shangdong Quangang Pharmaceutical Co., China), 10% inactivated human serum, 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37℃. Monoclonal antibody (MAb) against CD3

Table 1 Distribution of demographic and clinical characteristics in two groups

Demographic and clinical features	*n*	Groups, *n* (%)		χ^2	*P*
		Group Ⅰ	Group Ⅱ		
Sex					
Men	122	62 (76.5)	60 (80)	0.273	0.601
Women	34	19 (23.5)	15 (20)		
Age (yr)					
≤ 45	14	7 (8.6)	7 (9.3)		
45 < age ≤ 60	71	36 (44.5)	35 (46.7)	0.047[a]	0.977
> 60	71	38 (46.9)	33 (44.0)		
Tumor site[b]					
Gastric cardia					
Yes	58	30 (37.0)	28 (37.3)	0.002	0.970
No	98	51 (63.0)	47 (62.7)		
Gastric body					
Yes	64	35 (43.2)	29 (38.7)	0.332	0.564
No	92	46 (56.8)	46 (61.3)		
Gastric antrum					
Yes	36	17 (21.0)	19 (25.3)	0.414	0.520
No	120	64 (79.0)	56 (74.7)		
Tumor size[c] (cm)					
< 5	76	43 (59.7)	33 (55.0)	0.299	0.585
≥ 5	56	29 (40.3)	27 (45.0)		
Histological type[c]					
Differentiated	52	28 (35.4)	24 (64.6)	0.002	0.962
Poorly-differentiated	94	51 (38.5)	43 (64.2)		
Invasion[c]					
Yes	112	55 (76.4)	57 (89.1)	3.745	0.053
No	24	17 (23.6)	7 (10.9)		
Lymph node metastasis[c]					
Yes	100	55 (76.4)	45 (70.3)	0.643	0.423
No	36	17 (23.6)	19 (29.7)		
Relapse					
Yes	98	58 (28.4)	40 (46.7)	5.566	0.018
No	58	23 (71.6)	35 (53.3)		
Pathological grade					
1	2	1 (1.3)	1 (1.3)		
2	27	18 (22.2)	9 (12.0)	2.976	0.395
3	92	44 (54.3)	48 (64.0)		
4	35	18 (22.2)	17 (22.7)		
Tumor stage					
Ⅰ	14	9 (11.1)	5 (6.7)		
Ⅱ	22	12 (14.8)	10 (13.3)	2.129[a]	0.546
Ⅲ	102	49 (60.5)	53 (70.7)		
Ⅳ	18	11 (13.6)	7 (9.3)		

[a]cmh2 χ^2-test; [b]Two cases of gastric cancer involving gastric cardia and body; [c]Missed cases.

(100 μg/L, Antibody Diagnostic Inc., USA) and IL-1α (1.0 × 10^5 U/L, Promega) were added after 24 h culture. Supernatant was aspirated and the cells were cultured in Medium Ⅱ in the absence of INF-γ after another 48 h culture. The cells were transferred to Kolle flasks (Nunc, Denmark) and cultured in the same medium after 1 wk. The medium was changed every 3 d. The cytotoxic activity was assayed as previously described[17,21]. Patients in group Ⅰ were treated with oxaliplatin (120 mg/m^2 D1, 5-Fu 400 mg/m^2 CIV 24 h D1-5, CF 200 mg/m^2 D1-5) as previously described[22] with the doses adjusted according to the toxicity. Patients in group Ⅱ received CIK cells immunotherapy as previously described[17] after 6 cycles of chemotherapy, with 1.0 × 10^9 CIK cells transfused into the patients within 1 h.

Statistical analysis

All data were loaded into the Epidata3.0 database with double-check, and analyzed with the SAS software package (version 9.13; SAS Institute, Cary, NC, USA). The data were expressed as mean ± SD. χ^2-test or cmh2 χ^2-test was used to compare the difference in balance between the concerned clinical indexes and to find the confounding factors between the two groups. Survival data were analyzed using Kaplan-Meier method and log-rank test to estimate the median survival time, 2- and 5-year survival rates, and to determine if the survival curves for the two groups were different.

