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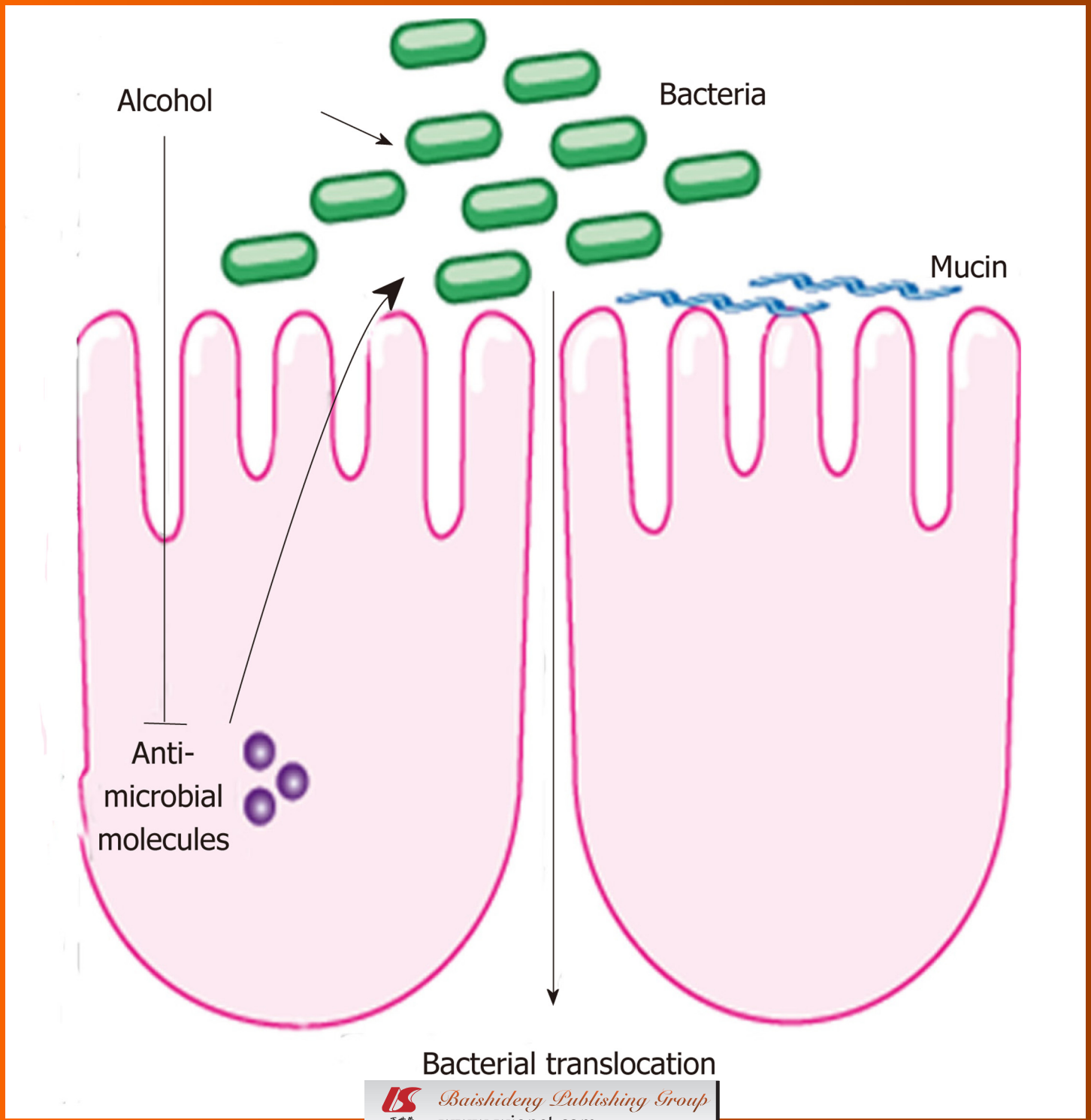

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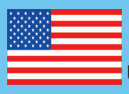

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EDITORIAL

Bacterial translocation and changes in the intestinal microbiome associated with alcoholic liver disease

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Abstract

Alcoholic liver disease progresses through several stages of tissue damage, from simple steatosis to alcoholic hepatitis, fibrosis, or cirrhosis. Alcohol also affects the intestine, increases intestinal permeability and changes the bacterial microflora. Liver disease severity correlates with levels of systemic bacterial products in patients, and experimental alcoholic liver disease is dependent on gut derived bacterial products in mice. Supporting evidence for the importance of bacterial translocation comes from animal studies demonstrating that intestinal decontamination is associated with decreased liver fibrogenesis. In addition, mice with a gene mutation or deletion encoding receptors for either bacterial products or signaling molecules downstream from these receptors, are resistant to alcohol-induced liver disease. Despite this strong association, the exact molecular mechanism of bacterial translocation and of how changes in the intestinal microbiome contribute to liver disease progression remains largely unknown. In this review we will summarize evidence for bacterial translocation and enteric microbial changes in response to alcoholic liver injury and chronic alcoholic liver disease. We will further describe consequences of intestinal dysbiosis on host biology. We finally discuss how therapeutic interventions may modify the gastrointestinal microflora and prevent or reduce alcoholic liver disease progression.

Key words: Alcoholic liver disease; Microbiome; Dysbiosis; Bacterial translocation; Steatohepatitis

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INTRODUCTION

Alcohol abuse is one of the leading causes of chronic liver disease. In its early stage, alcoholic liver disease is characterized by fatty infiltration of the liver, also known as steatosis. Forty-four percent of patients consuming modest amounts (40-80 g/d) of alcohol exhibit fatty liver, while it is even more common in heavy drinkers[1]. With steatosis alone, the liver continues to function well and few patients present with any clinical symptoms[2]. The most effective therapy for alcoholic steatosis is ces-

sation of alcohol consumption. However, if this cannot be achieved, subsequent inflammation and alcoholic hepatitis can take place. This ultimately results in liver fibrosis, which is an accumulation of scar tissue in the liver parenchyma that distorts the hepatic architecture. As hepatic fibrogenesis progresses to cirrhosis, disruption of the synthetic and metabolic functions of the liver occurs. Increased resistance to portal blood flow results in portal hypertension, the clinical consequence of which includes ascites, esophageal varices, and splenomegaly through shunting of blood to portal caval anastamoses. Cirrhosis is an end-stage disease and one of the leading causes of morbidity and mortality in the world, with liver transplantation as the sole remedy for survival.

Annually, over 27 000 people die from cirrhosis in the United States[3]. Half of all cirrhotic patients die within 2 years of diagnosis. Mortality from alcoholic liver disease (ALD) has been declining in recent years, likely due to improvements in clinical management of complications of ALD including portal hypertension and bleeding from esophageal varices[4]. A significant percentage of cirrhotic patients succumb to bacterial infections with infection-attributed mortality of 30% to 50%[5-7]. Mortality as a consequence of infection is increased 20-fold in patients with cirrhosis. These infections include spontaneous bacterial peritonitis, bacteremia, pneumonia, and urinary tract infections. The most common pathogens involved are *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumonia*, *Klebsiella spp*, and *Escherichia coli*[8,9]. The origin of these organisms is thought to be the enteric microflora, but may also be associated with nosocomial infections due to the higher number of invasive procedures performed in this patient population[10].

In the following article, we will review bacterial translocation and the changes in the intestinal microbiome associated with ALD. For each topic we will discuss the data from experimental and preclinical animal models of alcohol-induced liver injury followed by current evidence in patients with ALD.

BACTERIAL TRANSLOCATION

Bacterial translocation is defined as the passage of viable indigenous bacteria or their products, such as lipopolysaccharide and bacterial DNA, from the gut to extraintestinal sites, notably the mesenteric lymph nodes and the systemic circulation. In particular, lipopolysaccharide (LPS) is the cell wall molecule derived from gram-negative bacteria, and has been found to be increased in alcoholics with fatty liver disease or patients with alcoholic cirrhosis[11-13]. The presence of bacterial DNA in an animal model of cirrhosis has also been established as a surrogate marker of bacterial translocation, associated with marked inflammatory response in the host[14]. This migration of bacteria and bacterial products has been implicated in spontaneous bacterial peritonitis and sepsis in patients with end-stage liver disease[15]. In addition, this phenomenon is also considered to play a key role in the pathogenesis of liver fibrosis in experimental animal models of alcohol-induced liver injury[16-18].

Bacterial translocation and alcoholic liver disease

There have been several studies in animal models examining the association between bacterial translocation and alcohol administration. Some studies have demonstrated no significant difference in bacterial translocation to the mesenteric lymph nodes or the systemic circulation after alcohol administration for 2 wk[19,20]. However, one report provides evidence for the translocation of viable bacteria in rats fed alcohol as early as 14 d[21]. Bacterial products, including endotoxin, have also been seen translocating in experimental animal models of alcohol consumption. A positive correlation between alcohol ingestion and increased systemic levels of endotoxin have been observed[22-24]. Plasma levels of peptidoglycan, which makes up about 70% and 20% of Gram-positive and Gram-negative bacterial cell walls respectively, are also increased following acute administration of alcohol in rats[25]. Discrepancies in these studies might be explained by differences in species, treatment length, alcohol dose, and the model used to administer alcohol (drinking water or diet, gastric gavage, intragastric feeding tube).

The presence of endotoxemia in liver disease has also been seen in human studies. LPS is significantly higher in patients with alcoholic cirrhosis compared to patients with cirrhosis from other causes[11,12]. Endotoxemia has also been shown to increase with more severe liver dysfunction (Child-Pugh class) in patients with cirrhosis[26]. Furthermore, endotoxin is also present in patients with mild forms of alcoholic hepatitis that do not have evidence of fibrosis or cirrhosis[27,28]. These studies suggest that bacterial translocation occurs early in ALD, and that the degree of translocation of bacterial products may be related to the severity of liver injury present. There are many possibilities for why this occurs. As liver disease progresses, reduced hepatic clearance of toxins may result in higher systemic levels of translocated bacteria and bacteria products. Likewise, alcohol may directly injure the defensive intestinal barrier, contribute to intestinal dysmotility, result in bacterial overgrowth, and change the intestinal microflora.

Bacterial translocation contributes to the progression of alcoholic liver disease

Bacterial translocation not only results in severe infections in cirrhotic patients, but also leads to the progression of alcohol-induced liver injury and fibrosis. The enteric microflora may therefore play a role in augmenting the progression of liver disease. One mechanism by which this occurs is through the activation of the innate immune system in the liver. The innate immune system has developed phylogenetically conserved pattern recognition receptors including the Toll-like receptors (TLR), which recognize distinct microbial products, known as pathogen-associated molecular patterns (PAMPs). PAMPs not only include LPS, but also bacterial peptidoglycan, double-stranded RNA, and unmethylated DNA[29]. Ligand binding to PAMPs trigger intracellular

signaling cascades which activates downstream transcription and expression of a variety of genes involved in the immune and inflammatory host response. PAMPs stimulated by commensal enteric microflora are not necessarily interpreted as disease states but rather contributes to maintenance of homeostasis. For a detailed view of the role of the innate immune system in health and disease, we would refer to recently published reviews[30].

Animal studies have suggested integral roles for LPS signaling in the pathogenesis of ALD. In particular, TLR4, the cellular LPS receptor, plays an important role in the innate immune response to bacterial translocation. Selective intestinal decontamination with antibiotics have shown to reduce plasma endotoxin levels and prevents liver injury in animal models of ALD[31,32,22]. Moreover, mice deficient in CD14, the cellular co-receptor for LPS, are also resistant to alcohol-induced liver injury[17]. Similar findings were also noted in these mice with experimental hepatic injury induced by bile duct ligation[33]. However, the strongest evidence that supports the role LPS plays in ALD are studies with TLR4 mutant C3H/Hej mice and TLR4 deficient mice. These genetically modified animals demonstrate marked reductions in hepatic steatosis, inflammation, and necrosis in models of ALD, as compared to wildtype control mice[16,34]. Similar to TLR4, TLR9 is another pattern recognition receptor that is activated by CpG motifs specific to bacterial DNA. TLR9-deficient mice have been shown to be resistant to experimentally induced liver fibrosis[35].

In patients, TLR4 is identified as one of seven genes associated with an increased risk of developing cirrhosis in patients with chronic hepatitis C[36]. TLR4 D299G and T399I single nucleotide polymorphisms are associated with protection from hepatic fibrosis by reduced TLR4-mediated inflammatory and fibrogenic signaling[37].

Taken together, these studies highlight the important roles translocated bacteria and their products play in both hepatic fibrogenesis and infections in patients with chronic liver disease. Bacterial translocation must happen early for initial liver injury and fibrogenesis, while bacterial translocation in end-stage liver disease is partly responsible for the resulting infections and mortality.

Disruption of the intestinal barrier function as mechanism for bacterial translocation

The mechanism behind bacterial translocation with alcohol ingestion is not clear. Tight junctions normally join together at the apicolateral membranes of enterocytes, providing a mucosal barrier against paracellular diffusion of intestinal contents. Damage to these protective barriers may result in structural deficiencies that enable bacterial translocation[38-41,28].

Several studies have examined oxidative stress on the intestinal mucosa as a possible etiology for barrier dysfunction. Acute ingestion of alcohol has been shown to alter the epithelial barrier in the colonic mucosa of rats *via* ethanol oxidation into acetaldehyde by the enteric microflora with subsequent downstream activation of mast cells[42]. There is also an increase in tissue oxidative stress in jejunal, ileal, and colonic mucosa seen as early as 4 wk after alcohol administration in rats[43]. Furthermore, LPS is known to activate macrophages resulting in their subsequent release of pro-inflammatory cytokines, which in turn induce liver damage[44]. These cytokines, such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), iNOS, and IL-6, have been shown to be elevated in the distal ileum of mice administered alcohol for 14 d[20]. Cytokines such as IL-1β and TNF are known to cause a disruption of tight junctions[45].

In one study, distension of the intercellular spaces below these tight junctions was observed in duodenal biopsies of cirrhotic patients[46]. Alcoholic patients without liver cirrhosis that cease alcohol consumption demonstrated higher intestinal permeability in 3 d by way of a chromium-51-EDTA absorption test. These finding persisted beyond 2 wk in patients with evidence of liver cirrhosis, despite abstaining from alcohol[47]. Taken together, this suggests that underlying liver disease may prolong the damaging effects of alcohol on the intestinal epithelium.

Alcohol may also exert its effects on the intestinal epithelium indirectly through its oxidized metabolite, acetaldehyde. This has been shown both in cultured Caco-2 cell monolayers, as well as biopsy specimens of the intestines, where acetaldehyde disrupts tight junctions and adherens junctions with an associated rise in tyrosine phosphorylation[48,49,52].

QUANTITATIVE AND QUALITATIVE CHANGES OF THE ENTERIC MICROFLORA

In addition to the direct and indirect toxic effects of alcohol to the intestinal epithelial barrier, the quantitative and qualitative changes of the enteric microflora themselves may contribute to bacterial translocation. For example, animal studies demonstrate a correlation between bacterial overgrowth and bacterial translocation. Experimentally induced bacterial overgrowth results in bacterial translocation, liver injury, and inflammation[50]. In addition, as we have discussed above, selective intestinal decontamination improves experimental alcoholic steatohepatitis. This might be mediated by a decrease in the intestinal bacterial burden, which subsequently results in a reduction of bacterial translocation and alcoholic steatohepatitis. Furthermore, in non-cirrhotic patients that have small-intestinal bacterial overgrowth with colonic flora, increased intestinal permeability has been observed[51].

Intestinal bacterial overgrowth in alcoholic liver disease

Bacterial overgrowth and translocation have been observed in animal models of end-stage liver disease[52,53]. In cirrhotic rats with bacterial translocation, there is a higher prevalence of bacterial overgrowth. The prevalence of overgrowth in cirrhotic rats was 67%, with 47%-78% of animals also exhibiting bacterial translocation[14,54-56]. Following experimentally-induced cirrhosis *via* bile duct

ligation, there is an increase in number of gram-negative bacteria in the cecum of animals, which includes *E. Coli, Enterococci, Klebsiella, Pasteurella, Proteus, Pseudomonas*, *and Shigella*.

Intestinal bacterial overgrowth also occurs more frequently in patients with ALD. The number of aerobic and anaerobic bacteria has been found to be higher in jejunal aspirates in patients with chronic alcohol abuse[57,58]. Small intestinal bacterial overgrowth has also been found in cirrhotic patients with increasing prevalence corresponding with the severity of liver dysfunction - as high as 58%-75% in cirrhotics classified Child-Pugh C[59-62]. Serum antibodies to microbial components are also found more frequently in patients with more advanced liver disease[63]. The cultured flora from cirrhotic patients include *Bacteroides spp*., *E. Coli*, *Corynebacerium spp.*, ***Klebsiella spp.***, *Stomatococcus*, *Streptoccoci*, and *Veillonella spp*. It is interesting to note that the majority of these bacteria come from oropharyngeal sources, with exception to *Bacteroides*, *E. Coli* and *Klebsiella*. Of interest *Bifidobacterium*, often used in commercial probiotics, was reduced in the fecal flora of cirrhotics[64].

The reason for bacterial overgrowth in liver disease is not known. One hypothesis suggests that impaired bile flow results in bacterial proliferation and mucosal injury in the small intestines. Conjugated bile acids induce expression of antimicrobial proteins angiogenin 1 and RNAse family member 4 in the enterocytes, which then prevents bacterial overgrowth and promotes epithelial cell integrity in experimental mouse models[65]. As liver dysfunction progresses, less conjugated bile acids are produced and available in the small intestines, which may then contribute to small intestinal bacterial overgrowth.

Dysmotility has also been proposed as a mechanism for stasis and bacterial overgrowth in patients with cirrhosis. Alcohol has been shown to reduce gastrointestinal motility, which may result in a higher number of luminal bacteria[66]. A delayed transit time, characterized by alteration of the migrating motor complex in the small intestine, has been observed in cirrhotics[67-69]. A recent cross-sectional study also demonstrated a higher prevalence of small intestinal bacterial overgrowth with prolonged orocecal transit time in cirrhotic patients with hepatic encephalopathy[70]. Further evidence linking overgrowth and intestinal transit time includes the use of the pro-motility agent cisapride, which reversed small intestinal dysmotiliy and bacterial overgrowth in patients with cirrhosis[71]. These patients also exhibited further clinical improvement in Child-Pugh scores and hepatic encephalopathy.

Moreover, a higher gastric pH may possibly contribute to bacterial overgrowth and translocation. Hypochlorhydria has been associated with an increase in bacteria in the jejunum in patients with cirrhosis[61,72]. A retrospective case-control study also noted that cirrhotic patients with spontaneous bacterial peritonitis had a significantly higher pre-hospital use of proton pump inhibitors compared with controls[73]. Less gastric acid secretion with higher transit times from intestinal dysmotility enables bacterial colonization of the upper gastrointestinal tract. With a reduction in protective conjugated bile acids, cirrhotic patients are predisposed to bacterial overgrowth and translocation.

Intestinal microbiome changes in alcoholic liver disease

The enteric microbial communities are complex and harbor 10 different bacterial phyla with more than 15 000 species-level bacterial phylotypes[74]. Firmicutes and Bacteroidetes make up the vast majority of these phylotypes in mice[75] and humans[76,77]. In healthy individuals, the microflora maintains a symbiotic relationship with the human intestine. This balance of number, distribution, and composition is regulated by the innate and adaptive immune system including host antimicrobial proteins secreted from paneth cells and intestinal epithelial cells. Dysbiosis is known as the disruption and enteric disequilibrium between the microbiota and colonized host, and has been associated with disease including inflammatory bowel disease[78].

One of the first animal studies in alcoholism and enteric dysbiosis utilized length heterogeneity polymerase chain reaction fingerprinting, and demonstrated differing enteric microbiota composition in rats after 10 wk of intragastric feeding of alcohol[79]. A subgroup of animals given probiotics or prebiotics prevented dysbiosis in animals treated with alcohol. A recent animal study examined changes in the intestinal microbial community with the use of deep DNA pyrosequencing of the bacterial 16S rRNA during the early stages of ALD[75]. Following continuous intragastric feeding of alcohol for 3 wk, there was a relative predominance of the Bacteroidetes and Verrucomicrobia phylotypes in mice fed alcohol compared with an abundance of Firmicutes bacteria in the control group. Interestingly, *Lactobacillus* was significantly reduced in alcohol fed mice, which now explains the beneficial effect of probiotic *Lactobacillus* in alcoholic steatohepatitis shown in several reports[79,80,81]. In addition, *Akkermansia muciniphila*, a gram-negative anaerobic bacteria belonging to the bacterial phylum Verrucomicrobia[82], was strongly induced in animals fed alcohol for 3 wk. *Akkermansia muciniphila* is a common bacterial component of the human intestinal tract and has been found to degrade mucin in pure culture[83,84]. By degrading the intestinal mucus as part of the innate immune system, bacterial translocation might be facilitated. However, a causal relationship between an increase in intestinal *Akkermansia muciniphila* and bacterial translocation needs to be investigated in ALD in future studies. Antimicrobial effector molecules secreted by enterocytes and paneth cells not only protect against pathogenic organisms, but also play a role in maintaining a symbiotic relationship between the host and the commensal enteric microflora. A suppression of bactericidal protein expression regenerating islet derived (Reg)-3b and Reg3g were observed in the small intestines following alcohol administration in mice, suggesting a possible mechanism for qualitative and quantitative changes within the microbiome (Figure 1). Furthermore,

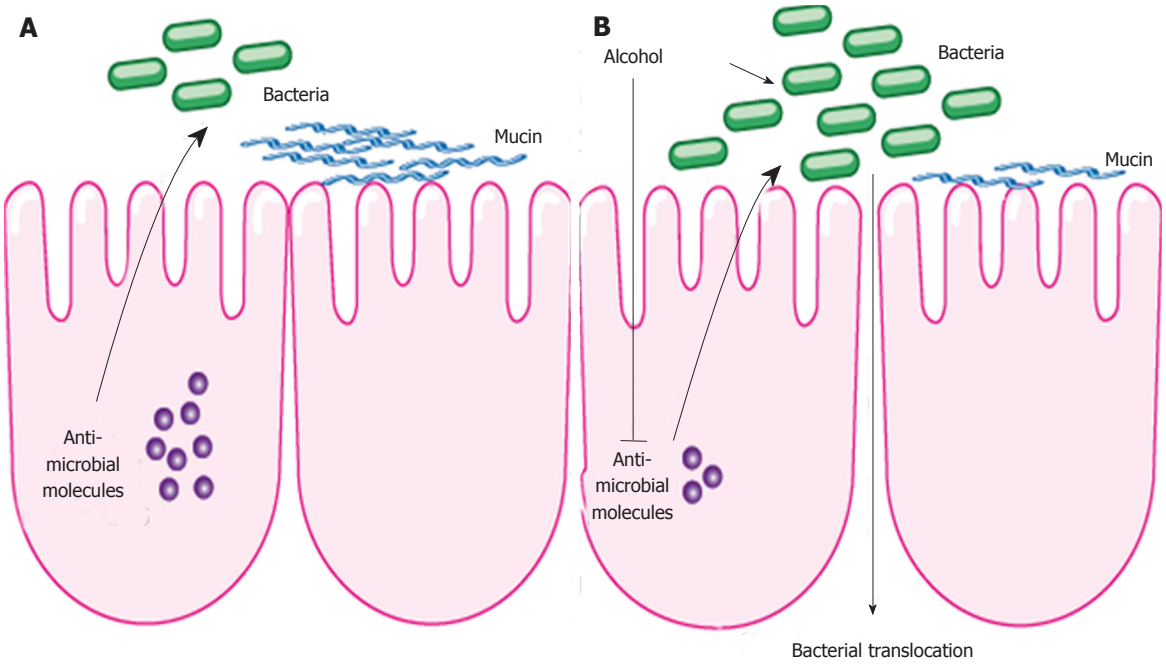

Figure 1 Intestinal changes following alcohol administration. A: In health, antimicrobial molecules as part of the innate immune response are secreted by intestinal epithelial cells and kill enteric bacteria. B: Alcohol suppresses the expression of these molecules resulting in intestinal bacterial overgrowth and dysbiosis. This might contribute to bacterial translocation observed after alcohol. Alcohol might also exert a direct effect on the intestinal microflora.

the treatment with prebiotics partially restored Reg3g protein levels, while mitigating bacterial overgrowth and attenuating the severity of alcoholic steatohepatitis[76].

Although changes in the enteric microbial composition has not been studied in patients with alcohol abuse, intestinal dysbiosis as a consequence of chronic alcohol consumption is speculated to be a possible precursor to bacterial translocation and a contributing factor to the initiation and progression of liver disease. It is likely that changes to the microbiota modulate mucosal barrier function and antimicrobial regulators within the host intestinal epithelium.

ARE ANTIBIOTICS, PROBIOTICS, PREBIOTICS OR SYNBIOTICS USEFUL IN ALCOHOLIC LIVER DISEASE?

The beneficial effect of antibiotics on alcohol-induced liver disease in animal models has been discussed above. In patients, antibiotics have mostly been used to decontaminate the intestine for the treatment of hepatic encephalopathy in end-stage liver disease. Antibiotic treatment for up to 6 mo has also been associated with improvement in liver function and Child-Pugh classification of patients with alcoholic cirrhosis[71]. Antibiotic-induced changes in the composition of the gastrointestinal microflora can influence the susceptibility of the host to specific enteric pathogens, including the induction of antibiotic resistance to other microorganisms. Antibiotics disturb the normal mechanisms of microbial community regulation, compromising the mucosal innate immune defense mechanism, which can result in pathogen colonization and antibiotic resistance[85].

Probiotics are dietary supplements of live microbes consumed by the host that benefit the health, and includes the microorganisms *Lactobacillus* and *Bifidobacterium*[86]. These microbes are thought to enhance production of anti-inflammatory cytokines, stimulate the secretion of antibacterial proteins, and alter the intestinal microflora, ultimately reducing production and translocation of endotoxin[87]. Pretreatment of animals with *Lactobacillus* decreases plasma LPS and reduces the severity of liver injury. As discussed above, *Lactobacillus* was depleted after 3 wk of intragastric alcohol administration in mice[75]. Feeding a gram-positive probiotic lactobacillus strain (species GG) with concomitant displacement of gram-negative bacteria also protected rats from ethanol-induced liver injury with a decrease in systemic endotoxin levels[80,81]. Thus, a possible mechanism for preventing ALD is reversing the enteric dysbiosis associated with alcohol abuse.

There have been multiple studies examining the benefits of probiotics in patients with ALD. Treatment of 20 patients with alcoholic liver cirrhosis with a probiotic mixture containing *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus bulgaricus* (VSL#3) for up to 4 mo, results in decreased plasma markers for oxidative stress and markedly reduced liver enzymes[88]. A randomized prospective trial demonstrated that probiotic treatment (*Bifidobacterium*

bifidum and *Lactobacillus plantarum*) for five days results in restoration of colonic bowel flora and improvement of serum liver tests in patients with alcoholic liver injury[89]. As a defective innate immune response to enteric pathogens likely contributes to increased infections in patients with cirrhosis, another mechanism that explains the beneficial effects of probiotics could be a restoration of neutrophil function. And in fact, administration of *Lactobacillus casei* for 4 wk restores neutrophil phagocytic activity in patients with alcoholic cirrhosis[90]. However, care must be applied when probiotics are administered to patients with a preexisting gut barrier leakage due to a higher risk of infection. In patients with predicted severe acute pancreatitis, probiotic prophylaxis with a combination of six different strains of probiotic strains (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum*, and *Bifidobacterium infantis*) did not reduce the risk of infectious complications and was associated with an increased risk of mortality[91].

Prebiotics are complex carbohydrates that cannot be digested by pancreatic or intestinal enzymes and are ultimately metabolized by the gut microflora. Common prebiotics are fructo-oligosaccharides (FOS), galacto-oligosaccharides, or lactulose[86]. The later is of special interest, as it is commonly used in patients with decompensated cirrhosis for the treatment and prevention of hepatic encephalopathy. As mentioned earlier, oats supplementation as a prebiotic not only prevents alcohol-induced dysbiosis following 10 wk of alcohol treatment, but reduces gut leakiness with subsequent reduction in endotoxin levels and attenuation of liver damage in rats[79,92]. Similarly, FOS reduces intestinal bacterial overgrowth and alcoholic steatohepatitis following intragastric feeding of alcohol for 3 wk in mice[75]. The beneficial effect of prebiotics is suggested to prevent quantitative and qualitative changes in the microbiome associated with alcohol use. At this point, it is not entirely clear whether prebiotics might prevent intestinal barrier leakage independent from changes to the microbiome. Prebiotics are very promising for future use in patients with ALD and further clinical studies are needed.

Synbiotics are combinations of prebiotics and probiotics. Pretreatment with synbiotics (*Lactobacillus acidophilus, Lactobacillus helveticus* and *Bifidobacterium* in an enriched medium) effectively protects against endotoxemia and bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption in rats[93].

CONCLUSION

Alcoholic liver disease remains a leading cause of morbidity and mortality worldwide. The progression of alcoholic liver disease to fibrosis and cirrhosis is partly mediated through an inflammatory response to the translocation of bacteria and endotoxin. Bacterial translocation is also a contributing factor to the complications arising from alcoholic liver disease including spontaneous bacterial peritonitis, hepatic encephalopathy, portal hypertension, and sepsis. There are many possible mechanisms for which this occurs. Bacterial overgrowth of pathogenic organisms and intestinal dysmotility both occur in alcoholic liver disease, and may predispose the intestines for bacterial translocation. The oxidative stress resulting from exposure of the intestines to alcohol and its metabolites disrupts the integrity of the intestinal wall, increasing permeability to gut-derived endotoxin. There have also been recent advances in diagnostic technologies for research in molecular genetics. The use of 454 pyrosequencing has enabled the qualitative and quantitative examination of the enteric microbiome. In an experimental model of alcoholic liver disease, shifts in the composition of the intestinal flora have been observed along with downregulation of intestinal antimicrobial proteins. These changes can modulate mucosal barrier function by disrupting the innate immune system and alter antimicrobial regulators in the host intestinal epithelium. This process can facilitate bacterial translocation and more animal studies will be needed to firmly establish this mechanism. There are few therapeutic options for patients suffering from alcoholic liver disease, aside from abstinence and liver transplantation for end-stage liver disease. There is some limited evidence on the use of antibiotics, probiotics, and prebiotics to attenuate disease activity in patients with alcoholic liver disease by altering the intestinal microflora. However, large scale clinical studies examining the potential benefits of probiotics and prebiotics are still needed before routine use of these supplements can be recommended.

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REVIEW

Recent advances in liver preconditioning: Thyroid hormone, n-3 long-chain polyunsaturated fatty acids and iron

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Abstract

Liver preconditioning (PC), defined as an enhanced tolerance to injuring stimuli induced by previous specific maneuvers triggering beneficial functional and molecular changes, is of crucial importance in human liver transplantation and major hepatic resection. For these reasons, numerous PC strategies have been evaluated in experimental models of ischemia-reperfusion liver injury, which have not been transferred to clinical application due to side effects, toxicity and difficulties in implementation, with the exception of the controversial ischemic PC. In recent years, our group has undertaken the assessment of alternate experimental liver PC protocols that might have application in the clinical setting. These include thyroid hormone (T_3), n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), or iron, which suppressed liver damage due to the 1 h ischemia-20 h reperfusion protocol. T_3, n-3 LCPUFA and iron are hormetic agents that trigger biologically beneficial effects in the low-dose range, whose multifactorial mechanisms of action are discussed in the work.

Key words: Liver preconditioning; Thyroid hormone; n-3 polyunsaturated fatty acids; Iron

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INTRODUCTION

Liver functioning is characterized by a multiplicity of processes that include most of the pathways for intermediary metabolism, biotransformation of xenobiotics, plasma protein biosynthesis, excretion and secretion of various types of molecules. With the exception of hyperglycemic conditions, the high energy requirements to support liver functions are primarily met by fatty acid oxidation, making the liver highly dependent on O_2 supply and susceptible to hypoxic or anoxic conditions. Liver damage underlying cellular death is associated with cholestasis, viral hepatitis, drug-induced injury, obesity[1] and ischemia-reperfusion (IR) episodes, including liver transplantation, hepatic resection, low-blood pressure conditions and abdominal surgery requiring hepatic vascular occlusion[2-5]. IR injury is a phenomenon in which cellular damage due to hypoxia is exacerbated following restoration of O_2 and nutrient supply[2-5]. In these situations, different types of ischemia can occur in the liver: namely, (1) warm ischemia inducing hepatocyte and sinusoidal endothelial cell (SEC) death, a feature of hepatic trauma, hypovolemic shock and inflow occlusion during

liver surgery; and (2) cold ischemia leading to SEC death observed in liver transplantation after harvesting and preservation, which might involve rewarming ischemia during vascular anastomosis[6]. IR liver injury is due to numerous contributory factors, including Kupffer cell activation, oxidative stress and up-regulation of pro-inflammatory cytokine signaling, which often determine hepatic failure[1-6]. Considering that IR liver injury is a major complication in clinical practice due to its complexity in terms of molecular and cellular mechanisms, strategies reducing IR injury have been extensively studied[4-8].

In general terms, organ preconditioning (PC) is defined as an increased tolerance to IR injury afforded by previous specific maneuvers triggering beneficial functional and molecular changes, a phenomenon initially described by Murry *et al*[9] in the heart. PC strategies evaluated in experimental models of IR liver injury include: (1) pharmacological approaches targeting tumor necrosis factor-α (TNF-α) response, mitochondrial dysfunction, reactive oxygen species (ROS) production, microcirculatory disorders or neutrophil infiltration; (2) gene therapy directed to up-regulation of proteins abrogating ROS production, apoptosis and nuclear factor-κB (NF-κB) activation or down-regulating of intercellular adhesion molecule-1 and P-selectin expression reducing neutrophil recruitment; and (3) surgical strategies such as ischemic preconditioning (IP) or other strategies underlying moderate oxidative stress development (for specific references see[4-8,10]). The latter group of PC maneuvers includes development of hyperthermia[11], hyperbaric oxygen therapy[12] or the administration of the model oxidants *tert-butyl* hydroperoxide[13], doxorubicin[14] and ozone[15]. However, due to toxicity, side effects and difficulties in implementation, these experimental PC strategies have not been transferred to clinical application, with the exception of IP[6,16]. Although IP proved to be useful in human liver resections[17-19] and in human liver transplantation[20-22], this PC maneuver remains controversial[23-25]. For these reasons, our group has recently undertaken the evaluation of alternate experimental liver PC strategies that might have application in the clinical setting; namely, administration of thyroid hormone (L-3,3',5-triiodothyronine, T_3)[26], n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA)[27] or iron[28], prior to an IR protocol.

THYROID HORMONE LIVER PRECONDITIONING

Liver PC by *in vivo* T_3 administration is based on the calorigenic action of thyroid hormones leading to stimulation and maintenance of basal thermogenesis[29], a response that is carried out through genomic and non-genomic signaling mechanisms[1,30]. In the liver, this effect is evidenced by enhancement in the rate of O_2 consumption, with consequent increment in ROS generation[31,32] by mechanisms primarily triggered in hepatocytes and in Kupffer cells (Figure 1). ROS produced in Kupffer cells activate redox-sensitive transcription factors such as NF-κB, signal transducer and activator of transcription 3 (STAT3) or activating protein 1 (AP-1), as shown by suppression of T_3-induced DNA binding of these proteins by *in vivo* pretreatment with the antioxidants α-tocopherol and N-acetylcysteine or the Kupffer cell inactivator gadolinium chloride[1,30]. Under these conditions, activation of NF-κB and AP-1 in Kupffer cells is associated with up-regulation of the expression of genes for the cytokines TNFα, interleukin (IL)-1 and IL-6[33], with enhanced synthesis and release into hepatic sinusoids (Figure 1). Interaction of Kupffer cell-derived TNF-α with TNF receptor 1 may trigger two responses in hepatocytes[33]: namely, (1) NF-κB activation *via* inhibitor of κB kinase (IKK) phosphorylation leading to the expression of antioxidant (manganese superoxide dismutase, inducible nitric oxide synthase), anti-apoptotic (Bcl2) and type I acute-phase (haptoglobin) proteins[34,35]; and (2) AP-1 activation *via* c-Jun N-terminal kinase (JNK) phosphorylation leading to up-regulation of hepatocyte proliferation[36] (Figure 1). In addition, interaction of Kupffer cell-derived IL-6 with IL-6 receptor through its binding to the gp130 receptor subunit[37] may activate Janus kinase (JAK)/STAT3 system and the transcription of both type I (haptoglobin) and type II (β-fibrinogen) acute-phase protein genes[35] (Figure 1). Activation of NF-κB, STAT3 and AP-1 by Kupffer cell-derived TNF-α and IL-6 may be reinforced by ROS generated within hepatocytes by different enzymatic mechanisms triggered by T_3 (Figure 1). These cytoprotective responses could be contributed by additional processes triggered by T_3 administration: including (1) post-transcriptional up-regulation of the acute-phase protein ferritin through increased iron-induced displacement of iron regulatory protein from the iron-responsive element in ferritin mRNA[38]; and (2) transcriptional up-regulation of uncoupling proteins *via* the classical genomic pathway[39], which have been proposed to decrease the pro-oxidant potential of the liver[38-40].

Recently, *in vivo* T_3 administration to rats was shown to activate hepatic nuclear factor erythroid 2-related factor 2 (Nrf2), as evidenced by the increased cytosol-to-nuclear translocation observed[41]. Liver Nrf2 activation induced by T_3 appears to be a redox-dependent process due to its abolishment by N-acetylcysteine pretreatment, which may be contributed by Nrf2 phosphorylation related to p38 activation[41]. This would represent a novel and alternate cytoprotective mechanism of T_3 action against free-radical and electrophile toxicity[41], in addition to that afforded by NF-κB, STAT3 and AP-1 up-regulation[34-36] (Figure 1), considering that Nrf2 controls the expression of antioxidant components, detoxification enzymes or membrane transporters (Figure 1) and interplays with NF-κB affording anti-inflammatory responses[42].

Redox activation of NF-κB, STAT3, AP-1 and Nrf2 up-regulating transcription of protective genes represents an additional non-genomic mechanism of T_3 action to those reported for different cellular processes[43], which is dependent upon the genomic pathway enhancing energy metabolism with ROS production. These observations

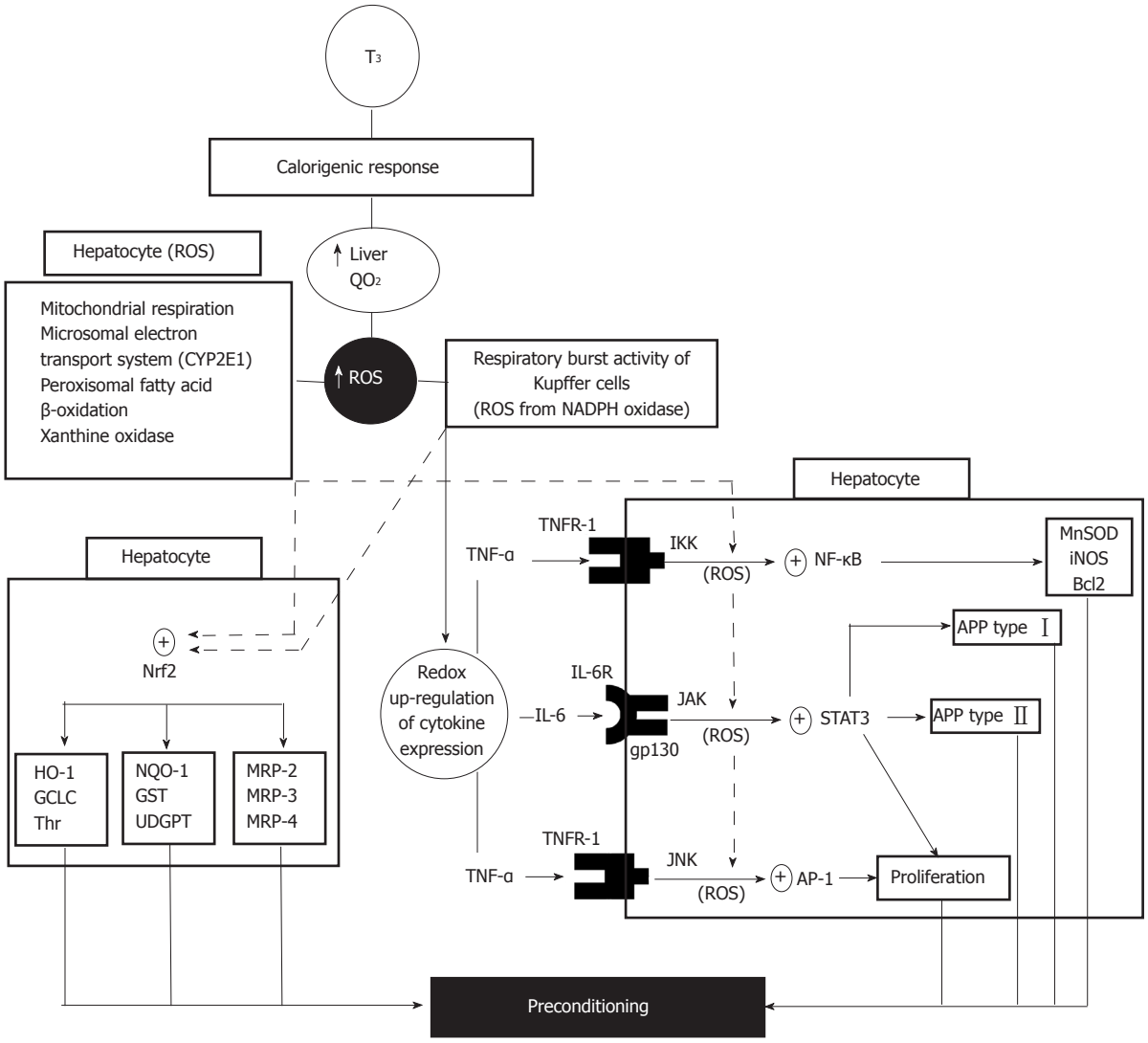

Figure 1 Redox signaling in T_3 liver preconditioning is mediated by activation of transcription factors nuclear factor-κB, activating protein 1, signal transducer and activator of transcription 3, and nuclear factor erythroid 2-related factor 2 triggering antioxidant, anti-apoptotic, acute-phase and proliferative responses. AP-1: Activating protein 1; APP: Acute-phase protein; CYP2E1: Cytochrome P450 isoform 2E1; GCLC: Glutamate cysteine ligase catalytic subunit; GST: Glutathione-S-transferase; HO-1: Heme-oxygenase 1; IKK: Inhibitor of IκB kinase; iNOS: Inducible nitric oxide synthase; IL-6: Interleukin-6; IL-6R: Interleukin-6 receptor; JAK: Janus kinase; JNK: c-Jun N-terminal kinase; MnSOD: Manganese superoxide dismutase; MRP: Multidrug resistance protein; NF-κB: Nuclear factor-κB; NQO-1: NAD(P)H quinone oxidoreductase 1; Nrf2: Nuclear factor-erythroid 2 related factor 2; QO_2: Rate of oxygen consumption; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3; Thr: Thioredoxin; TNF-α: Tumor necrosis factor-α; TNFR1: Tumor necrosis factor-α receptor 1; UDPGT: UDP-glucuronyl transferase.