When the frequency of CIK cells immunotherapy and the survival time were introduced into the Cox model, the interaction item was significantly associated with the death of gastric cancer patients (wald-χ^2 = 4.946, P = 0.0261). A two-stage time-dependent Cox model was established to precisely estimate the hazard risk (HR) and 95% confidence interval (95% CI) of the association between the frequency of CIK cells immunotherapy and the death of gastric cancer patients. Because the median survival time of gastric cancer patients was about 36 mo, 36 mo was used as the optimum cutoff point.

The first stage Cox model involved 154 patients with a survival time of over 36 mo who were defined as survivors. Otherwise, the survival status was the same as the original definition.

The second stage Cox model only involved 56 patients with a survival time longer than 36 mo, and their survival status was defined as the original definition.

Pearson correlation test was performed between Schoenfeld residual of the frequencies of CIK cells immunotherapy and the survival time of gastric cancer patients to determine whether the frequency of CIK cells immunotherapy is a time-dependent variable in the two Cox models[23].

RESULTS

Distribution of demographic and clinical characteristics in two groups

No statistical difference was found in sex and age of the patients, tumor site, histological type, invasion depth, lymph node metastasis, pathological grade, tumor size and stage between the two groups. However, the number of patients was significantly greater in group Ⅱ with recurrent disease than in group Ⅰ (46.7% *vs* 28.4%, χ^2 = 5.566, P = 0.018) (Table 1), suggesting that more patients with relapse should receive CIK cells immunotherapy.

Demographic and clinical characteristics of patients after CIK cells immunotherapy

No statistical difference was observed in sex and age of the patients, tumor site, histological type, invasion depth, lymph node metastasis, pathological grade or tumor size after CIK cells immunotherapy (0, 1-10, 11-25, and over 25 frequencies). However, a significant difference was found in cancer recurrence and stage after CIK cells immunotherapy (Table 2).

Table 2 Distribution of demographic and clinical characteristics in group Ⅱ

Demographic and clinical features	*n*	Frequency of CIK cells immunotherapy, *n* (%)				χ^2	*P*
		0	1-10	11-25	> 25		
Sex							
Men	122	62 (76.5)	39 (84.8)	13 (86.7)	8 (57.1)	5.573	0.134
Women	34	19 (23.5)	7 (15.2)	2 (13.3)	6 (42.9)		
Age (yr)							
⩽ 45	14	7 (8.6)	5 (10.9)	1 (6.6)	1 (7.1)	1.153	0.979
45 < age ⩽ 60	71	36 (44.4)	20 (43.5)	7 (46.7)	8 (57.1)		
> 60	71	38 (47.0)	21 (45.6)	7 (46.7)	5 (35.8)		
Tumor site[a]							
Gastric cardia							
Yes	58	30 (37.0)	17 (37.0)	5 (33.3)	6 (42.9)	0.290	0.962
No	98	51 (63.0)	29 (63.0)	10 (66.7)	8 (57.1)		
Gastric body							
Yes	64	35 (43.2)	19 (41.3)	5 (33.3)	5 (35.7)	0.691	0.875
No	92	46 (56.8)	27 (58.7)	10 (66.7)	9 (64.3)		
Gastric antrum							
Yes	36	17 (21.0)	10 (21.7)	4 (26.7)	5 (35.7)	1.614	0.656
No	120	64 (79.0)	36 (78.3)	11 (73.3)	9 (64.3)		
Tumor size[c] (cm)							
< 5	76	43 (59.7)	17 (45.9)	7 (58.3)	9 (81.8)	4.834	0.184
⩾ 5	56	29 (40.3)	20 (54.1)	5 (41.7)	2 (18.2)		
Histological type[c]							
Differentiated	52	28 (35.4)	12 (30.0)	6 (40.0)	6 (50.0)	1.760	0.624
Poorly-differentiated	94	51 (64.6)	28 (70.0)	9 (60.0)	6 (50.0)		
Invasion[c]							
Yes	112	55 (76.4)	38 (90.5)	10 (90.9)	9 (81.8)	4.226	0.238
No	24	17 (23.6)	4 (9.5)	1 (9.1)	2 (18.2)		
Lymph node metastasis[c]							
Yes	100	55 (76.4)	31 (75.6)	7 (58.3)	7 (63.6)	2.371	0.499
No	36	17 (23.6)	10 (24.4)	5 (41.7)	4 (36.4)		
Relapse							
Yes	98	58 (71.6)	31 (67.4)	8 (53.3)	1 (7.1)	15.633	0.0004
No	58	23 (28.4)	15 (32.6)	7 (46.7)	13 (92.9)		
Pathological grade							
1	2	1 (1.2)	1 (2.2)	0 (0.0)	0 (0.0)	2.976[b]	0.3953
2	27	18 (22.2)	8 (17.4)	0 (0.0)	1 (7.1)		
3	92	44 (54.3)	25 (54.4)	12 (80.0)	11 (78.6)		
4	35	18 (22.2)	12 (26.0)	3 (20.0)	2 (14.3)		
Tumor stage							
Ⅰ	14	9 (11.1)	3 (6.5)	0 (0.0)	2 (14.2)	13.66[b]	0.0386
Ⅱ	22	23 (28.4)	3 (6.5)	1 (6.7)	6 (42.9)		
Ⅲ	102	38 (46.9)	34 (73.9)	13 (86.7)	6 (42.9)		
Ⅳ	18	11 (13.6)	6 (13.1)	1 (6.6)	0 (0.0)		