constitute the basis for liver PC by T_3, with integration of different T_3-signaling inputs to achieve metabolic and redox balance (Figure 1) that are required to deal with the cytotoxic mechanisms underlying IR liver injury. Increased hepatocyte proliferation compensating for liver cells lost due to IR-induced necrosis[36] is an additional PC response due to the mitogenic action of T_3, leading to direct hyperplasia[44]. In agreement with these views, IR-induced (1) drastic enhancement in liver oxidative stress status and TNF-α response; (2) loss of DNA binding capacity of NF-κB and STAT3, implying loss of cytoprotective potential; and (3) major increment in hepatic AP-1 activation, which constitutes a crucial determinant of hepatotoxicity under conditions of reduced NF-κB activation and enhanced TNF-α response, are normalized by T_3 treatment[26]. Similar T_3 actions involving other physiological functions have been described, including (1) NF-κB activation in lymphocytes from thyroxin-treated rats[45] or hyperthyroid patients[46] in association with higher oxidative stress status and potentiation of humoral immune response; and (2) JNK/STAT3 activation by T_3 in a nutritional model of non-alcoholic steatosis in rats, with complete regression of fat accumulation[47].

n-3 LONG-CHAIN-POLYUNSATURATED FATTY ACID LIVER PRECONDITIONING

Dietary fatty acids, especially LCPUFA, are essential for growth and development in mammals including man,

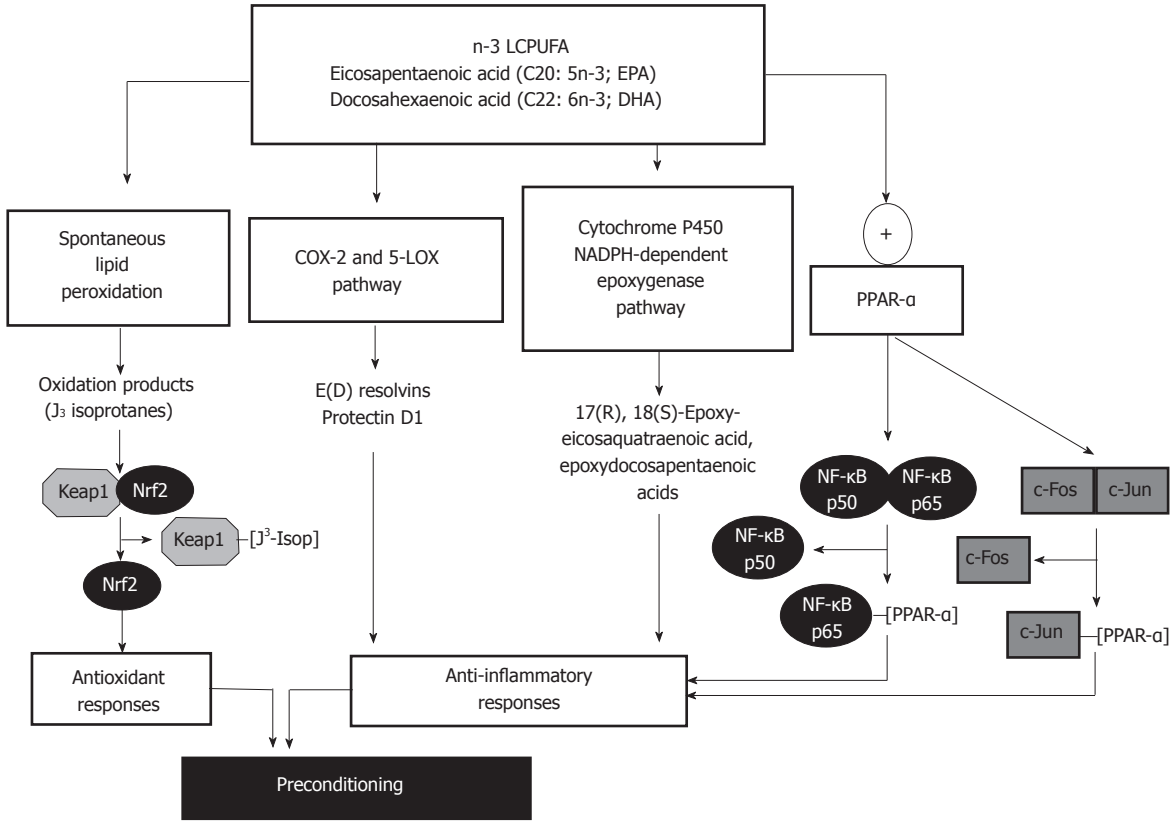

Figure 2 *n-3* long-chain polyunsaturated fatty acid -induced liver preconditioning is associated with antioxidant and anti-inflammatory responses triggered by oxidative products and peroxisome proliferator-activated receptor -α activation. n-3 LCPUFA: n-3 long-chain polyunsaturated fatty acid; **COX-2: Cyclo-oxy-**genase 2; Keap1: **Kelch-like ECH-associated protein 1; 5-LOX: 5-lipoxygenase; NF-κB: Nuclear factor-κB; Nrf2: Nuclear factor-erythroid 2 related factor 2; PPAR-α:** Peroxisome proliferator-activated receptor-α.

both n-6 and n-3 LCPUFAs being important as structural components of cellular lipids and substrates for the synthesis of physiological mediators[48]. Among the n-3 series of LCPUFAs, eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA), produced from α-linolenic acid (C18:3n-3), have been associated with multiple positive health effects[48,49] and proposed for the prevention of non transmissible chronic diseases[50] or against heart[51] and liver[27] IR injury. It is considered that attainment of a given n-6/n-3 ratio is crucial for prevention and treatment of several diseases, as a potential sensor for the activation of mechanisms involved in inflammatory processes[52] such as liver IR injury[1-6].

Recently, liver PC against IR injury was reported in rats subjected to fish oil (270 mg/kg EPA plus 180 mg/kg DHA) or saline (controls) administration for 7 d, prior to the 1 h ischemia-20 h reperfusion protocol[27]. *In vivo* n-3 LCPUFA supplementation significantly enhanced liver n-3 LCPUFA content and decreased n-6/n-3 LCPUFA ratios, with prevention of IR-induced liver injury, suppression of oxidative stress, recovery of pro-inflammatory cytokine homeostasis, and NF-κB functionality lost during IR[27]. Several molecular mechanisms can be invoked to explain liver PC by n-3 LCPUFA, including antioxidant and anti-inflammatory responses (Figure 2).

Considering the high susceptibility of n-3 LCPUFAs to free-radical attack with further decomposition[53], these fatty acids readily undergo *in vitro* non-enzymatic lipid peroxidation with formation of cyclopentenone-containing J-ring isoprostanes (J_3-isoprostanes)[54]. Gao *et al*[54] reported that J_3-isoprostanes from EPA and DHA oxidation react with sulfhydryl groups in recombinant Keap1, a Cul-containing E3 ubiquitin ligase (Cul3)-Ring box 1 complex responsible for Nrf2 ubiquitination and degradation[55]. This interaction alters Keap1 structure, leading to loss of binding to Cul3, Nrf2 stabilization and nuclear translocation, with expression of the antioxidant enzymes heme-oxygenase-1 and glutamate cysteine ligase, as assessed in cultured HepG2 cells[54] (Figure 2). In agreement with these findings, *in vivo* EPA supplementation in mice was shown to up-regulate the expression of other Nrf2-dependent antioxidant proteins, namely, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and catalase, with significant increases in liver glutathione content and diminution in lipid peroxidation rate[56].

Both EPA and DHA have been reported as effective anti-inflammatory and tissue protective mediators[48-50], effects that may underlie different mechanisms of action (Figure 2). These include various aspects of eicosanoid metabolism generating n-3 LCPUFA-derived mediators produced in the resolution phase following acute inflam-

mation. EPA and DHA can be metabolized by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) to generate E-series and D-series of resolvins *in vivo* and *in vitro*, respectively (Figure 2), which exhibit anti-inflammatory effects compared with those derived from arachidonic acid[57]. DHA can also be metabolized by 5-LOX to produce protectins, protectin D1 being the most potent anti-inflammatory isomer[58] (Figure 2). Resolvins E1, E2 and protectin D1 exert their anti-inflammatory action mainly through inhibition of neutrophil infiltration in target tissues[57,59]. In the case of resolving E1, its binding to G-protein-coupled receptors chemokine-like receptor-1 and leukotriene B_4 receptor attenuates the pro-inflammatory effects of NF-κB and leukotriene B_4 signaling, respectively[60]. Although the influence of resolvins and protectins has not been evaluated in IR liver injury, mouse kidney subjected to bilateral IR leads to endogenous mobilization and higher blood levels of DHA, with enhanced production of D-series of resolvins and protectins[61]. Moreover, pretreatment with exogenous resolvins was able to protect from IR kidney injury[61]. Interestingly, *in vivo* DHA supplementation is protective against liver necroinflammatory injury in mice subjected to carbon tetrachloride intoxication, a condition enhancing hepatic formation of the DHA-derived metabolites 17S-hydroxy-DHA (17S-HDHA) and protectin D1[62]. These findings and the protective effect of DHA and 17S-HDHA against *in vitro* hydrogen peroxide toxicity in hepatocytes establish a significant protective role of n-3 LCPUFA supplementation in well-known animal models of liver injury, which amplifies formation of DHA-derived anti-inflammatory lipid mediators in the liver[62] (Figure 2). In addition to EPA and DHA metabolism by the COX2/5-LOX pathway, these fatty acids may undergo oxygenation by cytochrome P450 NADPH-dependent epoxygenases (Figure 2), with production of multiple epoxyeicosaquatraenoic acid and epoxydocosapentaenoic acid regioisomers, respectively[57,63], which might have anti-inflammatory effects[57]. The finding that IR elicited a net decrease in the content of n-3 LCPUFA in the liver of EPA plus DHA supplemented rats over that in non-supplemented animals[27], support the contention that *in vivo* n-3 LCPUFA protection may be related to utilization of the fatty acids in lipid peroxidation, COX-2/5-LOX and cytochrome P450-dependent epoxygenation pathways. However, n-3 LCPUFA β-oxidation and replacement for n-6 LCPUFA in membrane phospholipids cannot be discarded.

In addition to the above discussed mechanisms related to the anti-inflammatory responses of n-3 LCPUFA involving oxidative processes, EPA and DHA may directly alter intracellular signaling pathways associated with transcription factors peroxisome proliferator-activated receptors (PPAR)-α/PPAR-γ and NF-κB/AP-1. The mechanism is based on the findings that LCPUFA, fatty acid derivatives and eicosanoids act as natural ligands for PPARs leading to their activation[64], which physically interact with both the p65 component of NF-κB and the c-Jun component of AP-1 (Figure 2), thus interfering with NF-κB and AP-1 transactivation of inflammatory genes[65]. Alternate mechanisms triggered by PPAR-α activation include: (1) enhancement of IκB-α mRNA and protein expression and its nuclear abundance, with diminution in NF-κB DNA binding activity[66]; (2) decreased IκB-α degradation, probably due to diminished phosphorylation[67]; and (3) up-regulation of antioxidant enzymes[56,68] (Figure 2) with reduction of the oxidative stress status, leading to loss of NF-κB activation and inflammatory cytokine production[68]. n-3 LCPUFA-induced re-establishment of inflammatory cytokine homeostasis under IR conditions[27,69] is accompanied by improvement of hepatic microcirculation, as a contributory factor protecting the liver against IR injury[70,71]. Although the relevance of n-3 LCPUFA supplementation in conditions underlying IR liver injury in humans has not been evaluated, several clinical studies have reported that supplementation with fish oil, seal oil or purified n-3 LCPUFA reduces hepatic lipid content in obese non-alcoholic fatty liver disease patients[72-76], exhibiting substantial depletion of n-3 LCPUFA content[77]. In addition, n-3 LCPUFA administration improved circulating liver function markers[72-76], serum triacylglycerol (TAG)[73,74] and tumor necrosis factor-α[73] levels, and hepatic microcirculatory function[72].

IRON LIVER PRECONDITIONING

Iron is an essential micronutrient and bio-catalyst of oxidation-reduction reactions that are related to its chemistry promoting electron exchange under aerobic conditions, being crucial for mitochondrial oxidative phosphorylation and other processes requiring enzymes/proteins with iron as a cofactor[78,79]. At the different cell compartments, iron is bound to low-molecular-weight molecules, giving a steady-state concentration of labile iron within the cell. This labile iron pool corresponds to a low-molecular-weight pool of weakly chelated iron that readily passes through the cell, representing a minor fraction of total cellular iron (3%-5%)[79]. The cellular labile iron pool is in equilibrium with (1) iron taken from the diet, delivered into bloodstream, and incorporated into cells through transferring-receptors; (2) iron export; (3) iron reversibly incorporated into heme and non-heme proteins; and (4) iron stored in ferritin, which constitutes a major and safe fraction of the iron that entered into the cell (Figure 3)[79].

The intracellular labile iron pool has been associated with physiological, pharmacological and toxicological iron functions. Iron is able to catalyze the conversion of by-products of respiration [superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)] into hydroxyl radical ($HO^{\bullet}$) *via* the Fenton reaction or the Fe^{2+}-assisted Haber-Weiss reaction (Figure 3)[79], thus enhancing the oxidative stress status of the cell. Rats subjected to a sub-chronic iron administration protocol (six doses of 50 mg iron-dextran/kg, ip every second day during 10 d) showed significant enhancement in total iron and in the labile iron pool of

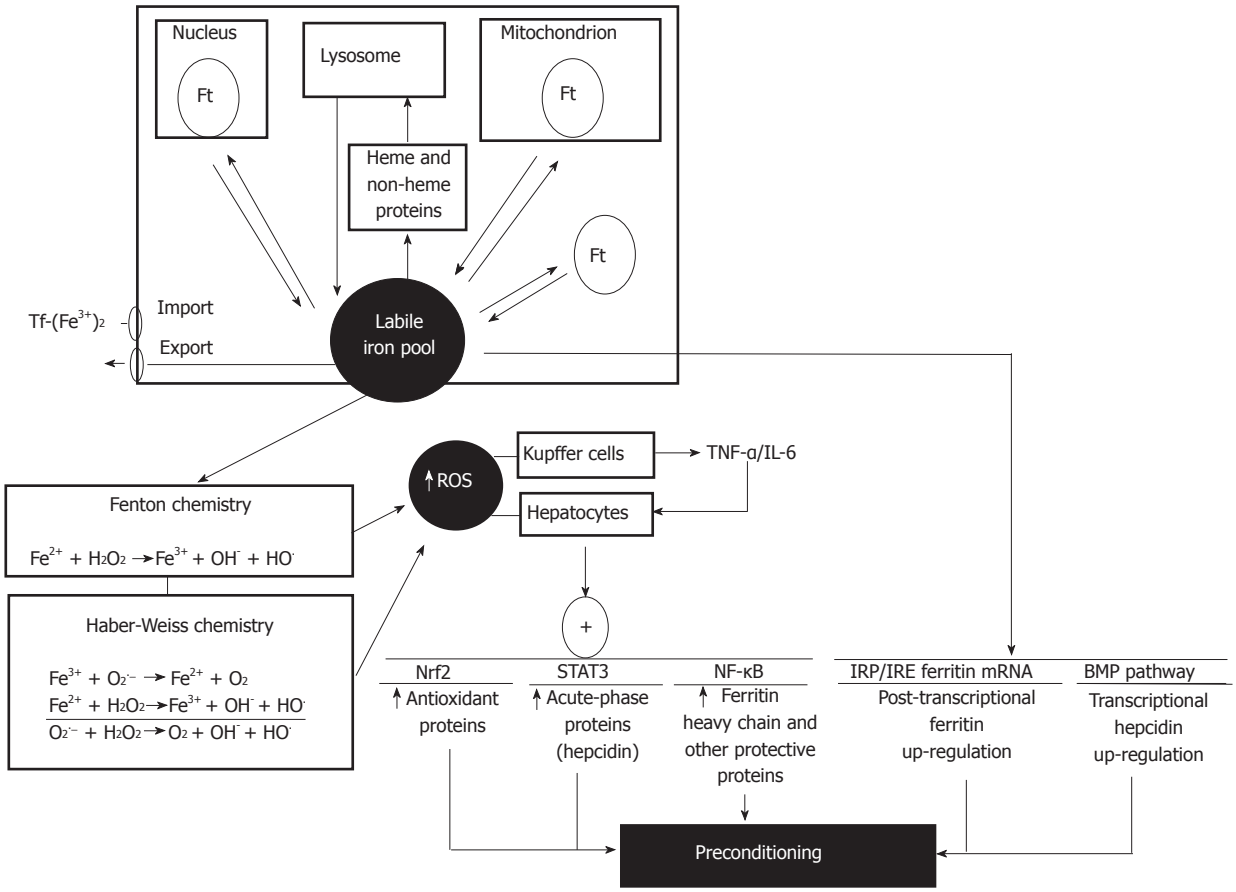

Figure 3 Redox signaling in iron (Fe)-induced liver preconditioning is elicited by the cellular labile Fe pool triggering nuclear factor-erythroid 2, signal transducer and activator of transcription 3, and nuclear factor-κB activation and iron-regulatory protein / iron-responsive element post-transcriptional up-regulation with antioxidant and acute-phase responses. BMP: Bone morphogenetic protein; Ft: Ferritin; H_2O_2: Hydrogen peroxide; $HO^{\cdot}$: hydroxyl radical; IL-6: Interleukin-6; IRE: Iron-responsive element; IRP: Iron-regulatory protein; Nrf2: Nuclear factor-erythroid 2 related factor 2; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3; $O_2^{\cdot-}$: Superoxide radical; Tf: Transferring; TNF-α: Tumor necrosis factor-α.

the liver, with consequent up-regulation of ferritin content, thus establishing a transient oxidative stress condition without development of hepatotoxicity[28]. Under these conditions, a significant protection was afforded by iron administration against liver IR injury, as evidenced by diminution in serum transaminase levels and normal liver architecture observed in iron supplemented animals subjected to IR compared to non-supplemented rats[28]. Iron liver preconditioning against IR could be due to cellular iron metabolism over the 72 h time-period between *in vivo* iron administration and the settlement of IR *in vitro*, with consequent ferritin up-regulation sequestering large amounts of administered iron to avoid liver injury, and expansion of the labile iron pool increasing the oxidative stress status that limits the further pro-oxidant challenge of IR. In addition, suppression of the TNF-α response and reversion of the changes in signal transduction and gene expression induced by IR were achieved by *in vivo* iron administration, with recovery of NF-κB activation and NF-κB-related expression of haptoglobin lost during IR (Figure 3)[28]. Haptoglobin is an anti-inflammatory and antioxidant acute-phase protein participating in the acute-phase response of the liver, a reaction restoring homeostasis by contributing to defensive and adaptive capabilities[80].

From the mechanistic viewpoint, development of transient oxidative stress in the liver of iron supplemented animals may be related to stimulation of different processes in Kupffer cells and hepatocytes. *In vivo* iron overload alters the functional status of Kupffer cells by increasing the respiratory burst activity without modifying phagocytosis, an effect that is probably related to O_2 equivalents used by NADPH oxidase to produce $O_2^{\bullet-}$ and H_2O_2, which may be further subjected to Fenton/Haber-Weiss reactions (Figure 3)[81]. Promotion of biomolecules oxidation and activation of nitric oxide synthase[82] may also contribute to this effect of iron. Iron-induced respiratory burst of Kupffer cells with enhanced ROS production[81] may have a role in NF-κB signaling, as shown by the activation of IKK and NF-κB DNA binding, leading to enhanced TNF-α promoter activity and TNF-α release from cultured Kupffer cells[83] (Figure 3). As proposed for T_3 liver preconditioning (Figure 1), TNF-α released from Kupffer cells may trigger protective signaling cascades in hepatocytes, thus achieving protection against IR liver injury. Interestingly, ferritin heavy

chain was identified as an essential mediator of the antioxidant and protective actions of NF-κB, as assessed in cultured NIN-3T3 cells[84]. This protein is induced downstream of NF-κB, providing a transcriptional regulatory mechanism for ferritin induction through iron-mediated ROS generation[85] (Figure 3), which represents a potential approach for anti-inflammatory therapy[84]. Up-regulation of ferritin expression by iron is also under post-transcriptional regulation, a mechanism involving the interaction of iron regulatory proteins with the iron-responsive elements in ferritin mRNA, to enhance ferritin synthesis and concentrate excess iron[84,86], avoiding cytotoxicity (Figure 3). Besides, iron overload up-regulates hepcidin expression, an acute-phase protein produced by hepatocytes that controls the dietary absorption, storage and tissue distribution of iron, which exhibits a significant correlation with serum ferritin levels[87]. The mechanism of hepcidin action involves internalization and degradation of ferroportin, a hepcidin-receptor and iron channel, that diminishes intestinal iron absorption, iron mobilization from hepatocytes, and iron recycling by macrophages, leading to iron entrapment in ferritin at enterocyte, macrophage and hepatocyte levels[87]. Although regulation of liver hepcidin transcription by iron involving the bone morphogenetic protein (BMP) pathway is not completely understood[87], IL-6/STAT3 signaling is a key effector of hepcidin expression during inflammatory conditions[88], a redox-sensitive pathway controlling the expression of several other acute-phase proteins (Figure 3). In addition to NF-κB and STAT3, liver Nrf2 signaling may also contribute to iron-induced preconditioning, considering (1) the enhancement in the expression of liver Nrf2 protein and catalase, glutathione-S-transferase and heme-oxygenase-1 mRNA in mice subjected to iron overloading[89]; and (2) the significant diminution in hepatic glutathione levels and in glutamate cysteine ligase activity observed in Nrf2$^{(-/-)}$ mice treated with ferric nitrilotriacetate over wild-type animals[90] (Figure 3).