[a]Tumor sites in some cases were repeated; [b]Because the theoretical value is less than 1, χ^2 was performed for patients who received cytokine-induced killer (CIK) cells immunotherapy at the frequencies of 11-25 and > 25, and for those who underwent CIK cells immunotherapy at the frequencies of 1-10, 11-25 and > 25; [c]Missed cases.

Survival time of patients in two groups

The survival time of gastric cancer patients in group Ⅱ was much longer than that of those in group Ⅰ (χ^2 = 10.907, P = 0.001, Figure 1). The median survival time of patients in group Ⅱ was also longer than that of those in group Ⅰ (49 mo *vs* 27 mo).

Two- and 5-year survival rates of patients in two groups

The 2- and 5-year survival rates of patients in group Ⅱ were significantly higher than those of patients in group Ⅰ (73.5% *vs* 52.6%, 40.4% *vs* 23.9%, P <0.05) (Table 3).

Survival time of patients after CIK cells immunotherapy

Because the CIK cells immunotherapy seemed effective against gastric cancer, whether the frequency of CIK cells immunotherapy affects its efficacy was determined. The survival curve was obviously higher for the patients after CIK cells immunotherapy plus chemotherapy than after chemotherapy alone. The survival time of gastric cancer patients was significantly longer after CIK cells immunotherapy than after chemotherapy (χ^2 = 14.534, P = 0.002, Figure 1).

Time-dependent Cox model analysis of CIK cells immunotherapy and prognosis of patients

The frequency of CIK cells immunotherapy was a strong time-dependent variable. A significant difference was observed in the survival time and the frequency of CIK cells immunotherapy between the two models (χ^2 = 27.990, P

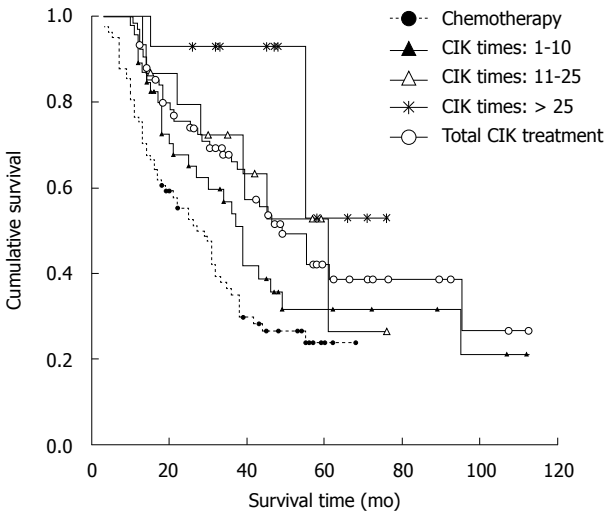

Figure 1 Survival rates of patients after cytokine-induced killer cells immunotherapy plus chemotherapy and chemotherapy alone. CIK: Cytokine-induced killer.