CONCLUDING REMARKS

T_3, n-3 LCPUFA and iron can be considered as hormetic agents[91,92], which are defined as compounds inducing a dose-response phenomenon characterized by biologically beneficial effects in the low-concentration (dose) range (organ preconditioning)[26-28] and harmful responses at high concentrations (doses) or after prolonged exposure (thyrotoxicosis[93], gastrointestinal upset/increase bleeding time[48] and hemochromatosis[94], respectively). Organ preconditioning by these hormetic agents is not restricted to the liver[26-28], considering that (1) thyroid hormone-induced preconditioning against IR injury is also observed in the heart[95,96], with a pattern of protection comparable to that of ischemic preconditioning[97]; (2) beneficial effects of n-3 LCPUFA have been demonstrated in rheumatoid arthritis, inflammatory bowel disease, coronary artery disease, asthma and sepsis, conditions with inflammation as a key component of their pathology[48], in addition to neuroinflammation in all major central nervous system diseases[98]; and (3) protective effects of iron are reported in cardiomyocytes and heart[99-101], oligodendroglia cells[102] and neurones[103]. T_3, n-3 LCPUFA or iron liver preconditioning are suitable to be introduced in the clinical setting, considering that these hormetins are known to be well tolerated in the treatment of hypothyroidism[104], non-alcoholic fatty liver disease[72-76] and other diseases[48], or anemia[105,106], respectively. Interestingly, there is evidence to conclude that n-3 LCPUFAs potentiate the effects of certain drugs, thereby allowing a reduction of their required dose, thus avoiding adverse effects[48]. In agreement with this view, combined n-3 LCPUFA (300 mg/kg for 3 consecutive days) plus T_3 (0.05 mg/kg) administration prevented rat liver IR injury, whereas separate protocols lack protection[107], when compared with the preconditioning action afforded by separate n-3 LCPUFA (300 mg/kg for 7 consecutive days)[27] or T_3 (0.1 mg/kg)[26]. Data discussed in this article warrants further experimental and clinical research in the future, to support the incorporation of T_3, n-3 LCPUFA and iron preconditioning strategies or their combinations in human liver resections and in human liver transplantation using reduced-size grafts from living donors.

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REVIEW

G6PT-H6PDH-11βHSD1 triad in the liver and its implication in the pathomechanism of the metabolic syndrome

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Abstract

The metabolic syndrome, one of the most common clinical conditions in recent times, represents a combination of cardiometabolic risk determinants, including central obesity, glucose intolerance, insulin resistance, dyslipidemia, non-alcoholic fatty liver disease and hypertension. Prevalence of the metabolic syndrome is rapidly increasing worldwide as a consequence of common overnutrition and consequent obesity. Although a unifying picture of the pathomechanism is still missing, the key role of the pre-receptor glucocorticoid activation has emerged recently. Local glucocorticoid activation is catalyzed by a triad composed of glucose-6-phosphate-transporter, hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase type 1 in the endoplasmic reticulum. The elements of this system can be found in various cell types, including adipocytes and hepatocytes. While the contribution of glucocorticoid activation in adipose tissue to the pathomechanism of the metabolic syndrome has been well established, the relative importance of the hepatic process is less understood. This review summarizes the available data on the role of the hepatic triad and its role in the metabolic syndrome, by confronting experimental findings with clinical observations.

Key words: Metabolic syndrome; Liver; Glucocorticoid; Glucose-6-phosphate-transporter; Hexose-6-phosphate dehydrogenase; 11β-hydroxysteroid dehydrogenase type 1

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INTRODUCTION

The metabolic syndrome (MS) is a multicomponent clinical entity with a prevalence of about 20%-25%. Several definitions of MS have been suggested, including those of the World Health Organization[1,2], the European group for the study of insulin resistance[3], the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III)[4] and the International Diabetes Federation[5,6]. Although these definitions share many common features, some criteria are different. Ac-

cording to the most widely accepted diagnostic definition of NCEP ATP III, metabolic syndrome is diagnosed if three or more of the following parameters are present: waist circumference greater than 102 cm in men and 88 cm in women, serum triglyceride level higher than 150 mg/dL (1.7 mmol/L), HDL-C level lower than 40 mg/dL (1.04 mmol/L) in men and lower than 50 mg/dL in women, blood pressure higher than 130/85 mmHg and fasting glucose level at least 110 mg/dL (6.1 mmol/L). The most important complication of metabolic syndrome is the increased (approximately doubled) risk of the development of ischemic heart disease.

The metabolic syndrome is much more than the simple sum of the symptoms. It is a multifactorial disease, which is not due to a single genetic defect and lacks a unique pathomechanism. Its complex etiology likely includes inherited predisposition, intrauterine effects, lifestyle factors and excessive calorie intake. Central (omental) adiposity and the pro-inflammatory conditions in the adipose tissue have emerged as crossing points of these etiological factors.

The phenotype and symptoms (e.g. serum lipid pattern and derangements of carbohydrate metabolism) of the metabolic syndrome are remarkably similar to those of Cushing syndrome, which is caused by excessive glucocorticoid production or medication. This clinical observation leads to the hypothesis that glucocorticoids might play a role in the pathogenesis of the metabolic syndrome. Although plasma cortisol levels are normal, both cortisol excretion and total body cortisol production were found to be increased in patients with abdominal obesity[7]. Preclinical data on rodent models proved the role of glucocorticoids in obesity[8]. The absence of elevated serum cortisol levels both in human and rodent metabolic syndrome suggests the existence of a local glucocorticoid effect in the background of this phenomenon. The activity of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) has appeared in the focus of the pathogenesis recently.

BIOCHEMISTRY OF THE GLUCOSE-6-PHOSPHATE TRANSPORTER-HEXOSE-6-PHOSPHATE DEHYDROGENASE-11βHSD1 SYSTEM

11βHSD1 is a luminal enzyme of the endoplasmic reticulum (ER), which is expressed in many organs and tissues. The enzyme expressed in the liver and adipose tissue plays presumably the most important role in the pathogenesis of the metabolic syndrome. Its main function is the regulation and enhancement of local glucocorticoid effect at tissue level. 11βHSD1 catalyzes the reversible interconversion of cortisone and cortisol *in vitro*, by using nicotinamide adenine dinucleotide phosphate $(NADP)^+$ or NADPH as a cofactor, which makes the activity sensitive to modifications in cofactor supply. The fact that 11βHSD1 acts exclusively as a reductase *in vivo* suggests a high luminal NADPH/$NADP^+$ ratio in the ER. This ratio is generated by hexose-6-phosphate dehydrogenase (H6PDH), another luminal enzyme. H6PDH seems to be the major, if not the only, enzyme responsible for $NADP^+$ reduction in the ER lumen[9]. This tandem enzyme catalyzes the first two steps of the pentose-phosphate pathway, i.e. the formation of 6-phosphogluconate from glucose-6-phosphate. Besides their colocalization and direct physical interaction[10], cooperativity between 11βHSD1 and H6PDH was proved by biochemical[11], as well as by genetic[12] approaches. The activities of the two enzymes are linked by cofactor sharing, i.e. they mutually generate cofactors for each other. Their physical interaction and functional cooperation allow cortisone reduction despite the otherwise oxidative environment in the ER lumen. In agreement with *in vivo* observations, the existence of a dominantly reduced intraluminal pyridine nucleotide pool was reported in the ER[13,14].

The substrate supply for and the specificity of H6PDH are ensured by glucose-6-phosphate transporter (G6PT), an ER membrane protein. In the ER of hepatocytes, adipocytes and neutrophil granulocytes (and possibly a number of other cells), 11βHSD1 can be considered as a component of a complex system, which also includes H6PDH and G6PT (Figure 1).

The stringent cooperation of the members of the G6PT-H6PDH-11βHSD1 system can convert metabolic effects to an endocrine response; thus, the triad can act as a nutrient sensor[15,16]. Intracellular glucose-6-phosphate accumulation can accelerate the concerted action of the G6PT-H6PDH-11βHSD1 triad, which promotes intracellular glucocorticoid activation. Beyond its physiological sensor role, the triad also detects overnutrition. It can participate in the pathomechanism of gluco-, lipo-, and glucolipotoxicity[15,17-20]. Excessive glucose and fatty supply activates the unfolded protein response and induces ER stress by an unknown mechanism; local glucocorticoid activation might represent an alternative signaling pathway[15,16].

As it can be supposed from the variety of symptoms of human metabolic syndrome, the G6PT-H6PDH-11βHSD1 triad present in different cell types and tissues can contribute to the development of this complex disease in various ways and to different extents. The existence of the triad has been proved in hepatocytes[11], neutrophil granulocytes[19] and adipocytes[21] and the system is presumably present also in other cell types. Its exact role in the pathogenesis of the metabolic syndrome has been best clarified in adipose tissue.

TISSUE SPECIFIC EXPRESSION AND COOPERATION OF THE G6PT-H6PDH-11βHSD1 TRIAD

The hepatic and adipose G6PT-H6PDH-11βHSD1 triad plays a crucial role in the pathogenesis of metabolic syndrome (Figure 2). Blood circulation, especially the portal venous system, keeps the triads of different localization connected by transporting glucocorticoid metabolites. Glucocorticoid supply is an important determinant of

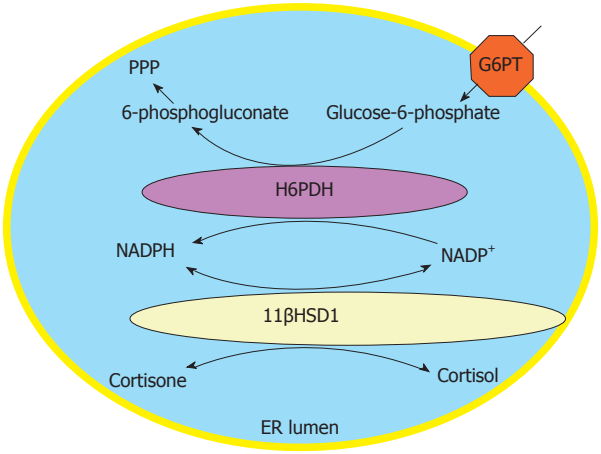

Figure 1 The glucose-6-phosphate transporter - hexose-6-phosphate dehydrogenase - 11β-hydroxysteroid dehydrogenase type 1 triad. Local glucocorticoid activation is catalyzed by a triad of the endoplasmic reticulum, composed by glucose-6-phosphate-transporter, hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase type 1. The role of their cooperation is the enhancement of local glucocorticoid effect. PPP: pentose phosphate pathway; G6PT: Glucose-6-phosphate transporter; H6PDH: Hexose-6-phosphate dehydrogenase; 11βHSD1: 11β-hydroxysteroid dehydrogenase type 1; NADP: Nicotinamide adenine dinucleotide phosphate; ER: Endoplasmic reticulum.

the activity. Glucocorticoids are synthesized *de novo* in the adrenal gland; however, glucocorticoid precursors can also derive from type 2 isoform of 11β-hydroxysteroid dehydrogenase (11βHSD2) activity. 11βHSD2 is present primarily in the kidney[22] and other mineralocorticoid target tissues, such as colon and salivary gland[23,24]. Its physiological role is to prevent the action of glucocorticoids through mineralocorticoid receptor by the conversion of cortisol to inactive cortisone (Figure 2).

It has been recently proved that omental 11βHSD2 activity is also an important substrate supply for hepatic 11βHSD1 activity[25]. Another possible mechanism for the omental cortisone production has been forwarded: 11βHSD1 activity changes during the differentiation of preadipocytes. The existence of 11βHSD1 in adipose tissue was proved more than ten years ago with the conversion of radioactively labeled cortisone to cortisol in abdominal adipose tissue. Both the activity and expression of the enzyme were higher in comparison with subcutaneous adipocytes[26]. Abdominal obesity is known as the Cushing's disease of the omentum[27]. The existence of the G6PT-H6PDH-11βHSD1 system was found in adipocytes as well[21].

However, the enzyme was suggested to function in a bidirectional manner in adipose tissue; the direction is determined by the developing stage of the preadipocyte or adipocyte. While cortisol oxidation dominates in preadipocytes, cortisone reduction is predominant in matured adipocytes. Inactive glucocorticoid metabolites play an important role in the formation and hence in the localization of adipose tissue: they inhibit the development of adipocytes from preadipocytes. If 11βHSD1 acts as a dehydrogenase, it inactivates cortisol and corticosterone, which leads to the inhibition of preadipocyte proliferation[28,29]. When preadipocytes start to differentiate, the reductase activity of the enzyme is progressively increased, which leads to an accelerating cortisol production that drives adipocyte differentiation[30,31,27]. This mechanism leads to the special localization of adipose tissue in Cushing syndrome and in the metabolic syndrome. The omentum contains preadipocytes that only start to differentiate under excessive cortisol effects leading to central or visceral adiposity. Increase in H6PDH expression and its association with the increased 11βHSD1 activity was suggested to be present in the background of adipocyte differentiation[32].

Adipocyte differentiation is enhanced in 11βHSD1 overexpressing mice as well[33]. Direction of enzyme activity depends on the developmental stage, as it was proved in rodent adipocyte cell lines[34,35]. However, recent findings showed that adipose H6PDH activity is constantly high in human adipose-derived mesenchymal stem cells during differentiation, which suggests that other factors can be responsible for the dehydrogenase-to-reductase switch in 11βHSD1 activity[36].

HEPATIC G6PT-H6PDH-11βHSD1 SYSTEM AND THE PATHOGENESIS OF THE METABOLIC SYNDROME

As the main site of lipid and carbohydrate metabolism, the liver has a crucial role in the pathogenesis of the metabolic syndrome. Metabolic effects of glucocorticoids, as well as the alterations caused by excessive blood glucocorticoid concentrations are widely known. However, the local enhancement of glucocorticoid effect in liver and its role in the metabolic syndrome has been recently investigated.

Glucocorticoid effect in the liver

The presence of glucocorticoid receptor and of the above detailed components of the prereceptorial glucocorticoid activating system has been reported in the liver. Besides the adrenal gland, other organs also contribute significantly to cortisol production. It has been recently proved that the main site of splanchnic cortisol production in obese nondiabetic human is the liver[25]. Receptorial glucocorticoid effects are well known in the liver: their main outcome is mobilization of nutrients from the depots supporting the maintenance of normoglycemia in stress and starvation[37]. Stimulation of gluconeogenesis *via* increasing the expression of phosphoenolpyruvate carboxykinase, the rate-limiting enzyme, is one of the most important elements of this action. It also activates glucose production from glycogen *via* induction of glucose-6-phosphatase.

11βHSD1 and its functioning in the liver

11βHSD1 expression and enzyme activity have been described both in rodent and human liver. The first description of 11βHSD1 in human liver showed the highest

enzyme activity around the vena centralis[38]. Direction, rather than localization, of the enzyme activity has a major importance in pathophysiology. Although in isolated and perfused rat liver, both reductase and dehydrogenase activities were described[38,39], in human and rat liver cell cultures, the enzyme was found to be acting exclusively as a reductase. Reductase activity of 11βHSD1 that can be detected in intact cells is responsible for its main pathophysiological effects: decrease in insulin sensitivity and stimulation of hepatic gluconeogenesis *via* enhancement of local glucocorticoid effect. Both transgene 11βHSD1 deficient mice and the selective inhibition of the enzyme[40] proved that decreased glucocorticoid effect caused by the impaired enzyme activity leads to increased insulin sensitivity in hepatocytes.

Hepatic 11βHSD1 in the metabolic syndrome –11βHSD1 transgenic animals

Growing data suggest the role of hepatic 11βHSD1 in the development of abnormalities in carbohydrate and lipid metabolism that occur in the metabolic syndrome. Therefore, the expression and activity of this enzyme are promising therapeutic targets for the future.

The impact of hepatic 11βHSD1 on enhancing local glucocorticoid effect and in the pathogenesis of the metabolic syndrome was proved by using 11βHSD1 knockout mice[41]. 11βHSD1-nul mice develop normally, become fertile, and their blood pressure is in the normal range. After adrenalectomy, the 11βHSD1 knockout mice are unable to convert 11-dehydrocorticosterone to corticosterone. Despite the elevated serum 11-dehydrocorticosterone levels, the intracellular glucocorticoid regeneration is decreased so its antagonism on insulin effect is impaired as well. This is manifested in the impairment of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activities, which leads to a decrease in stress-induced hyperglycemia. The observation that 11βHSD1 knockout mice are resistant to the development of the metabolic syndrome, despite high-fat feeding, is another evidence for the role of the enzyme in the pathogenesis. Although the serum corticosterone level is mildly elevated in these animals, the intracellular level is significantly decreased. Their serum lipid profile shows a cardioprotective pattern: serum triglyceride level is decreased and the serum HDL level is increased[42]. This pattern could be influenced neither by high-fat feeding nor by permanent hyperglycemia induced by chronic stress. Expression of the enzymes of gluconeogenesis is decreased, while expression of the enzymes of lipid peroxidation is highly increased. These data show that 11βHSD1 inactivation protects against the development of the pathophysiological alterations that are responsible for the metabolic syndrome.

The role of 11βHSD1 in the regulation of gluconeogenesis was proved by the selective inhibition of the enzyme as well. The seven-day-long carbenoxolone (non-selective 11βHSD inhibitor) treatment of rodents significantly decreased phosphoenolpyruvate carboxykinase and glucose-6-phosphatase expression at mRNA level in hyperglycemic KKAγ mice strain, which leads to a decrease in the circulating serum insulin level[43]. Selective inhibition of the enzyme leads to increased insulin sensitivity in type 2 diabetes mellitus model mice[44].

In contrast, transgenic mice overexpressing 11βHSD1 selectively in the liver show all symptoms of the metabolic syndrome except for obesity. Transgenic mice overexpressing 11βHSD1 selectively in the adipose tissue show all symptoms of the metabolic syndrome: these animals are obese, have hypertension, insulin resistance and dyslipidemia[33,45]. The corticosterone, serum leptin, tumor necrosis factor-alpha (TNFα) concentrations and lipoprotein lipase mRNA level in their adipose tissue are elevated. Hypertension is due to elevated angiotensinogen level. These data show that overexpression of 11βHSD1 in adipocytes is mainly responsible for the development of the typical obesity in metabolic syndrome, while hepatic 11βHSD1 contributes to the establishment of the metabolic alterations of the disease.

These data suggest, independently from the exact mechanism, that decreased or abolished activity of 11βHSD1 protects against the development of the metabolic syndrome. On the other hand, overexpression of 11βHSD1 is an underlying condition in the pathomechanism of the disease.

Hepatic 11βHSD1 in animal models of type 2 diabetes and hereditary hyperlipidemia

Studies on expression and activity in hereditary hyperlipidemic and diabetic animal models provided important results strongly supporting the contribution of the enzyme in the pathogenesis of the metabolic syndrome. Investigations on other model animals of type 2 diabetes and the hereditary hyperlipidemic model revealed the participation of 11βHSD1 in the pathogenesis, as also observed in transgenic animals.

Experiments on type 2 diabetic Goto-Kakizaki rats helped to clarify the role of 11βHSD1 in the development of the metabolic syndrome. Goto-Kakizaki rats show all of the metabolic, hormonal and vascular abnormalities of human type 2 diabetes. The animals, unlike many other type 2 diabetes models, have a lean phenotype. Hepatic 11βHSD1 expression and enzyme activity are elevated, but expression and activity of the adipose enzyme are decreased[14]. These data suggest that this combination of expression and enzyme activity of 11βHSD1 can lead to and is responsible for the lean phenotype. To the contrary, Zucker fat rats, the obese model of human metabolic syndrome, show an altered pattern of 11βHSD1 activity and expression compared to the control Zucker lean animals and to Goto-Kakizaki rats. In Zucker fat rats, expression of 11βHSD1 is increased in the adipose tissue but decreased in the liver. The reason for this difference in enzyme activities remains unclear: the role of insulin, growth factors and cytokines were proposed but none of them could be proved[46,22].

Besides the liver and adipose tissue, the hippocampus also shows decreased 11βHSD1 activity in obese Zucker rats: this phenomenon correlates to the abnormalities in

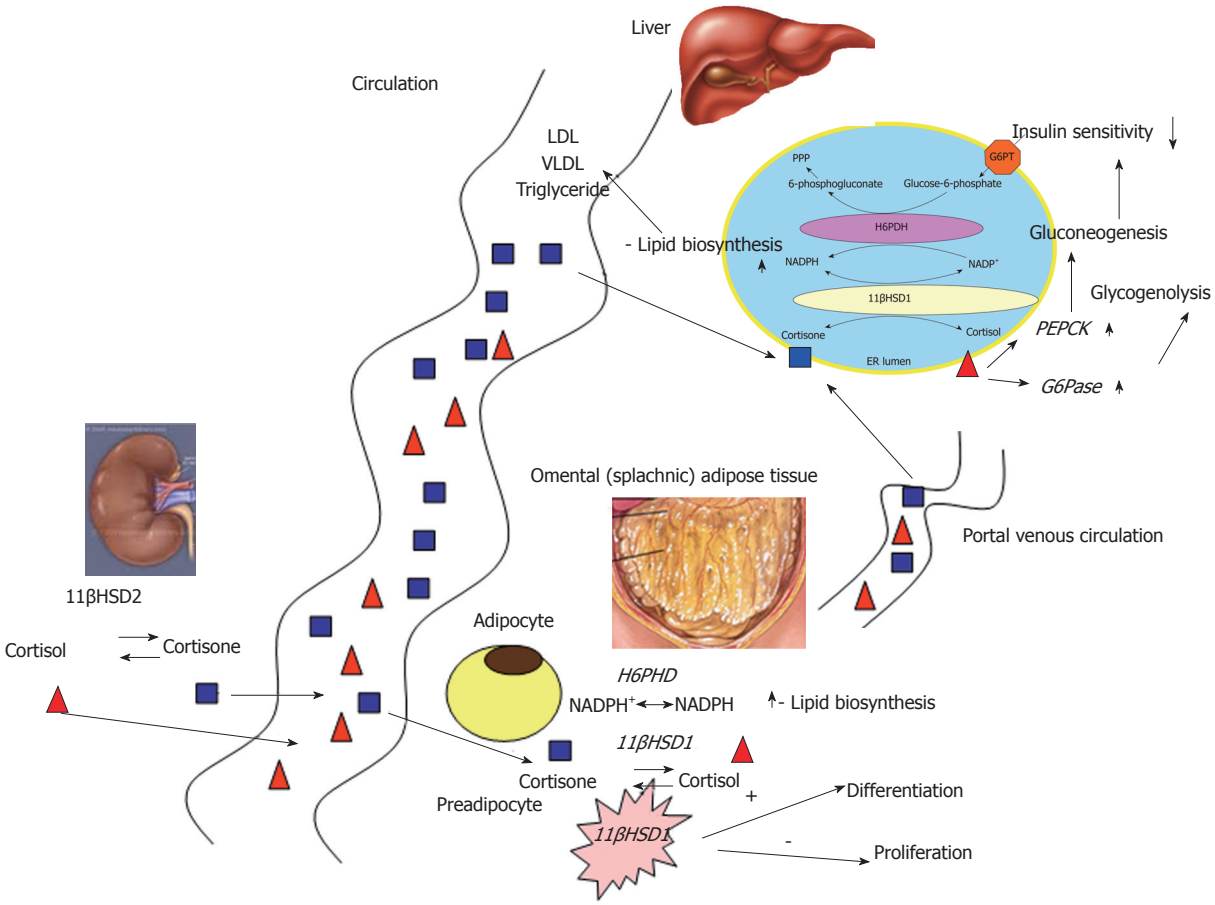

Figure 2 The participation of the glucose-6-phosphate transporter-hexose-6-phosphate dehydrogenase -11β-hydroxysteroid dehydrogenase type 1 system in the pathogenesis of the metabolic syndrome. The different, tissue specific localization of G6PT-H6PDH-11βHSD1 triad contributes to the pathogenesis of the metabolic syndrome both by its effect on local, tissue specific lipid and carbohydrate metabolism and by the systemic connection between the liver and the omental adipose tissue. Renal 11βHSD2 activity plays a crucial role in the maintenance of systemic connection. PPP: pentose phosphate pathway; G6PT: Glucose-6-phosphate transporter; H6PDH: Hexose-6-phosphate dehydrogenase; 11βHSD1: 11β-hydroxysteroid dehydrogenase type 1; NADP: Nicotinamide adenine dinucleotide phosphate; ER: Endoplasmic reticulum; VLDL: Very low density lipoprotein; PEPCK: Phosphoenolpyruvate carboxykinase.

hypothalamus-hypophysis-adrenal axis that is known to have a role in human obesity[47].

Several data from different hyperglycemic and hyperinsulinemic mice strains suggest a possible protective effect of the downregulation of 11βHSD1 against the metabolic syndrome. The expression of hepatic 11βHSD1 is decreased in the leptin deficiency model ob/ob mice[48]. However, in type 2 diabetes model db/db mice, hepatic 11βHSD1 activity is elevated. This is followed by enhanced glucocorticoid receptor expression, elevated phosphoenolpyruvate carboxykinase level and increased insulin sensitivity[49]. In the KKAγ polygenic type 2 diabetes model, adult animals have hyperglycemia, hyperinsulinemia and glucose intolerance. Its hepatic 11βHSD1 expression and activity are decreased compared to the non-diabetic control. In a polygenic metabolic syndrome animal model[50], the body fat content of the "fat" animals was 21%, while in the lean animals it was 4%. The fat animals developed the metabolic syndrome with insulin resistance, fatty degeneration of the liver and hypertension. In fat animals, the expression of adipose 11βHSD1 and serum glucocorticoid levels are impaired, but the expression of the hepatic enzyme is increased. Despite a high-fat diet, the metabolic syndrome does not develop in lean animals. Hereditary hypertriglyceridemic Prague rats show elevated 11βHSD1 activity. Lipid content of the liver is highly elevated compared to normal control. 11βHSD1 inhibition seems to have no effect on hepatic lipid content[51]. Inhibition of 11βHSD1 in diet-induced obesity model rat leads to reduced hepatic very low density lipoprotein secretion, which improves hypertriglyceridemia[52]. Carbenoxolone treatment of hereditary hypertriglyceridemic rat strain shows the same alteration in lipid metabolism[53].

These data show that the changes of hepatic 11βHSD1 activity in obese animals may be a protective mechanism against the development of metabolic abnormalities that lead to type 2 diabetes. Downregulation of hepatic 11βHSD1 in obese rats is due to elevated serum glucocorticoid levels rather than to insulin resistance alone; therefore, it cannot be prevented by oral antidiabetic drugs (thiazolidinediones or metformin) in Zucker rats[46]. Nevertheless, the exact underlying mechanisms remain to be elucidated. It is also clarified in type 2 diabe-

tes and metabolic syndrome model animals that hepatic 11βHSD1 enzyme is mainly responsible for the metabolic abnormalities (insulin resistance, serum lipid profile alterations *etc*), while the adipose enzyme is responsible for the phenotypical alterations (abdominal obesity) characteristic to the disease.

Human hepatic 11βHSD1 in the metabolic syndrome

The role of 11βHSD1 in human metabolic syndrome is uncertain in many points and controversial data appear in the literature[54]. Serum glucocorticoid levels in patients with metabolic syndrome are in normal range or only mildly elevated, which supports the role of local enhancement of glucocorticoid action by 11βHSD1. In human individuals, the whole body 11βHSD1 activity can only be monitored indirectly and noninvasively, by the measurement of 24 h urine tetrahydrocortisol and tetrahydrocortisone levels and cortisone/cortisol ratio. Many authors reported correlation between body mass index and 11βHSD1 activity: some of them found a positive[55-57], while others reported a negative correlation[58]. Some authors did not find correlation between BMI and enzyme activity[59-61]. However, increased urinary cortisone/cortisol ratio was found in women with increased abdominal fat compared to peripheral distribution of adipose tissue[62].

The role of different tissue specific 11βHSD1 enzymes was investigated in the metabolic syndrome: just like the non-human models, the hepatic and adipose tissue enzymes are the most important. Ethical burdens limit the possibilities of measuring different tissue-specific 11βHSD1 enzymes in human individuals: adipose enzyme can be examined *via* fat biopsies of the subcutaneous fat depot, but human studies based on sample taking from the omental tissue, as well as liver biopsies, are rarely available and do not provide sufficient data.

The activity of hepatic 11βHSD1 negatively correlates to body mass index in human individuals. Impairment of enzyme activity leads to decreased hepatic glucose production and increased insulin sensitivity[55]. It has been recently proved that hepatic 11βHSD1 activity negatively correlates with abdominal adipose tissue area, and the expression positively correlates with phosphoenolpyruvate carboxykinase expression[63]. The lack of impairment of hepatic 11βHSD1 activity in type 2 diabetic obese individuals[64] is an intriguing observation. The absence of hepatic 11βHSD1 downregulation in obese diabetics emphasizes its possible role in pathogenesis. This raises the hypothesis that inhibition of 11βHSD1 in obese people who develop impaired glucose tolerance could protect from progression to type 2 diabetes.

Regulation of 11βHSD1 in liver

Many factors can influence hepatic 11βHSD1 activity. In rodent liver, estrogen and insulin decrease the activity of the enzyme[65,66]. Growth factors (TGFβ, bFGF, EGF, HGF) seem to be ineffective[38]. Reductase activity of the enzyme was inhibited by insulin and IGF-1, while increased by dexamethasone in a rat hepatoma cell line[67]. The stimulating effect of interleukin (IL)-1, IL-2, IL-5, IL-6, IL-13, leptin, estradiol and gonadotropins on 11βHSD1 activity was proved as well[54].

In the human hepatoma cell line, TNFα, IL-1β increases and the clinically used oral antidiabetic PPARγ agonist significantly decreases the transcriptional activity of hepatic 11βHSD1 gene[68]. This phenomenon was further supported by studies on transgenic mice overexpressing TNFα: 11βHSD1 mRNA level and activity are elevated in their livers[69].

CEBPα transcription factor regulates hepatic 11βHSD1[70]. The exact mechanism of this regulatory process was recently revealed: TNFα-induced transcription of *11βHSD1* gene acts *via* the p38 MAPK pathway in HepG2 cell line[69]. Besides CEBPα, CEBPβ has an important role in the control of basal 11βHSD1 transcription too. Thyroxin influences both the transcription and the activity of the enzyme[71,72]. Metyrapone inhibits 11βHSD1 activity in sheep liver[73]. The mechanism underlying the increase of 11βHSD1 activity in liver cirrhosis remains unclear [74].

The exact roles of these numerous factors having impact on 11βHSD1 activity in cell lines and different animal models have not been totally clarified in the pathogenesis of metabolic syndrome. Some of them provide a possible new therapeutic option in the future or help to understand the exact mechanism of clinically used antidiabetic and antihyperlipidemic drugs.

Options for therapeutic intervention at hepatic 11βHSD1

Carbenoxolone is the most widely examined non-selective inhibitor of 11βHSD. Results on animal models and human individuals proved its positive effect on metabolic alterations: it increases insulin sensitivity, decreases hepatic gluconeogenesis, serum cholesterol and improves triglyceride profile[75-78].

Although human data are not available yet, inhibition of hepatic 11βHSD1 with newly developed targeted drugs (compound 544 Merck, BVT2733 biovitrum) seems to be effective in animal models: both compounds increase hepatic insulin sensitivity and decrease serum triglyceride and cholesterol levels. Besides this effect, compound 544 Merck decreases fasting glucose level, food intake and adipose tissue mass[79]. BVT2733 seems to be effective in lowering hepatic gluconeogenesis and plasma glucose and insulin levels[80,43,44,54]. These compounds have been applied mainly in the preclinical phase or human phase 1 drug development studies, so little data are available about their efficacy and safety. Their possible clinical application is now a future goal in the pharmaceutical industry.

H6PDH

The exact role of H6PDH in obesity and metabolic syndrome is not fully elucidated. It is known that diet and macronutrient composition can influence glucocorticoid metabolism, which might be due to the impact of carbohydrate intake on the activity of the pentose phosphate

pathway. Sucrose ingestion increases both 11βHSD1 and H6PDH mRNA levels in mesenteric fat, while it decreases 11βHSD1 and increases H6PDH mRNA levels in liver. These observations support the hypothesis that increased 11βHSD1 activity in the adipose tissue contributes to sucrose-induced obesity[81].

The symptoms observed in H6PDH knockout mice and a decreased negative feedback on the hypothalamic–pituitary–adrenal axis show the importance of H6PDH in the regulation of 11βHSD1 as well as in the pathogenesis of the disease[82,83]. As a consequence of diminished H6PDH activity, 11βHSD1 enzyme activity switches from a reductase to a dehydrogenase in the livers of H6PDH knockout mice, which leads to glucocorticoid inactivation. Since 11βHSD1 activity greatly influences hepatic glucose output, this switch causes many alterations in glucose homeostasis of H6PDH knockout mice. Compared to wild-type mice, H6PDH knockout animals have a reduced weight gain, a peripheral fasting hypoglycemia, an improved glucose tolerance, and elevated plasma corticosterone concentrations. Both fed and fasted H6PDH knockout mice have normal plasma insulin levels; however, insulin and plasma glucose levels are reduced 4 h after fasted animals are refed, indicating an improved insulin sensitivity. There is a preserved induction and activity of the glucocorticoid-responsive gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the livers of fasted H6PDH knockout mice. Glycogen storage is elevated in the liver of fed H6PDH knockout mice, with enhanced glycogenesis. These data suggest a partial retention of glucocorticoid sensitivity in the liver[84].

It can be hypothesized that the switch in 11βHSD1 activity from reductase to dehydrogenase caused by the lack of H6PDH activity leads to a protection against type 2 diabetes at high-fat feeding. This mechanism might be responsible for the above-mentioned metabolic alterations in H6PDH knockout animal.

G6PT

11βHSD1 activity is significantly reduced in G6PT-deficiency (GSD1b) while remarkably increased in glucose-6-phosphatase deficiency (GSD1a), which strongly supports the contribution of G6PT to metabolic sensing[85]. Inhibition of G6PT with chlorogenic acid leads to decreased cortisol production in the liver, *via* the change of cofactor supply of 11βHSD1[11]. These data suggest that pharmacological inhibition of G6PT can be a hopeful therapeutic option in the metabolic syndrome and in type 2 diabetes.

CONCLUSION

The hepatic G6PT-H6PDH-11βHSD1 triad plays a crucial role in the pathogenesis of the metabolic syndrome. The system integrates the metabolic and redox homeostasis of the cell with a prereceptorial hormone activation. Metabolic alterations characteristic to the disease, especially those of carbohydrate and lipid metabolism, are at least partly due to derangements of this metabolic sensor. The growing experimental data obtained from pharmacological or genetic manipulations with the components of the triad collectively indicate that the triad is a hopeful therapeutic target in the prevention and/or treatment of the metabolic syndrome.

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ORIGINAL ARTICLE

Identification of cellular genes showing differential expression associated with hepatitis B virus infection

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Abstract

AIM: To investigate the impact of hepatitis B virus (HBV) infection on cellular gene expression, by conducting both *in vitro* and *in vivo* studies.

METHODS: Knockdown of HBV was targeted by stable expression of short hairpin RNA (shRNA) in huH-1 cells. Cellular gene expression was compared using a human 30K cDNA microarray in the cells and quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) (qRT-PCR) in the cells, hepatocellular carcinoma (HCC) and surrounding non-cancerous liver tissues (SL).

RESULTS: The expressions of HBsAg and HBx protein were markedly suppressed in the cells and in HBx transgenic mouse liver, respectively, after introduction of shRNA. Of the 30K genes studied, 135 and 103 genes were identified as being down- and up-regulated, respectively, by at least twofold in the knockdown cells. Functional annotation revealed that 85 and 62 genes were classified into four up-regulated and five down-regulated functional categories, respectively. When gene expression levels were compared between HCC and SL, eight candidate genes that were confirmed to be up- or down-regulated in the knockdown cells by both microarray and qRT-PCR analyses were not expressed as expected from HBV reduction in HCC, but had similar expression patterns in HBV- and hepatitis C virus-associated cases. In contrast, among the eight genes, only *APM2* was constantly repressed in HBV non-associated tissues irrespective of HCC or SL.

CONCLUSION: The signature of cellular gene expression should provide new information regarding the pathophysiological mechanisms of persistent hepatitis and hepatocarcinogenesis that are associated with HBV infection.

Key words: Hepatitis B virus; Differential gene expression; Hepatocellular carcinoma; Gene expression signature; Adipose most abundant 2

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INTRODUCTION

Hepatitis B virus (HBV) is a major causative agent of chronic liver diseases that lead to the development of hepatocellular carcinoma (HCC) worldwide[1]. Vaccination against HBV has been proven efficacious for the prevention of virus transmission and has markedly reduced the carrier rate[2]. Several anti-viral agents, such as entecavir and interferon-α, also exert therapeutic effects by reducing virus titer[3]. Unfortunately, these preventative and therapeutic treatments are not widely administered, especially in areas with HBV epidemics[4]. Small molecule therapies and interferon treatments suffer from a number of drawbacks, including the selection of drug-resistant mutants, toxicity and limited efficacy[5,6]. Furthermore, the increase in international travel has introduced different genotypes of HBV to the world's populations, which may not be efficiently protected by the vaccine developed against the endemic genotype[7]. The World Health Organization reported that an estimated 350 million people worldwide are chronically infected with HBV and a significant proportion of chronic infections terminate in HCC, leading to more than half a million deaths annually (http://www.who.int/mediacentre/factsheets/fs204/en/). Thus, the need to elucidate the detailed pathophysiology of HBV infection is great.

Recent advancements in technology allow us to evaluate the proteome or transcriptome during pathogenic processes, providing new insight in terms of host-pathogen interactions. So far, *in vivo* cellular reactions associated with HBV infection have mainly been evaluated by comparing HBV-associated HCC [HCC(B)] with other liver tissues. Kim *et al*[8] reported a characteristic protein profile of HCC(B) in comparison with hepatitis C virus (HCV)-associated HCC [HCC(C)]. Differential gene expression profiles have also been reported in HCC(B) in comparison with corresponding surrounding liver tissues (SL)[9] or HCC(C)[10]. Although reduced tumorigenicity after knockdown of HBx protein has been reported in PLC/PRF/5 HCC cells[11], it is unclear whether HBV still has significant effects on cellular gene expression once the cells have been transformed because, at the time of HCC development, tumor cells no longer allow efficient viral expression[12,13]. In addition, it is reasonable to assume that malignant transformation causes a significant alteration of the gene expression signature and may overcome the impact of HBV on the profile. Thus, a simple HCC-oriented observation may not accurately reflect the cellular events induced by HBV infection.

Artificial control of HBV expression is another approach to studying differential cellular gene expression. Otsuka *et al*[14] reported that, in comparison with parental cells, several cellular genes were specifically up- or down-regulated in HepG2.2.15 cells, which are derived from HepG2 cells by transfecting them with plasmids containing HBV DNA, leading to the production of HBV proteins. Alteration of cellular gene expression has also been reported in HepG2.2.15 cells after knockdown of HBV through RNA interference (RNAi)[15]. Furthermore, microarray analysis has revealed differential cellular gene expression between wild-type and HBV transgenic mouse livers[16,17]. There are concerns, however, that the methodologies employed may have direct effects on cellular gene expression. There are inconsistencies in the genes that have been reported to be altered as a result of HBV infection, not only among studies using different models of HBV infection, but also using the same methodologies[18].

In this report, we elucidate the differentially expressed cellular genes associated with HBV infection by sequentially applying two processes: (1) selection of candidate genes by knockdown of HBV expression using RNAi in cells derived from a HBV-associated case; and (2) quantification of the selected gene expression in various liver tissues from both HBV-infected and non-infected patients. The advantage of our approach and the pathophysiological implications of our results are discussed.

MATERIALS AND METHODS

Design and construction of shRNA

Seventeen HBV genome sequences from GenBank were aligned and analyzed to identify the conserved regions containing at least nineteen contiguous nucleotides spanning within the region that was shared by all four open reading frames. Nineteen nucleotides following AA were common for all genotypes except for F and H, which are quite rare in Japan, and were further analyzed by BLAST to ensure that the sequence does not have significant homology with known human genes. Finally, the selected sequence, 5'-TGTCAACGACCGACCTTGA-3', was designed to form a hairpin structure when transcribed and cloned into pSUPER.retro (OligoEngine, WA, United States), which generates 3'-UU overhanging transcripts without a poly-A tail under the control of the polymerase-III H1-RNA gene promoter. Plasmids containing the target sequence or the same sequence with an A to G transition at the ninth nucleotide were designated pSUPER.HB4 or pSUPER.HB4G, respectively.

Cell culture and transfection

huH-1 cells; JCRB0199, were obtained from the JCRB cell repository and transfected with 2 μg of plasmids using Effectene transfection reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In brief, 5.0×10^5 huH-1 cells were seeded into a 60 mm dish a day before transfection and the plasmids were mixed with 16 μL of enhancer followed by the addition of 50 μL Effectene reagents. After mixing with medium, the mixture was applied onto the culture plate. The stable transformants with pSUPER.retro, pSUPER.HB4 and pSUPER.HB4G were named huHpS, huHB4 and huHB4G, respectively.