< 0.0001). However, it could not stratify the theoretical hypothesis of proportional hazard model. Hence, we tried to improve the analyzing results of the Cox model by dividing the patients into two stages with a relatively short survival time. Because of the relatively short interval time, the frequency of CIK cells immunotherapy may be a time-independent variable and can satisfy the assumption of proportional hazard model. The median survival time (36 mo) of the patients was used as the optimum cut-point.

In the first stage Cox model, the frequency of CIK cells immunotherapy was not a time-dependent variable, thus refusing the hypothesis of non proportional hazard model by Pearson correlation test between Schoenfeld residual of CIK cells immunotherapy frequency and survival time ($r = 0.04$, $F = 0.10$, $P = 0.751$). After the patients who remained alive beyond 36 mo after treatment were defined as survivors, the frequency of CIK cells immunotherapy was significantly associated with the decreasing risk of death in gastric cancer patients after adjustment for sex and age of the patients, tumor stage and relapse (HR = 0.54, 95% CI: 0.36-0.80) (Table 4).

In the second stage Cox model, the frequency of CIK cells immunotherapy was not a time-dependent variable ($r = 0.307$, $F = 1.98$, $P = 0.176$).When the second stage Cox model involving only 56 patients with a survival time of over 36 mo was fitted, no association was observed between the frequency of CIK cells immunotherapy and the survival time of gastric cancer patients (HR = 1.09, 95% CI: 0.63-0.89) (Table 5).

DISCUSSION

In China, gastric cancer patients are usually diagnosed at a relatively advanced stage with metastasis to other organs[24]. A number of treatment modalities are available for gastric cancer, such as surgical resection combined with chemotherapy[25,26], radiotherapy[27,28], thermotherapy[29,30] and/or traditional Chinese medicine[31,32]. However,

Table 3 Two- and five-year survival rates of gastric cancer patients in two groups

Groups	Survival rate (%)	95% CI	u-value	*P*
2-yr				
Chemotherapy	52.6	41.7-63.6	2.721	0.007
CIK treatment	73.5	63.3-83.7		
5-yr				
Chemotherapy	23.9	13.5-34.3	1.913	0.0526
CIK treatment	40.4	27.1-53.7		

CIK: Cytokine-induced killer; 95% CI: 95% confidence interval.

the 5-year survival rate of advanced gastric cancer patients is still very poor[33]. It has been shown that cellular immunotherapy can promote host anti-cancer immunity, thus prolonging the survival time of gastric cancer patients[34]. Treatment of gastric cancer with autologous CIK cells is one of the promising cellular immunotherapies[35].

The results of the present study demonstrate that CIK cells immunotherapy plus chemotherapy for gastric cancer has more potential benefits than chemotherapy alone. Therefore, the effect of adjuvant cellular therapies for gastric cancer has drawn more attention of oncologists. It has been shown that adjuvant radiotherapy and chemotherapy for gastric cancer after curative resection can improve the disease-free and overall survival time of gastric cancer patients[25,36]. Residual tumor cells after chemotherapy can be removed by the host immune system[37]. However, several cycles of chemotherapy can decrease the immune functions of gastric cancer patients[38], and have been suspected to be one of the reasons for the high relapse rate of gastric cancer after postoperative systemic chemotherapy. Immunotherapy can directly kill cancer cells and boost the immune responses against the tumor[39]. Therefore, immunotherapy should be beneficial for gastric cancer patients, and not conducive to the growth of tumor cells.

In the present study, CIK cells were obtained upon culturing PBMC in the presence of IFN-γ, IL-2, anti-CD3MAb, and IL-1α[13]. This method allows us to generate a large number of CIK cells. In addition, the anti-tumor cell activity of CIK cells is stronger than that of anti-tumor effector cells[16]. The effector cells in our culture are believed to be $CD3^+CD56^+$. CIK cells have a higher survival rate, proliferation capacity, and killing activity than their target cells[40,41], and can secrete a variety of cytokines, which further enhance the cytotoxicity of immune effector cells[42] and change the tumor microenvironment to favor cancer eradication. In addition, CIK cells can kill both autologous and allogeneic tumor cells[43,44], as well as multi-drug resistance cells and FasL-positive cells[45,46]. Accordingly, CIK cells immunotherapy combined with chemotherapy may have a synergistic effect.

Several studies[17,47-49] showed that CIK cells immunotherapy can significantly improve the immune functions of cancer patients, such as an increase in $CD3^+CD56^+$ level. However, the clinical data are not enough to demonstrate the effectiveness of CIK cells immunotherapy. The results of this retrospective study, based on the follow-up

Table 4 First stage time-dependent multivariate Cox model analysis of cytokine-induced killer cells immunotherapy at different frequencies and prognosis of the patients[1,2] (*n* = 154[3])

Variables[4]	β	$s_β$	Wald-χ^2	*P*-value	HR	95% CI
No. of CIK infusion	-0.620	0.200	9.592	0.002	0.54	0.36-0.80
Sex	0.294	0.298	0.969	0.325	1.34	0.75-2.41
Age	0.508	0.200	6.492	0.011	1.66	1.12-2.46
Tumor stages	0.739	0.202	13.377	0.0003	2.10	1.40-3.11
Relapse (yes or no)	3.363	0.719	21.848	< 0.0001	28.87	7.05-118.25

[1]All patients with survival time of longer than 36 mo (median value for total patients) were defined as the survivors; [2]Pearson correlation test between Schoenfeld residual of cytokine-induced killer (CIK) cells immunotherapy frequency and survival time (r = 0.01, F = 0.01, P = 0.936); [3]Missed patients; [4]Variable value definition in the Cox model (frequency of CIK cells immunotherapy: 0 = 0 time, 1 = 1-10 times, 2 = 11-25 times, 3 = more than 25 times; Sex: 1 = man, 2 = woman), age (0 = ≤ 45 yr, 1 = about 60 yr, 2 = over 60 yr), tumor stage (0 = stage Ⅰ, 1 = stage Ⅱ, 2 = stage Ⅲ, 3 = stage Ⅳ), relapse (1 = yes, 0 = no). HR: Hazard risk; 95% CI: 95% confidence interval.

Table 5 Second stage time-dependent multivariate Cox model analysis of cytokine-induced killer cells immunotherapy at different frequencies and prognosis of patients[1,2] (*n* = 56)

Variables[3]	β	$s_β$	Wald-χ^2	*P*-value	HR	95% CI
Number of CIK infusion	0.089	0.280	0.102	0.750	1.09	0.63-1.89
Sex	0.676	0.619	1.191	0.275	1.97	0.58-6.62
Age	-0.318	0.442	0.518	0.472	0.73	0.31-1.73
Tumor stages	-0.471	0.341	1.909	0.167	0.62	0.32-1.22
Relapse (yes or no)	2.203	0.558	15.582	< 0.0001	9.05	3.03-27.02

[1]This model only involving the patients with a survival time longer than 36 mo. The survival rate of each patient was similar; [2]Pearson correlation test between Schoenfeld residual of cytokine-induced killer (CIK) cells immunotherapy frequency and survival time (r = 0.307, F = 1.98, P = 0.176); [3]Variable value definition in the Cox model (frequency of CIK cells immunotherapy: 0 = 0 time, 1 = 1-10 times, 2 = 11-25 times, 3 = more than 25 times; Sex: 1 = man, 2 = woman), age (0 = ≤ 45 yr, 1 = about 60 yr, 2 = over 60 yr), tumor stage (0 = stage Ⅰ, 1 = stage Ⅱ, 2 = stage Ⅲ, 3 = stage Ⅳ), relapse (1 = yes, 0 = no). HR: Hazard risk; 95% CI: 95% confidence interval.

of 156 gastric cancer patients, show that the frequency of CIK cells immunotherapy can significantly prolong the survival time of gastric cancer patients.