Evaluation of HBsAg and HBx expression

The cells of 1×10^6/mL were subjected to culture and supernatants were collected on days 2 and 4 and stored at -20 ℃ until use. HBsAg was quantified in the medium by

a chemiluminescence immunoassay using ARCHITECT HBsAg QT (Abbott Japan Co. Ltd., Chiba, Japan).

Hydrodynamic gene delivery was employed[19] to target HBx in HBx transgenic mice[20] by injecting 100 μg of pSUPER.retro, pSUPER.HB4 or pSUPER.HB4G. Immunohistochemistry was performed on the liver specimens, which were obtained 48 hours after delivery, by a standard avidin-biotin complex method[21] that involved incubating the sections with primary antibodies of rabbit polyclonal anti-HBx[22].

cDNA microarray analysis

Total RNA was isolated from huHpS and huHB4 cells using IsoGen (Nippon Gene Co. Ltd., Tokyo, Japan) and stored at -80 ℃. After amplification by T7 polymerase, cDNAs were labeled with the fluorescent dyes cy3 or cy5, and hybridized with AceGene human 30K cDNA microarrays (DNA Chip Research Inc. Yokohama, Japan), which contain 32 000 sequences verified by the mouse IMAGE consortium (http://image.hudsonalpha.org/). After washing, the arrays were scanned and the signal intensity of a spot was considered significant if the intensity was 200 times greater than the background; otherwise, the spot was flagged as "not found". Using TIGR MIDAS software (http://www.tm4.org/), the data obtained from qualified spots were normalized by applying the LOWESS algorithm. After normalization, the spots were judged to be inconsistent between a pair of flip-dye replicates and filtered out, if $\log_2(cy3/cy5)/\log_2(cy5/cy3)$ was outside the range from -1 to 1. We took a difference of spot intensities between huHB4 and huHpS of more than double or less than half as significant. The selected genes were fed to the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/home.jsp).

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNAs of huH-1 cells and liver tissues were purified with RNeasy Mini kits (QIAGEN KK, Tokyo, Japan) after digestion with RNase-free DNase I (Invitrogen Corporation, Carlsbad, United States) and were reverse transcribed for use in TaqMan Gene Expression Assays using a LightCycler (Roche Diagnostics, Mannheim, Germany). To quantify the expression of the candidate genes selected in microarray analyses and of the internal controls, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and beta 2-microglobulin (*β2M*), commercially available primer and probe sets were employed, whereas custom primers, 5'-CCCGTCTGTGCCTTCTCA-3' and 5'-GGTCGGTCGTTGACATTGCT-3', and a probe, 5'-CCGTGTGCACTTCGCT-3', sequences that are common to all four open reading frames, were designed by Custom TaqMan® Gene Expression Assay (Applied Biosystems Inc., Foster City, United States) for HBV expression. The results were analyzed using LightCycler software (Roche Diagnostics, Mannheim, Germany). A relative fluorescent intensity was calculated from a standard curve obtained by quantitative analyses using a serial dilution of total RNA from HepG2. Finally, a relative amount of each message was calculated as a ratio of threshold cycle after normalization against *HPRT1* or *β2M*.

Liver tissue samples

Liver tissue samples were obtained from surgical resections of HCC or other cancers from eighteen patients consisting of five HBV-positive cases, five HCV-positive cases, three neither HBV nor HCV positive cases, and five cases without chronic liver disease, which included one colon cancer, one common bile duct cancer and three rectal cancer cases. The three patients without viral hepatitis had autoimmune hepatitis, primary biliary cirrhosis and alcoholic liver cirrhosis. No cases showed positive reactions to HBsAg or anti-HBc, except for the HBV-positive cases. Two expert pathologists independently evaluated liver specimens. Each HBV-positive or HCV-positive group consisted of two chronic hepatitis cases and three cirrhotic cases. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki, as reflected in prior approval by the Niigata University Graduate School of Medical and Dental Sciences Human Research Committee.

Statistical analysis

Doubling times of the cells and quantity of HBsAg in cultured medium were compared using ANOVA analysis followed by post hoc Bonferroni's multiple comparison tests, whereas the Mann-Whitney test was employed to compare HBV expression between HCC(B) and SL(B). In the DAVID annotation system, Fisher's exact test was adopted to measure the gene enrichment in annotation terms by referencing the frequencies of 30 000 genes of the human genome. All analyses except for the functional annotation were performed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, United States) and a two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

Stable expression of shRNA is effective for knockdown of various HBV transcripts

We cloned huHB4G and huHB4, in which shRNA for the HBx coding region is continuously expressed in huH-1 cells with and without nucleotide replacement at the center from A to G, respectively. The cells transfected with plasmids with no insert were referred to as huHpS.

Morphologically, no remarkable differences were observed among the clones in culture, as shown in Figure 1A. The average doubling times in three independent cultures of huHpS, huHB4G and huHB4 were 40.9 ± 1.8 h, 46.8 ± 2.6 h and 45.1 ± 1.2 h, respectively, and were not significantly different between huHpS and huHB4, but were different between huHpS and huHB4G (P = 0.022). To evaluate the efficacy of the shRNA, HBsAg was quantified in the culture medium. Its concentrations

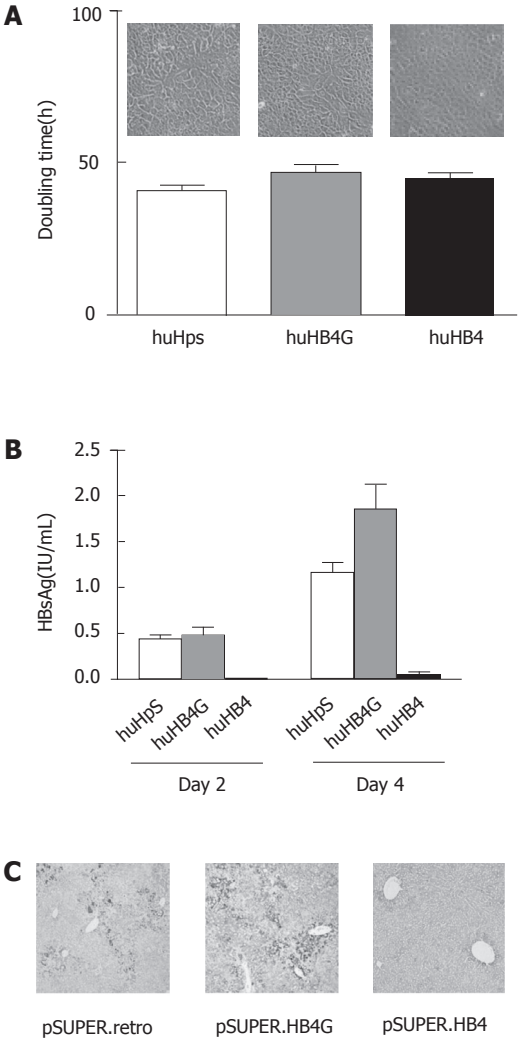

Figure 1 Effects of short hairpin RNA targeting hepatitis B virus on huH-1 cells. A sequence of nineteen nucleotides that is shared by all four open reading frames of HBV was cloned into pSUPER.retro and named pSUPER.HB4, which was further mutated at the ninth A to G and designated pSUPER.HB4G. Stable transformants of each plasmid in huH-1 cells were established as huHpS, huHB4 and huHB4G, respectively. A: Representative morphologies (upper panel) and average doubling times (lower graph) of each transformant. Each stable transformant was maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and microscopically observed after 48 h (original magnification: 10x). The cells were counted on days 2 and 4 to calculate the average doubling times of each cell line from three independent cultures (40.9 ± 1.8 h, 46.8 ± 2.6 h and 45.1 ± 1.2 h in huHpS, huHB4G, and huHB4, respectively). The observed doubling times were not significantly different between huHpS and huHB4 but were different between huHpS and huHB4G (P = 0.022); B: The concentrations of HBsAg in the culture medium from huHpS, huHB4G, and huHB4 were 0.44 ± 0.046 IU/mL, 0.48 ± 0.091 IU/mL and 0.010 ± 0.0016 IU/mL on day 2 and 1.16 ± 0.11, 1.86 ± 0.26, and 0.050 ± 0.036 IU/mL on day 4, respectively. On both days, HBsAg was significantly reduced in huHB4 compared with the other two clones, but it was increased in huHB4G compared with huHpS (P = 0.0001 and P < 0.0001, respectively). C: Immunohistochemistry for HBx protein in HBx transgenic mouse liver 48 h after hydrodynamic gene delivery of pSUPER.retro, pSUPER.HB4G or pSUPER.HB4. The positive signals were remarkably reduced in mice that received pSUPER.HB4 compared with mice that received the other vectors, but were rather overrepresented in pSUPER.HB4G compared with pSUPER.retro (original magnification: 4x).

were 0.44 ± 0.046 IU/mL, 0.48 ± 0.091 IU/mL and 0.010 ± 0.0016 IU/mL on the second day and 1.16 ± 0.11 IU/mL 1.86 ± 0.26 IU/mL and 0.050 ± 0.036 IU/mL on the fourth day, respectively (Figure 1B). On both days 2 and 4, HBsAg was significantly reduced in huHB4 compared with the other two clones, but was increased in huHB4G compared to huHpS (P = 0.0001 and P < 0.0001, respectively).

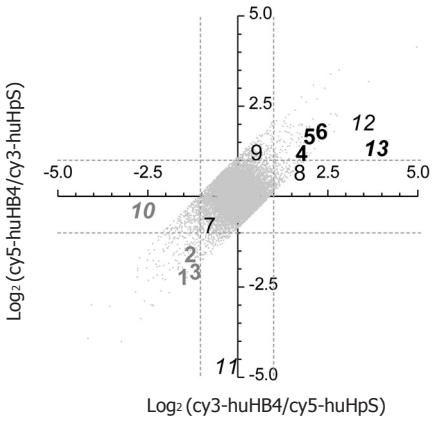

Figure 2 Log2 plot of flip-dye conversion in microarray analysis between huHpS and huHB4. Total RNAs from huHpS and huHB4 were labeled with cy3/cy5, or vice versa, and subjected to a human 30 K cDNA microarray. Log2 ratios of the intensities in each labeling combination were plotted after LOWESS normalization as long as signal intensities were consistent between dye-flipping with less than a twofold difference. In total, 13 012 spots were plotted, and the dotted lines indicate a log2 intensity ratio of 1 or -1. Numbers 1 to 13 indicate the spots that were randomly selected for further quantitative reverse transcription-polymerase chain reaction evaluation. Bold gray and black numbers represent the spots that were found to show a twofold higher or lower intensity in huHB4 compared with huHpS by both microarray and quantitative RT-PCR analyses. Italicized numbers indicate the flip-inconsistent spots. RT-PCR: Real-time reverse transcription-polymerase chain reaction.

Next, the plasmids were delivered to hepatocytes in HBx transgenic mice using hydrodynamic gene delivery. After 48 h, the mouse livers were harvested and an immunohistochemical analysis was performed for HBx protein. As shown in Figure 1C, the positive signals were remarkably reduced in mice that received pSUPER.HB4 compared with other mice and were rather overrepresented in pSUPER.HB4G compared to pSUPER.retro.

Cellular gene expression was affected by knockdown of HBV messages

Because pSUPER.HB4G is suggested to have the potential enhancing HBV expression, gene expression signatures were compared between huHB4 and huHpS using a human cDNA microarray. Of 29 953 effective spots, LOWESS normalization validated 19 923 and 20 926 signals in each dye-sample combination, leading to final validation of 18 288 spots for both dye combinations. Additionally, 5276 signals were eliminated based on a flip-dye inconsistency greater than twofold, resulting in 13 012 genes available for further evaluation (Figure 2). Among those, 145 and 103 genes were down- and up-regulated, respectively, by at least twofold in huHB4 cells compared to huHpS cells (Supplementary Table 1, Supplementary material online). In order to exclude genes

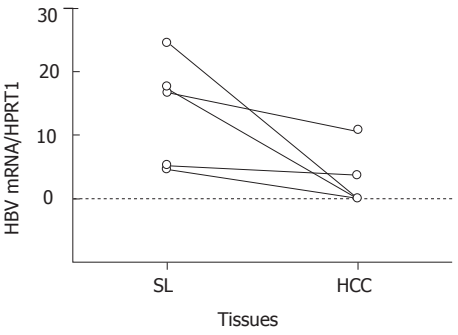

Figure 3 Quantity of hepatitis B virus mRNA in hepatocellular carcinoma and surrounding liver tissues. The total amount of hepatitis B virus (HBV) mRNA was quantified as a ratio relative to hypoxanthine phosphoribosyltransferase 1 message in hepatocellular carcinoma (HCC) and surrounding noncancerous liver tissues (SL) from five HBV-associated cases. In all cases, the message was repressed in HCC, and the average was significantly reduced from 13.7 ± 8.6 to 2.9 ± 4.7 (P = 0.032).

that might be repressed by off-targeting effects, the 13 012 genes were evaluated using a siRNA seed locator (http://www.dharmacon.com/seedlocator/default.aspx). A total of 1063 genes were found to have at least one match with our shRNA target sequence and ten genes were included in the 145 repressed genes. Thus, 135 repressed and 103 enhanced genes were selected as final candidates.

To confirm the microarray results, thirteen genes were randomly selected from the first and third quadrants of the $\log_2$(cy3-huHB4/cy5-huHpS)-$\log_2$(cy5-huHB4/cy3-huHpS) plot for the 18 288 LOWESS-validated genes and subjected to quantified by real-time reverse transcription-polymerase chain reaction (qRT-PCR) (Table 1). Of the thirteen genes, nine genes were chosen from the 13 012 flip-consistent genes, whereas the other four genes were selected from the 5276 flip-inconsistent genes. The flip-consistent genes were further divided into three groups of three genes each according to their intensity ratio of huHB4/huHpS: (1) less than 0.5 in both dye combinations; (2) more than 2 in both dye combinations; or (3) between 0.5 and 2 in at least one combination. In all flip-consistent genes, the relative quantities were not remarkably different between the two references of *HPRT1* and *β2M*, and gene expression patterns were quite similar between microarray and qRT-PCR analyses. On the other hand, the four flip-inconsistent genes showed various expression patterns in qRT-PCR analysis. Two genes were found to express less or more than twofold in huHB4, whereas the expression differences were approximately twofold for the other two genes. These results suggest that the final 238 candidate genes are highly likely to have altered gene expression of a more than twofold magnitude in both microarray and qRT-PCR analyses.

To mine functional annotation, a list of the candidate genes was uploaded to DAVID program. Of 238 genes, 62 up- and 85 down-regulated genes were annotated and identified as being enriched into four and five representative functional categories, respectively, based on the controlled vocabulary of the Gene Ontology Consortium, as shown in Table 2. The up-regulated genes were classified into groups of lipid synthesis, glycoprotein, biopolymer metabolism and hydrolase activity. Genes that function as signal peptides, protease inhibitors and cytokines and in sensory perception and transport were significantly enriched in the down-regulated genes.

Table 1 Microarray and quantitative reverse transcription-polymerase chain reaction for candidate genes

No.	Genes	Microarray[3]		qRT-PCR[4]		Accession number[5]
		cy3/cy5	cy5/cy3	/β2M	/HPRT1	
1	*CSTA*[1]	0.35	0.26	0.23	0.23	NM_005213
2	*APM2*[1]	0.46	0.31	0.22	0.23	NM_006829
3	*SLPI*[1]	0.47	0.31	0.08	0.09	NM_003064
4	*CTGF*[1]	3.36	2.026	3.49	3.93	NM_001901
5	*NADE*[1]	3.89	2.85	3.99	4.48	AF187064
6	*TTR*[1]	5.13	3.07	4.6	5.3	NM_000371
7	*ARL3*	0.59	0.52	0.54	0.62	NM_004311
8	*GCNT3*	3.2	1.97	1.74	1.95	NM_004751
9	*NRF-1*	1.42	2.16	0.98	1.12	L22454
10	*KIAA1808*[1,2]	0.16	0.85	0.34	0.4	AB058711
11	*HSPC159*[2]	0.9	0.04	0.66	0.77	NM_014181
12	*MAP2K6*[2]	11.08	3.76	1.68	1.89	NM_002758
13	*SKAP2*[1,2]	13.45	2.33	2.03	2.34	NM_003930

[1]huHpS > 2x huHB4 or 2x huHpS < huHB4, respectively, in both microarray and qRT-PCR; [2]flip-inconsistent spots; [3]a signal intensity ratio of huHB4/huHpS; [4]relative expression in huHB4 against in huHpS normalized by beta 2-microglobin (*β2M*) or hypoxanthine phosphoribosyl transferase 1 (*HPRT1*); [5]GenBank accession number. qRT-PCR: Quantified by real-time reverse transcription-polymerase chain reaction.

Evaluation of candidate gene expression in livers with various disorders

The differential expression in microarray analysis was validated by qRT-PCR using HCC and SL tissues from eighteen livers. Expression levels of the eight genes, which are listed as genes 1 to 6, 10 and 13 in Table 1, were quantified. Four genes were suppressed and the other four genes were enhanced more than twofold in huHB4. In HBV-associated tissues, HBV expression was also quantified. As shown in Figure 3, the average relative HBV expressions were 2.9 ± 4.7 and 13.7 ± 8.6 in HCC(B) and SL(B), respectively, and were a significantly decreased in HCC(B) (P = 0.032).

As shown in Figure 4A, all $\log_2$ ratios of SL(C), SL(NBNC) and SL(N) to SL(B) were distributed between -1 and 1, except for *CSTA*, *APM2*, *CTGF* and *TTR*, which showed expressions in SL(B) at magnitudes of 7.7-, 2.5-, 2.6- and 2.3-fold in comparison with SL(C), SL(C), SL(N) and SL(C), respectively. The reduced expression patterns in liver tissues without HBV infection are consistent with the results in huHB4 only for *CSTA* and *APM2*. In comparisons of HCC(C) and HCC(NBNC) with HCC(B), several genes showed expression differences of more than twofold that were consistent with differential expression in huHB4 (Figure 4B). Of the four genes that had low expression in huHB4, *APM2* also had low expression in both HCC(C) and HCC(NBNC), with magnitudes of 0.074 and 0.11, respectively. *SLPI* and

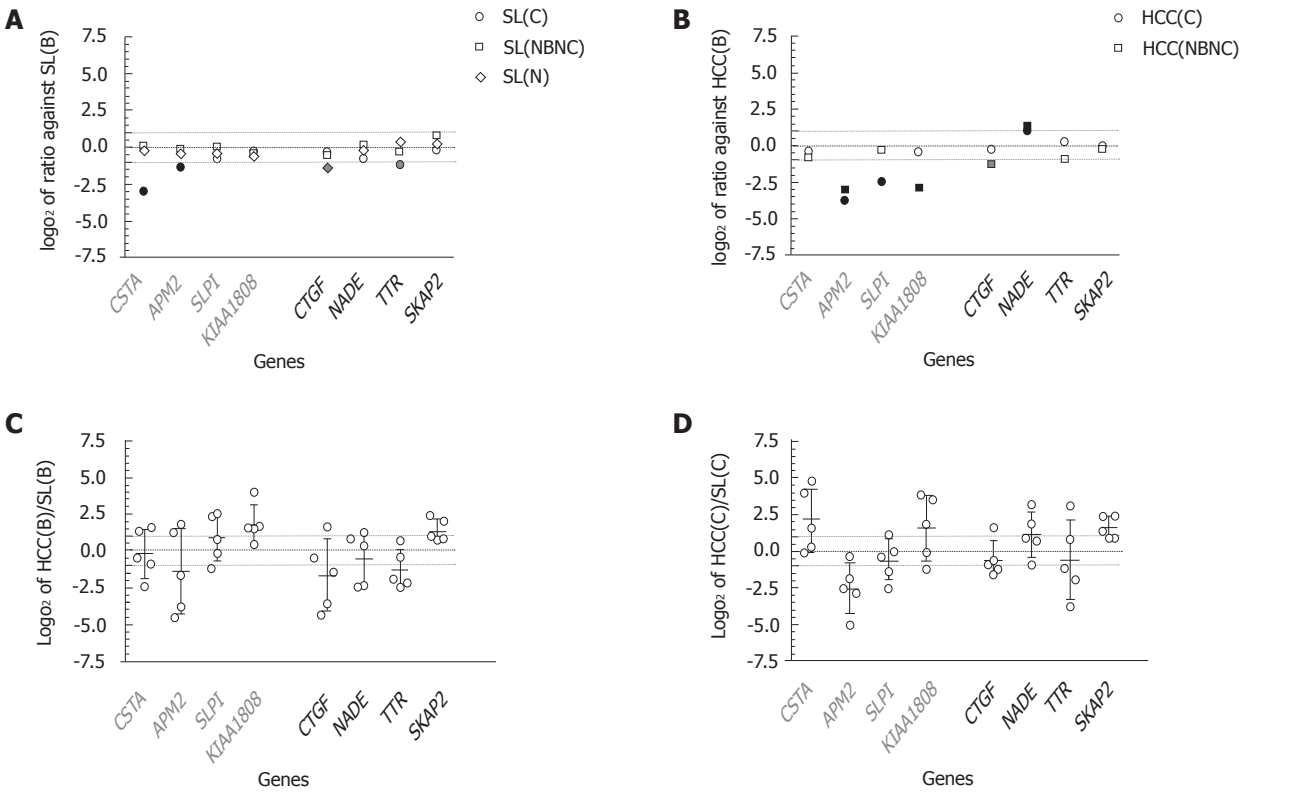

Figure 4 Expression profiles in various liver tissues with respect to genes differentially expressed in huHB4. Quantitative RT-PCR was performed using various liver tissues for eight genes that were confirmed to be differentially expressed with HBV knockdown in huH-1 cells by both microarray and quantitative RT-PCR. The genes included *CSTA*, *APM2*, *SLPI* and *KIA1808*, which are down-regulated, and *CTGF*, *NADE*, *TTR* and *SKAP2*, which are up-regulated in huHB4 knockdown cells. Relative amounts of each message are plotted as log2 ratios, and the dotted gray lines indicate 1 and -1 log2 ratios. Liver tissues include five HBV infected cases (B), five HCV infected cases (C), three neither HBV nor HCV infected cases (NBNC) and five cases without chronic liver diseases (N). A: **Comparison between** surrounding non-cancerous liver tissues (SL). The combinations include comparisons of SL(B) to SL(C), **SL(NBNC) and SL(N); B: Comparisons between HCC. The** combinations include comparisons of HCC(B) to HCC(C) and to HCC(NBNC). In A and B, the results are plotted in white if the ratio was between 1 and -1, whereas black and gray marks indicate consistency and inconsistency between the results from liver tissues and huHB4, respectively. C, **D: Log2 ratio between HCC(B) and** the corresponding SL(B), or HCC(C) and corresponding SL(C). **The error bars indicate mean ± SD. RT-PCR: Real-time reverse transcription-polymerase chain reac**tion; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; SL: Surrounding non-cancerous liver tissues.