However, the frequency of CIK cells immunotherapy may significantly decrease the risk of cancer-related death in gastric cancer patients. The common Cox model was not preferred in our analysis because the balance test showed that tumor relapse and stage were different in patients of the two groups. There are two reasons that support our conclusion. First, except for tumor relapse and stage, the balance test showed the following factors, including age and sex of the patients, tumor size, site and invasiveness, lymph node metastasis and pathological grade did not affect us to assign the gastric cancer patients into groups Ⅰ and Ⅱ. Second, due to the fact that the frequency of CIK cells immunotherapy was a time-dependent variable in the Cox model, a two-stage time-dependent Cox model adjustment was made for some confounding factors (tumor relapse and stage), thus the false results were avoided when the common Cox model was used to make our analysis more reliable.

After the postoperative adjuvant chemotherapy, most residual tumor cells sensitive to chemotherapy were removed. Chemotherapy may suppress the immune function and therefore immunotherapy is necessary for boosting immunity. It was reported that the number of $CD3^+$ cells and the $CD4^+/CD8^+$ ratio are significantly lower in most gastric cancer patients than in healthy controls after chemotherapy[17]. In the present study, the number of $CD3^+$ and $CD4^+$ cells was significantly increased, while the number of $CD8^+$ cells was declined and the $CD4^+/CD8^+$ ratio was increased after CIK cells immunotherapy, suggesting that CIK cells also have an immune modulating function in addition to their anti-tumor function[50]. Single CIK cells immunotherapy has an *in vivo* effect against gastric cancer for about one month[51]. In contrast, the number of $CD3^+$ cells and the $CD4^+/CD8^+$ ratio maintain at a high level after three cycles of CIK cells immunotherapy[17].

Our study has a few limitations. First, it was a retrospective cohort/observational study rather than a strictly-designed randomized trial. Since the patients were not assigned into CIK cells immunotherapy group and chemotherapy group, imbalance of certain clinical factors between the two groups could not be avoided. Second, there were some unknown potential reasons for choice of treatment regimens, which might affect our conclusion, despite the fact that the balance test was performed and some confounding factors were adjusted in the Cox model. Therefore, a randomized clinical trial is necessary to justify the benefit of CIK cells immunotherapy for gastric cancer. The benefit of radiochemotherapy and S1 chemotherapy for gastric cancers, established in a recent clinical trial[52], is important to determine whether CIK cells immunotherapy provides additional benefit when it is used in combination with radiotherapy and chemotherapy.

In conclusion, more frequencies of CIK cells are necessary for gastric cancer. The survival time of gastric cancer patients is significantly longer after chemotherapy plus CIK cells immunotherapy than after chemotherapy alone.

COMMENTS

Background

Gastric cancer is one of the most common causes of cancer-related death in China. Although standardized surgical resection and numerous adjuvant therapeutic modalities are available for gastric cancer, the postoperative survival rate of advanced stage cancer patients remains very low. In recent years, immune therapy for malignant tumors has become the fourth important tumor treatment modality following surgery, radiotherapy and chemotherapy. Cellular immunotherapy can promote host anti-cancer immunity, thus prolonging the survival time of gastric cancer patients. Treatment with autologous cytokine-induced killer (CIK) cells is one of the promising cellular immunotherapies.

Research frontiers

A number of adoptive cells immunotherapy have been reported, including using lymphokine activated killer cells, tumor infiltrating lymphocytes, and anti-CD3 monoclonal antibody-induced killer cells. However, their therapeutic efficacy is limited due to their low anti-tumor activities. CIK cells, a new type of anti-tumor effector cells, can proliferate rapidly *in vitro*, with a stronger anti-tumor activity and a broader spectrum of tumor targets than the reported anti-tumor effector cells. Moreover, CIK cells can regulate and enhance immune function.