KIAA1808 expression were also decreased in HCC(C) and HCC(NBNC) to 0.17- and 0.13-fold, respectively. On the other hand, only NADE exhibited differential expression larger than twofold, a pattern consistent with that seen in huHB4, and it was expressed 2.0- and 2.4-fold higher in HCC(C) and HCC(NBNC), respectively.

On the other hand, the comparison of gene expression between HCC and SL from the same individual suffering from HBV infection showed wide variation among cases (Figure 4C). Only *SKAP2* exhibited more than a twofold difference with relatively smaller variation and was expressed at 2.60 ± 1.07-fold higher in HCC(B). None of the eight candidates showed expression differences of more than twofold in all the five cases examined. Wide variation was also observed in HCV-associated cases, with a pattern similar to that seen in HBV-associated cases, as represented by *SKAP2* (Figure 4D).

DISCUSSION

Consistent with previous reports[12,13], we found that HBV expression was significantly reduced in HCC(B) compared to the corresponding SL(B) in all of our cases. In order to mimic physiological repression of HBV expression through hepatocarcinogenesis, a sustained induction of RNAi against HBV was employed in this study. A single target of shRNA for a shared sequence in all four transcripts from HBV successfully achieved substantial reduction of HBsAg and HBx protein expressions *in vitro* and *in vivo*, respectively, without affecting cell morphology or replication rate.

RNAi is an evolutionarily conserved mechanism of post-transcriptional gene silencing induced by dsRNA and has been widely studied to prove its efficacy in reverse functional genomics and for therapeutic use[23,24]. Unfortunately, it is important to remember that the modulation of gene expression through RNAi can be extended to unintended genes due to various processes including off-target effects[25]. Off-target effects, which are RNAi-mediated events affecting the expression levels of dozens to hundreds of genes, are quite difficult to completely eliminate because off-targeting can be mediated by complementarity even between a hexamer seed region of dsRNA and the 3' untranslated region of a target[26]. It has been reported that using different siRNA sequences that target HBV in HepG2.2.15 cells leads to different cellular gene expression profiles, suggesting a complicated influence of siRNA on cellular gene expression[18].

Table 2 Representative genes in enriched functional categories

Category	No. of genes	*P* value[1]	Genes	Accession number[2]
Up regulated				
Lipid synthesis	3	0.01	*3-hydroxy-3-methylglutaryl-coenzyme A reductase*	NM_000859
			7-dehydrocholesterol reductase	NM_001360
			Growth differentiation factor 1	M62302
Glycoprotein	15	0.019	*Integrin beta 3*	NM_000212
			Interleukin 1 alpha	NM_000575
			Vitronectin	NM_000638
Biopolymer metabolism	16	0.02	*Cell division cycle 7*	NM_003503
			Colony stimulating factor 1 receptor	NM_005211
			Connective tissue growth factor	NM_001901
Hydrolase activity	6	0.046	*Bile acid coenzyme A: amino acid n-acyltransferase*	NM_001701
			Inositol polyphoshpate-5-phosphatase	AF184215
			Poly(A)-specific ribonuclease	AJ005698
Down regulated				
Signal	17	0.001	*Cytotoxic T-lymphocyte-associated protein 4*	NM_005214
			Epithelial cell adhesion molecule	NM_002354
			Gamma-aminobutyric acid A receptor alpha 4	NM_000809
Protease Inhibitor	3	0.024	*Cystatin A*	BC010379
			Secretory leukocyte peptidase inhibitor	NM_003064
			Tissue factor pathway inhibitor	AF021834
Sensory perception	3	0.024	*Collagen type I alpha 2*	NM_000089
			GATA binding protein 3	BC006839
			USHER syndrome 1C	NM_005709
Transport	7	0.031	*Amyloid beta precursor protein binding family A member 2*	BC007794
			ATP-binding cassette subfamily D member 4	NM_020323
			Solute carrier family 17 member 2	AL138726
Cytokine activity	4	0.046	*Chemokine ligand 25*	AB046579
			Kit ligand	NM_000899
			Prolactin	NM_000948

[1]Probability of gene enrichment calculated using Fisher's exact test by referencing the frequency in 30 000 genes of human genome background; [2]GenBank accession number.

In this study, a seed locator program found at least one match in approximately 8% of 13 012 genes, even after the target sequence was carefully selected to make sure that no known cellular gene would show homology in its coding sequence with the target. Furthermore, pSUPER.HB4G unexpectedly up-regulated the expression of both HBsAg and HBx. It seems prudent to assume that cellular genes selected by knockdown experiments using RNAi against HBV involve genes directly affected by the RNAi process. In this regard, it is quite important to validate the results from *in vitro* experiments in liver tissues.

When human liver tissues were evaluated to find genes that are involved in hepatocarcinogenesis under HBV infection, gene expression profiles were generally compared between HCC(B) and SL(B). It is thought, however, that the transformation process itself requires a tremendous alteration of cellular gene expression, irrespective of etiology, that may outstrip the impact of HBV infection on the cellular gene expression. This concern seems to be relevant because qRT-PCR using liver tissues revealed that the differential expression patterns between HCC(B) and SL(B) were inconsistent with those between huHB4 and huHpS, but quite resembled the patterns between HCC(C) and SL(C). It would be ideal to evaluate the effect of HBV on cellular gene expression using non-tumorous liver tissues from the same individual at various time points with different levels of HBV expression, such as before and after natural seroconversion. In reality, however, it is quite difficult to collect liver specimens repeatedly in those settings.

To address this issue, HBV-associated liver tissues were compared with various liver tissues from the same individual and others. The major factors considered in comparisons among patients should be (1) with or without chronic liver diseases; (2) viral or non-viral liver diseases; and (3) cancerous or non-cancerous liver tissues. Thus, in the present study, gene expression levels in HCC(B) and SL(B) were compared with those in HCC(C), HCC(NBNC), SL(C), SL(NBNC) or SL(N). Because the presence or absence of viral infection presumably has a substantial impact on the cellular gene expression profile irrespective of virus species, the comparison between SL(B) and SL(NBNC) or between SL(B) and SL(N) does not reflect the specific influence of HBV infection, but of viral hepatitis in general. The cellular genes that are strictly regulated in connection with HBV infection should be differentially expressed, not only between HCC(B) and HCC(C), but also between SL(B) and SL(C). Among eight candidates, only *APM2* fulfilled those criteria in this study. Thus, *APM2*, which is confirmed to be repressed after reduction of HBV expression in both RNAi knockdown experiments and liver tissues, is highly likely to be differentially expressed in association with HBV infection.

APM2 is a gene located on chromosome 10 at q23.2 and was originally identified as the second most abundant transcript in adipose tissue following adiponectin, *APM1*[27]. It is reported, however, that *APM2* is expressed in a wide variety of tissues, including the liver[28], and is dysregulated in various cancers. Up-regulation of *APM2* has been reported in pancreatic intraepithelial neoplasms[29], breast cancer tissues from patients with poor prognoses[30], and cisplatin-resistant gastric cancer cell lines[31]. Furthermore, overexpression of *APM2* is reported to promote cisplatin resistance in a variety of cancer cell types[32]. Although the exact function of *APM2* is currently unknown, these observations suggest that *APM2* plays a role as an anti-apoptotic factor. HBV infection may induce sustained expression of *APM2*, leading to persistent viral infection and hepatocarcinogenesis. It would be interesting to determine whether HCC(B) is more resistant to cisplatin than HCC(C) and/or HCC(NBNC) because *APM2* expression is substantially higher in HCC(B).

In the present study, 32 000 initial genes were evaluated, and of these genes, only one gene, *APM2*, was selected as a highly possible candidate gene that is differentially expressed due to HBV infection. Although we have so far evaluated only eight genes of 238 microarray candidates using human tissues, the concordance between the results from the cell lines and liver tissues is insufficient. It is true that the results obtained from the cell lines under shRNA overexpression technically involve many artificial limitations. However, another possible explanation for the low concordance is that cellular gene expression is regulated as a functional unit rather than by each gene. Functional annotations of genes selected in this study through microarray analysis indicated that the reduction of HBV expression led to disproportionately higher rates of increases or decreases of cellular gene expression in certain functional categories. The categories of genes with significantly enhanced or repressed expression after knockdown of HBV included lipid synthesis and protease inhibitors, respectively. In terms of lipid synthesis, for example, it has been reported that there is an inverse correlation between HBV and apolipoprotein expressions[33], and it is the largest functional category of lipid biosynthetic pathways to show differential expression between HBV transgenic and wild-type control mice[17]. Consistently, differential expression of cellular proteins were investigated in association with HBV infection and indicated that lots of proteins were up or down regulated as groups of several functional categories, including metabolisms[34-37]. Unfortunately, however, few reports applied the technologies of proteome or transcriptome analyses to human liver tissues instead of HBV-associated cell lines. To clarify the clinical significance of functional annotation, it is necessary to conduct proteome or transcriptome analyses on a large scale using liver tissues in various conditions.

In conclusion, it is suspected that the comparison between HCC(B) and SL(B) is not ideal for determining which genes are differentially expressed as a result of HBV infection. Because chronic viral hepatitis should have significant impacts on cellular gene expression, just as cellular transformation does, differential expression should be confirmed by comparing HBV-associated cases with HCV- or other viral-associated cases in terms of both HCC and SL. In the present study, the *APM2* gene was selected from 32 000 human genes as the gene that was differentially expressed in those settings. In the future, differential cellular gene expression should be evaluated with respect to functional categories based on proteome and/or transcriptome analyses. The knowledge of cellular gene expression will help to elucidate the detailed mechanisms involved in chronic hepatitis B and HBV-associated hepatocarcinogenesis.

SUPPLEMENTARY MATERIAL

Supplementary Table 1 is available at Supplementary material online (http://www.wjgnet.com/1948-5182/g_info_20120428090827.htm).

COMMENTS

Background

Powerful preventive and therapeutic means have been developed for hepatitis B virus (HBV) infection, such as vaccination and nucleotide analogue, on the basis of our knowledge for HBV life cycle. Unfortunately, however, the World Health Organization reported that an estimated 350 million people worldwide are chronically infected with HBV and a significant proportion of chronic infections terminate in hepatocellular carcinoma (HCC), leading to more than half a million deaths annually. These dreadful situations request thorough understanding of the mechanisms for HBV to persuade sustained infection and hepatocarcinogenesis.

Research frontiers

A recent technological advancement makes it possible to analyze an impact of a specific molecule such as HBV on the proteome and/or transcriptome in host cells. It is not difficult to assume that a deviation of gene expression profile plays a crucial role for immunological escape and cancer progression in HBV infection. The knowledge of HBV-host cell interaction must provide a powerful tool to fight HBV infection.

Related publications

So far, an alteration of gene expression profile due to HBV infection has mainly been evaluated between non-infected parental cells and artificially-infected cells or between cells that were established from HBV positive case and HBV-knockdown cells. In terms of clinical materials, the comparisons between HBV-associated HCC and corresponding noncancerous liver tissues or between HBV-positive and HBV-negative cancer cells have been reported.

Innovations and breakthroughs

In the knockdown or artificial infection of HBV, it is quite difficult to completely eliminate the impact of knockdown/infection treatment itself on cellular gene

expression. Furthermore, it is not difficult to assume that a malignant transformation process induces a tremendous change in cellular gene expression profile, which may overtake the effects of host-pathogen interactions. In order to avoid these confounding factors, the research team led by Takeshi Suda from Division of Gastroenterology and Hepatology, Niigata University took a two-step approach. Firstly, pick up candidate genes in a HBV-positive cell line by knocking HBV expression down, using small hairpin RNA, then confirm differential expression of the candidates in various liver tissues, including both cancer and noncancer associated with HBV, hepatitis C virus (HCV) or no HBV/HCV. Through this approach, the authors finally selected one gene, which differentially expressed in association with HBV infection, regardless of cancerous or noncancerous tissues.

Applications

The new insight of cellular reaction evoked by HBV infection must provide a clue to better understanding the mechanisms of sustained viral infection and cancer development, and will be implicated in the development of novel therapeutic options.

Terminology

RNA interference is a process within living cells, in which double-stranded RNA directs a gene activity control with certain sequence specificity. shRNA is a small hairpin RNA, and short hairpin RNA is a sequence of RNA that makes a tight hairpin turn that can induce RNA interference. Off-targeting is a phenomenon where genes with incomplete complementarities with a target sequence are regulated in RNA interference. Dye flip is a strategy to account for dye bias in microarray experiments by labeling of DNA targets with the two dyes in reciprocal fashion. APM2 was discovered as the second most abundant transcript in adipose tissue following adiponectin and has been reported to be expressed in a variety of other tissues, including liver. *APM2* gene overexpression is associated with malignant transformation and resistant to a chemotherapeutic agent have been reported.

Peer review

The authors provided evidence indicating that silencing HBV mRNA altered cellular gene expressions in several functional categories including lipid synthesis. By stably targeting the common ORF region of the key HBV protein genes with shRNA, they observed a substantial decrease of HBsAg expression and up- or down-regulation of cellular genes, as revealed by DNA microarray. To verify *in vitro* assay results, they extended the observations to human liver tissues. The conclusions provide new insights into the mechanisms of HBV- or HCV-induced chronic liver diseases and hepatocarcinogenesis, and would be very useful for identification of novel drug targets against HBV infection.

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BRIEF ARTICLE

Non invasive continuous hemodynamic evaluation of cirrhotic patients after postural challenge

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Abstract

AIM: To assess whether Most Care is able to detect the cardiovascular alterations in response to physiological stress (posture).

METHODS: Non invasive hemodynamic was assessed in 26 cirrhotic patients compared to healthy subjects, both in the supine and standing positions.

RESULTS: In baseline conditions, when compared to healthy subjects, cirrhotic patients showed significantly lower values of dicrotic and diastolic pressures and systemic vascular resistance. While in the standing position, cirrhotic patients showed higher values of cardiac index, stroke volume index and cardiac cycle efficiency. When returning to the supine position, cirrhotic patients exhibited lower values of dicrotic and diastolic pressures and systemic vascular resistance in the presence of higher values of cardiac index, stroke volume index and cardiac cycle efficiency.

CONCLUSION: Most Care proved to be able to detect cardiovascular abnormalities bedside in the resting state and after postural challenge in cirrhotic patients.

Key words: Liver cirrhosis; Non-invasive hemodynamic; Pressure recording analytical method; Posture

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INTRODUCTION

Historically, liver cirrhosis has not been associated with any cardiac abnormalities, despite the fact that a hyperdynamic circulation was described in patients with cirrhosis over 50 years ago[1]. Beginning in the late 1980s, occasional reports of unexpected deaths due to heart failure following liver transplantation[2], transjugular intrahepatic portosystemic stent shunt (TIPS) insertion[3] and surgical portocaval shunts[4] led to growing interest in investigating cardiac dysfunction related to cirrhosis. Over the last 2 decades, there is accumulating evidence to suggest that the presence of cirrhosis per se is associated with significant cardiovascular abnormalities, irrespective of the cause of cirrhosis[5]. The majority of patients are diagnosed during phases of clinical decompensation of

cirrhosis in which they present with features of diastolic heart failure and/or high-output heart failure. Investigators have reported an increased risk of death due to cardiovascular complications following the insertion of a transjugular intrahepatic TIPS[6,7] and after liver transplantation[8]. Patients who experienced these complications had pre existing changes in cardiac performance that suggest a diagnosis of cirrhotic cardiomyopathy[9].

The identification of cardiovascular abnormalities has been made mainly by means of echocardiography[5,9]. Recently, non invasive technologies based on the analysis of the peripheral arterial waveform pulse contour methods are gaining popularity in various settings. Among them, Most Care [pressure recording analytical method, (PRAM)] has been recently introduced and it has been proved to be feasible and accurate in different clinical settings, both in stable and unstable patients[10-14]. Unlike echo-cardiography, it is operator-independent and it allows a beat-by-beat hemodynamic evaluation, lasting even for hours.

The aim of the present investigation was to assess whether the PRAM methodology which allows a non invasive beat-to-beat hemodynamic evaluation, is able to detect the cardiovascular alterations in response to physiological stress (posture) in 26 cirrhotic patients compared to healthy subjects.

MATERIALS AND METHODS

Twenty-six consecutive non-smoking patients with liver cirrhosis [indicated by Child-Pugh score in 17 A, 8 B, 6 C; 11 patients with ascites (AC), 15 without (CC); 19 males; mean age 59.45 ± 13.4 years, range 32-86 years] who attended outpatients at the Department of Internal Medicine, University of Florence, Italy, from March to July 2008, were included in the study. The diagnosis of cirrhosis was based on the patients' history, physical examination, liver ultrasound and laboratory findings, and was confirmed by transjugular liver biopsy when not contraindicated. Cirrhosis was hepatitis C virus-related in 24 patients and hepatitis B virus-related in 2. All patients had portal hypertension, as indicated by measuring hepatic venous pressure gradient. No patient had infection, organic cardiovascular, renal or pulmonary diseases, hypoxemia in clino- or orthostatic position; diabetes, type 1 hepatorenal syndrome[15], hepatocellular carcinoma or other malignancies; recent gastrointestinal bleeding or a North Italian Endoscopic Club score ⩾ 35[16]. No patient was taking β-blockers or nitrates in the 7 d preceding or during the study. Ten healthy subjects of comparable sex and age from the medical and laboratory staff of our unit constituted the control group healthy subjects. The investigation conforms to the principles outlined in the Declaration of Helsinki, was approved by the Local Ethical Committee and all subjects gave their informed written consent to participate in the study.

On the day of the study, after a 30 min resting period (equilibrium), data were obtained, in each subject, for a supine resting period (baseline phase) throughout the standing period (stress phase) and after return to the supine position (recovery phase). Each step lasted 10 min. The blood pressure (BP) measurements were assessed by digital photoplethysmography (Finapres)[11].

We performed the hemodynamic evaluation from the signal pressure obtained by Finapres from the analogical output. Analysis of the signal blood pressure was performed, in real time, beat-by-beat, by MostCare® monitor (Vytech, Padova, Italy). This device uses the hemodynamic estimation by the PRAM[10-14] of the systemic BP waves from the Finapres. The computed values of stroke volume PRAM were displayed in real time and the corresponding results beat-to-beat were recorded and stored for subsequent check of the data.

The data from each subject were reviewed and edited manually to remove artefacts (which consisted of the calibration intervals, devoid of pressure signal by the Finapres). Data from pressure signals were evaluated, averaging 1 min periods at halfway of the baseline, halfway of the stress phase, and halfway of the recovery phase, by averaging 30 s before and 30 s after the selected time.

PRAM sampling BP morphology wave at 1000 Hz and data were electronically stored. PRAM needs neither calibration nor pre-estimated parameters of patients (such as gender, age, anthropometric data). The hemodynamic variables obtained beat to beat by this monitoring system were: systolic, diastolic, dicrotic and mean arterial pressures (mmHg); cardiac index (CI; L/min/m^2); stroke volume index (SVI; mL/m^2); dP/dt$_{max}$ (mmHg/s)[11,14]; cardiac cycle efficiency (CCE; units)[10-14]; Systemic Vascular Resistance Index (SVRI; dyne*sec/cm^5); and Pulse Pressure variation (%).

PRAM methodology

The analysis of the wave form is based on the theory that in any given vessel, volume changes occur mainly because of radial expansion in response to variations in pressure[10]. This process involves the interplay of several physical parameters, including force of left ventricular ejection, arterial impedance counter acting the pulsatile blood inflow, arterial compliance and peripheral, small vessel resistance. These variables are tightly interdependent and simultaneously estimated by PRAM. Thus, any kind of flow that is perceived at the peripheral arterial level, whether pulsatile or continuous as in physiological conditions, can be evaluated. The PRAM technique, based on the analysis of the peripheral artery waveform morphology, has been extensively described elsewhere[10,12], in various clinical and experimental settings[10-14]. The PRAM analyzes and identifies the characteristic points of the pressure wave during each beat (diastolic, systolic, dicrotic and resonant points pressure during the systolic and end-diastolic phases), thus performing a beat-to beat hemodynamic assessment[10,12-14].