Innovations and breakthroughs

CIK cells immunotherapy can decrease levels of tumor markers, change immune functions, and achieve a short-term efficacy against gastric cancer. However, the relation between the frequency of CIK cells immunotherapy and its clinical efficacy has not been examined. In the present study, data obtained from 156 gastric cancer patients were used in fitting multivariate Cox model, showing that more frequencies of CIK cells immunotherapy improve the survival rate of gastric cancer patients.

Applications

The survival time of gastric cancer patients was significantly longer after chemotherapy plus CIK cells immunotherapy than after chemotherapy alone, and more frequencies of CIK cells immunotherapy benefited gastric cancer patients more. This strategy can be applied in treatment of gastric cancer.

Terminology

CIK cells are cytokine-induced killer cells and a new type of anti-tumor effector cells, which can proliferate rapidly *in vitro* with a stronger anti-tumor activity and a broader spectrum of tumor targets than the reported anti-tumor effector cells. Moreover, CIK cells can regulate and enhance immune function.

Peer review

This study showed beneficial effect of CIK cells immunotherapy on gastric cancer, thus improving the 2- and 5-year survival rates of gastric cancer patients. The study is well designed and the data are believable.

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World J Gastroenterol 2010 December 28; 16(48): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Chakshu Gupta, MD, FCAP, Pathology and Laboratory Medicine, Heartland Regional Medical Center, 5325 Faraon Street, St. Joseph, MO 64506, United States

Ralf Jakobs, Professor, Dr., Director, Department of Medicine C (Gastroenterology), Klinikum Ludwigshafen, Bremserstrasse 79, 67063 Ludwigshafen, Germany

Walter E Longo, Professor, Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven, CT 06510, United States

Giovanni Maconi, MD, Department of Gastroenterology, 'L.Sacco' University Hospital, Via G.B. Grassi, 74, Milan 20157, Italy

Kotaro Miyake, MD, PhD, Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Fabrizio Montecucco, MD, Assistant, Division of Cardiology, Department of Internal Medicine, University of Geneva, Avenue de la Roseraie 64, 1211 Geneva, Switzerland

Jong Park, PhD, MPH, MS, Associate Professor, Division of Cancer Prevention and Control, H. Lee Moffitt Cancer Center, College of Medicine, University of South Florida, 12902 Magnolia Dr. MRC209, Tampa, FL 33612, United States

Nadia Peparini, MD, PhD, Department of General Surgery "Francesco Durante", La Sapienza University, Viale del Policlinico, 155, Rome 00161, Italy

Ian C Roberts-Thomson, Professor, Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Noriko Suzuki, Dr., MD, PhD, Honorary Consultant, Wolfson Unit for Endopscopy, St Mark's Hospital, Watford Road, Harrow, Middlesex, HA1 3UJ, United Kingdom

Alberto Tommasini, Dr., MD, Professor, Laboratory of Immunopathology, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste 34137, Italy

Lucia Ricci Vitiani, Dr., Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, Rome 00161, Italy

Catherine Walter, MD, Department of Surgery, Cheltenham General Hospital, Sandford Road, Cheltenham, GL51 7AN, United Kingdom

Ming-Lung Yu, MD, PhD, Professor, Division of Hepatology, Department of Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1st Rd, Kaohsiung 807, Taiwan, China

Inti Zlobec, Dr., PhD, Institute for Pathology, University Hospital Basel, Schoenbeinstrasse 40, Basel, CH-4031, Switzerland

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World J Gastroenterol 2010 December 28; 16(48): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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World J Gastroenterol 2010 December 28; 16(48): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Instructions to authors

GENERAL INFORMATION

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Name of journal

World Journal of Gastroenterology

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ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Indexed and Abstracted in

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Acknowledgments

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Format

Journals

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Chinese journal article (list all authors and include the PMID where applicable)

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 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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 DOI:10.1046/j.1526-4610.42.s2.7.x]

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 DOI:10.1097/00003086-200208000-00026]

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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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