Statistical analysis

Data were entered in a dedicated data-base and processed by means of SPSS 13.0 statistical package (SPSS Inc. Chicago, IL). Data were expressed as mean ±SD and were

Table 1 Beat-to-beat, non invasive hemodynamic evaluation in healthy subjects

	SBP	DBP	DIC	HR	SVR	CCE	SVI	CI	dP/dT
Baseline	117 ± 13	65 ± 7	82 ± 8	68 ± 10	1252 ± 144	0.111 ± 0.12	0.0412 ± 0.009	2.81 ± 0.35	0.81 ± 0.14
Standing	111 ± 11	68 ± 15	84 ± 13	78 ± 11[a]	1504 ± 301[b]	-0.193 ± 0.223[b]	0.0305 ± 0.009[b]	2.34 ± 0.46[a]	0.72 ± 0.17
Recovery	121 ± 16	67 ± 8	86 ± 10	66 ± 10[a]	1260 ± 139[b]	0.040 ± 0.25	0.0424 ± 0.007[a]	2.86 ± 0.32[b]	0.82 ± 0.16

[a]$P < 0.05$; [b]$P < 0.01$ *vs* baseline. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; DIC: Dicrotic pressure; HR: Heart rate; SVR: Systemic vascular resistance; CCE: Cardiac cycle efficiency; SVI: Stroke volume indexed; CI: Cardiac index.

analysed by Student-*t* test for paired and unpaired measure. A two-tailed P value < 0.05 was considered statistically significant.

RESULTS

Healthy subjects

As depicted in Table 1, healthy subjects showed, when assuming the standing position, a significant increase in heart rate and SVR ($P < 0.05$ and $P < 0.01$, respectively) associated with a significant reduction ($P < 0.05$) in stroke volume index, cardiac index and CCE.

The standing *vs* recovery phase was associated with a significant reduction in heart rate ($P < 0.05$) and SVR ($P < 0.05$) and increase for CI and SVI ($P < 0.05$ and $P < 0.01$ respectively).

For all variables, there were no significant changes between phases, base-returning supine.

Cirrhotic patients

Cirrhotic patients exhibited, with the assumption of the standing position, an increase heart rate and SVR ($P < 0.05$ and $P < 0.01$, respectively) associated with a significant reduction in cardiac index and stroke volume index (Table 2). From the standing to the recovery phase, heart rate and SVR decreases ($P = 0.038$ and $P = 0.043$). The CI, SVI and CCE significantly increase from the standing position to recovery phase ($P = 0.037$, $P < 0.001$ and $P = 0.002$ respectively). From the comparison between the phases base *vs* recovery, there are no significant variations in our variables.

Comparison between healthy subjects and cirrhotic patients

In baseline conditions, when compared to healthy subjects, cirrhotic patients showed significant lower values of dicrotic and diastolic pressures and SVR, no difference in heart rate and higher CI, SVI and CCE (Table 3).

In the standing position, dicrotic pressure and SVR were lower in cirrhotic patients, who showed higher values of CI, SVI and CCE in cirrhotic patients compared to healthy subjects.

When returning to the supine position, cirrhotic patients exhibited lower values of dicrotic and diastolic pressures and SVR. In cirrhotic patients, there were no significant differences for the HR and systolic pressure. CI, SVI and CCE were higher when compared to healthy subjects (Table 3).

DISCUSSION

The main finding of the present investigation is that cardiovascular abnormalities, as indicated by increased cardiac output and reduced SVR, are detectable in cirrhotic patients when compared to healthy patients, both in the supine and in the standing position, bedside, by means of PRAM methodology, a non invasive beat-to-beat hemodynamic monitoring.

While previous studies investigating hemodynamics in liver cirrhosis were mainly performed by echocardiography[5,9], which is known to be operator-dependent, in the present study we used PRAM monitoring, which proved to be feasible since it is non invasive and does not need calibration.

Recent studies documented the clinical impact of cardiovascular abnormalities in cirrhotic patients[17], especially when submitted to surgical procedures, such as TIPS insertion[6,7,18] and liver transplantation[19]. Sampathkumar and colleagues[19] reported severe myocardial dysfunction in 1% of patients after liver transplantation, since during reperfusion, the return of a significant volume to the heart can unmask any diastolic dysfunction. When followed long-term for over 1 year, there is complete reversal of cardiac changes in post transplant patients.

Although cardiac complications are usually seen in patients with more advanced liver disease, adverse cardiac outcomes can still occur in patients who are not as ill[20] and overt manifestations of cardiovascular dysfunction often only become evident after a cirrhotic patient is exposed to physiological, surgical or drug-induced stress. In this context, PRAM methodology appears, according to our data, able to detect cardiovascular alterations in cirrhotic patients, both at rest and during postural change, by means of a bedside non invasive evaluation.

In the present investigation, we furthermore documented that cirrhotic patients, when evaluated by means of PRAM, showed an altered cardiovascular response to physiological stress (both assuming the standing position and returning to the resting state).

This is in keeping with previous studies[21-24] performed by means of echocardiography during active tilting. Laffi *et al*[21] assessed the inotropic and chronotropic response in a group of non alcoholic cirrhotic patients and reported a significantly decreased stroke volume in response to active tilting, leading to a reduced cardiac index despite a significant increase in heart rate. In 1999, Gentilini *et al*[22] observed that compensated cirrhotic

Table 2 Beat-to-beat, non invasive hemodynamic evaluation in cirrhotic patients

	SBP	DBP	DIC	HR	SVR	CCE	SVI	CI	dP/dT
Baseline	112 ± 16	53 ± 8	65 ± 11	69 ± 9	973 ± 169	0.29 ± 0.13	0.051 ± 0.008	3.57 ± 0.52	1.06 ± 0.23
Standing	117 ± 18	61 ± 9[b]	71 ± 10	79 ± 13[a]	1150 ± 281[a]	0.183 ± 0.17[a]	0.042 ± 0.009[b]	03.31 ± 0.63	1.01 ± 0.24
Recovery	116 ± 20	56 ± 11	69 ± 16	70 ± 10[a]	1047 ± 158	0.253 ± 0.12	0.0514 ± 0.001[a]	3.32 ± 0.42	1.02 ± 0.21

[a]$P < 0.05$; [b]$P < 0.01$ *vs* baseline. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; DIC: Dicrotic pressure; HR: Heart rate; SVR: Systemic vascular resistance; CCE: Cardiac cycle efficiency; SVI: Stroke volume indexed; CI: Cardiac index.

Table 3 Comparison between healthy subjects and cirrhotic patients

	SBP	DBP	DIC	HR	SVR	CCE	SVI	CI	dP/dT
Baseline									
HS *vs* C	NS	< 0.001	< 0.001	NS	< 0.001	< 0.001	0.01	< 0.001	< 0.001
Standing									
HS *vs* C	NS	NS	0.02	NS	< 0.001	0.02	0.01	< 0.001	< 0.001
Recovery									
HS *vs* C	NS	< 0.01	< 0.001	NS	< 0.001	NS	< 0.05	< 0.01	0.02

HS: Healthy subjects; C: Cirrhotic patients; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; DIC: Dicrotic pressure; HR: Heart rate; SVR: Systemic vascular resistance; CCE: Cardiac cycle efficiency; SVI: Stroke volume indexed; CI: Cardiac index.

patients, both normotensive and hypertensive, showed an altered cardiovascular response to postural challenge characterized by reduced left ventricular telediastolic volume and stroke volume and cardiac index associated with a marked activation of the renin-angiotensin system. Similarly, Bernardi *et al*[23] observed (by means of Doppler echocardiography) that while standing, hemodynamic patterns of cirrhotic patients and controls did not differ and increased cardiac index and reduced systemic vascular resistance were detectable in cirrhotic patients in the supine position compared to controls. The authors concluded that hyperdynamic circulation appeared, or was enhanced, by the lying position. An altered cardiovascular response was also documented in cirrhotic patients by Moller *et al*[24] after 30 degrees head-down tilting and after 60 degrees passive head-up tilting.

A possible limitation of the study is that the number of enrolled patients is small. However, the behavior of hemodynamic parameters, as assessed by PRAM methodology, was uniform in cirrhotic patients, so that differences were clearly detectable between patients and healthy subjects.

In conclusion, PRAM methodology proved to be able to detect cardiovascular abnormalities both in the resting state and after postural challenge by means of a non invasive continuous bedside hemodynamic evaluation in cirrhotic patients when compared to healthy subjects. This new methodology could therefore represent a feasible tool for a clinically more accurate non invasive continuous hemodynamic monitoring in these patients.

COMMENTS

Background

The presence of cirrhosis per se has been associated with significant cardiovascular abnormalities, irrespective of the cause of cirrhosis and an increased risk of death due to cardiovascular complications has been recently reported following surgical procedures in cirrhotic patients.

Research frontiers

So far, cardiovascular assessment in cirrhosis has been performed mainly by echocardiography, which is operator-dependent and does not allow a beat-to-beat evaluation. Recently non invasive technologies based on the analysis of the peripheral arterial waveform pulse contour methods are gaining popularity in various settings. Among them, Most Care [pressure recording analytical method (PRAM)] has been recently introduced and it has been proved to be feasible and accurate in different clinical settings, both in stable and unstable patients.

Innovations and breakthroughs

This is the first investigation assessing whether the PRAM methodology which allows a noninvasive beat-to-beat hemodynamic evaluation, is able to detect the cardiovascular alterations in response to physiologic stress (posture) in cirrhotic patients in respect to healthy subjects.

Applications

Cardiovascular abnormalities, as indicated by increased cardiac output and reduced systemic vascular resistance, are detectable in cirrhotic patients, when compared to healthy patients, both in the supine and in the standing position, bedside, by means of PRAM methodology. A continuous non invasive hemodynamic monitoring is therefore feasible by PRAM methodology in cirrhotic patients in stable conditions and during procedures.

Terminology

The terminology has been explained in the text.

Peer review

This study investigated the cardiovascular alternations in response to physiologic stress by PRAM methodology in 26 cirrhotic patients and 10 healthy subjects. The main findings of the study are that the cardiac output is higher and SVR lower in the cirrhotic patients than the healthy subjects by means of PRAM methodology both in the supine and standing position.

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CASE REPORT

SlimQuick™ - associated hepatotoxicity in a woman with alpha-1 antitrypsin heterozygosity

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Abstract

Green tea (*Camellia sinensis*)-associated hepatotoxicity is reported. However, the presence of alpha-1 antitrypsin MZ phenotype as a predisposing factor to green tea-associated drug-induced liver injury (DILI) is unknown. A previously healthy woman with alpha-1 antitrypsin MZ phenotype who took SlimQuick™, an herbal supplement containing green tea extract, developed severe hepatotoxicity requiring corticosteroid treatment. Green tea-associated hepatotoxicity is reviewed and alpha-1 antitrypsin MZ phenotype as a predisposing factor to green tea-associated DILI is discussed. Liver biopsy demonstrated marked inflammation with necrosis suggestive of toxic injury with diffuse alpha-1 antitrypsin globule deposition on immunostaining. Corticosteroid therapy resulted in rapid clinical improvement. Alpha-1 antitrypsin MZ phenotype may increase vulnerability to herbal hepatotoxicity.

Key words: SlimQuick™; Green tea, Hepatotoxicity; Drug-induced liver injury, Alpha-1-antitrypsin MZ phenotype

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INTRODUCTION

Green tea obtained from the leaves of *Camellia sinensis* is thought to improve health due to antioxidant and anti-carcinogenic effects[1-4]. Nonetheless, green tea has pro-oxidant effects, primarily due to epigallocatechin-3-gallate (EGCG)[2,3]. Lambert *et al*[5] reported dose-dependent hepatotoxicity in mice associated with pro-oxidant effects of high-dose EGCG. Hepatotoxicity associated with green tea extracts that contain high concentrations

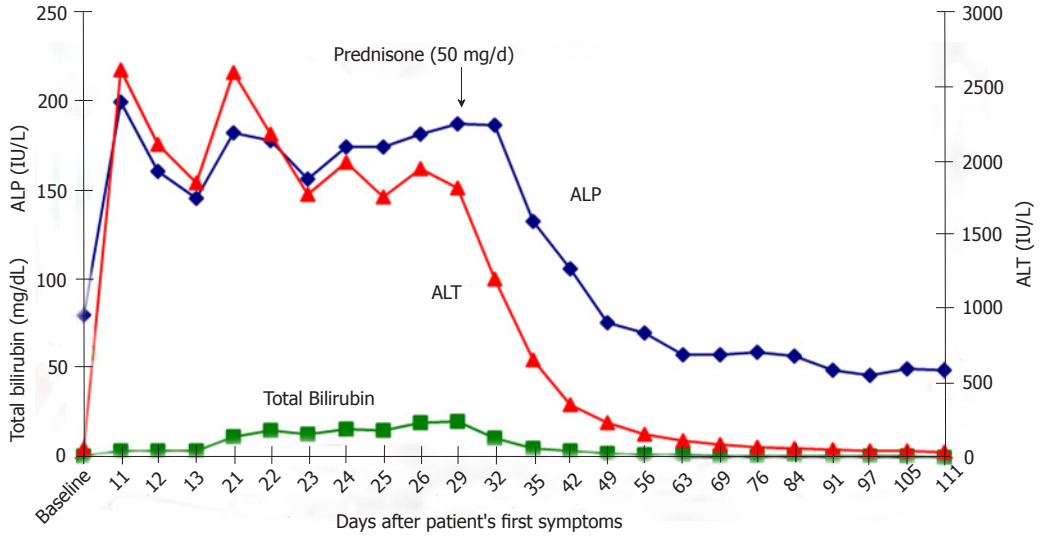

Figure 1 Two months earlier, our patient had normal serum alanine aminotransferase, alkaline phosphatase and total bilirubin (baseline). The peak total bilirubin level lagged 19 d behind the peak ALT and ALP levels. There was striking improvement in serum aminotransferase and total bilirubin levels after initiation of prednisone (50 mg/d). Laboratory values remained normal after prednisone therapy was discontinued. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

of EGCG is being reported[6-9]. Due to hepatotoxicity, Exolise® (Arkopharma, Carros, France), a weight-loss supplement was withdrawn from the market in France and Spain[8]. Exolise® contained EGCG[7,8]. The Drug-Induced Liver Injury Network indicated that in 6 of 28 cases of hepatotoxicity secondary to herbal and dietary supplements, green tea extract was the major component of the supplement[10]. Fong *et al*[6] reported 8 patients who developed drug-induced liver injury (DILI) due to Hydroxycut®, 3 of whom required liver transplantation. The authors concluded that green tea was likely the major ingredient in Hydroxycut® resulting in severe liver injury[6].

We report a young woman who developed severe hepatotoxicity while taking SlimQuick™ (Distributed by Wellnx Life Sciences, Wilmington, DE), an herbal weight-loss product containing green tea extract. This is important because we identified an often overlooked predisposition to DILI; the alpha-1 antitrypsin MZ phenotype[11].

CASE REPORT

A 24-year-old white woman presented to her primary care physician with complaints of dark urine, acholic stools, right upper quadrant pain and progressive fatigue. She reported taking two caplets of SlimQuick™ orally on an empty stomach 6 h apart twice per day for three months to improve energy for marathon training. She took no other dietary supplements or medications except for oral tetracycline 500 mg/d orally for eleven months for acne. She stopped both drugs eight days after the onset of symptoms. Past medical, surgical and family histories were unremarkable. Two months as well as 3 years earlier, serum alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin were normal (Figure 1). Eleven days after the symptoms began; ALT was 2615 IU/L, aspartate aminotransferase 2320 U/L, alkaline phosphatase 200 IU/L and total bilirubin level 4.0 mg/dL (Figure 1). A day later, the prothrombin time and international normalized ratio were 12 s and 1.2, respectively. Three weeks after her first symptoms, she was transferred to our institution for further evaluation. At presentation, vital signs were normal. The physical examination was normal except for icteric sclera and mild right upper quadrant abdominal tenderness. Serological tests for viral hepatitis, autoimmune hepatitis, Wilson disease and primary biliary cirrhosis were negative. The alpha-1 antitrypsin phenotype test was reported as MZ. Magnetic resonance imaging of the abdomen revealed hepatomegaly. Magnetic resonance cholangiopancreatography and abdominal ultrasound were normal. Transjugular liver biopsy revealed a hepatic venous pressure gradient of 3 mmHg. Histopathology demonstrated severe inflammatory infiltrate involving both the portal tracts and lobules (Figure 2). The infiltrate included plasma cells, lymphocytes, and neutrophils with prominent eosinophils (Figure 2). There was also focal necrosis with loss of hepatic parenchyma, with accompanying architectural collapse and proliferation of bile ductules (Figure 2). Alpha-1 antitrypsin staining with specific immunoperoxidase highlighted minute globules, predominantly within periportal hepatocytes, as well as diffuse background staining in hepatocytes (Figure 3). These globules were much smaller than those typically associated with alpha-1 antitrypsin disorder in homozygotes.

Because the patient demonstrated no laboratory or clinical improvement three weeks after stopping SlimQuick™ and liver biopsy was consistent with marked inflammation with necrosis, treatment with Prednisone (50 mg/d) was initiated. All laboratory parameters rapidly improved within one week of therapy (Figure 1). Prednisone was tapered over 4 wk and stopped. On follow-up, the patient's liver tests were normal and she was asymptomatic.

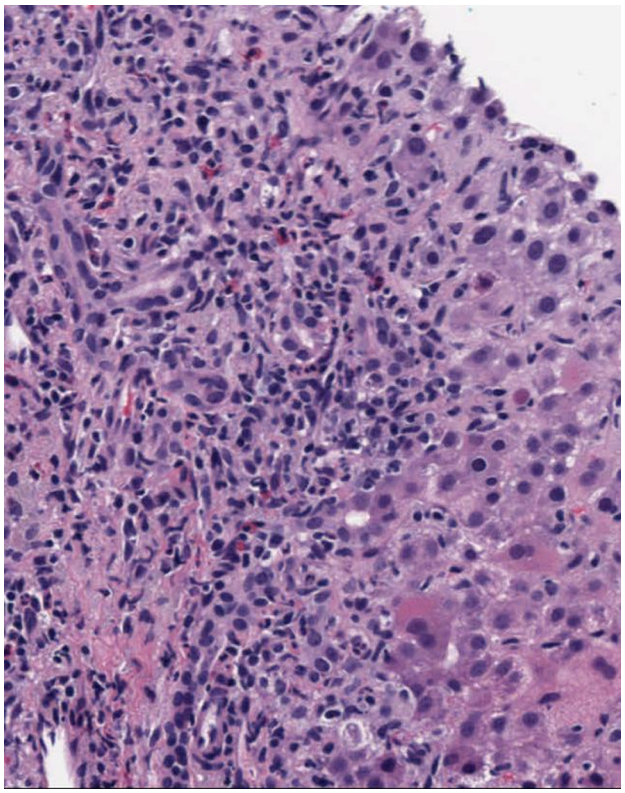

Figure 2 Portal tract and lobules with marked mixed inflammation and parenchymal loss (hematoxylin and eosin staining, X100).

DISCUSSION

The major ingredient in SlimQuick™ caplets is green tea extract (*Camellia sinensis* leaf) containing 135 mg of EGCG. EGCG has pro-oxidant effects that can cause hepatotoxicity when administered at high doses[3,5,8]. SlimQuick™ caplets also contain other ingredients including rhodiola (*Rhodiola rosea*), chaste tree (*Vitex agnus castus*), Juniper (*Juniperus communis*), soy (*Glycine max*), Asian ginseng (*Panax ginseng*), Japanese knotweed (*Polygonum cuspidatum*) extracts, brown seaweed (*Fucus vesiculosus*), dandelion (*Taraxacum officinale*), yerba mate (*Ilex Paraguariensis*), uva-ursi (*Arctostaphylos uva ursi*), phytosterols (*Glycine max*), l-theanine, caffeine, vitamins D, K, B6 and B12, folate, and calcium. Boehm *et al*[1] reviewed 51 studies, including 27 case-control, 23 observational and 1 randomized controlled trial related to green tea consumption and concluded that drinking 3 to 5 cups of green tea per day provided at least 250 mg catechins per day and might be considered safe. We calculated that our patient was exposed to green tea extract that contained catechin in amounts higher than these suggested safe levels. Moreover, she was also directed to take it while fasting. This augments the likelihood of hepatotoxicity in animals and fasting humans achieve 5-fold higher catechin concentrations compared to fed controls[7]. Lambert *et al*[5] reported moderate to severe hepatic necrosis in 60% and mild necrosis in 40% of mice treated with two oral doses of 750 mg/kg EGCG per day.

We performed an Ovid Medline search that extended from 1948 until October 22, 2010 by combining key words "drug-induced liver injury" or "hepatotoxicity" and both common and scientific names of herbal ingredients of SlimQuick™. We also searched the Natural Medicines Comprehensive Database[12] for hepatotoxicity associated with any ingredients of SlimQuick™ other than green tea extract. Based on our search, we identified only two herbal ingredients in SlimQuick™ other than green tea extract associated with hepatotoxicity in animals or humans. Nowak *et al*[13] reported a radiographer exposed to hydroquinone fumes who developed toxic hepatitis. Hydroquinone is a component of uva ursi[14]. However, Nowak *et al*[13] associated toxic hepatitis with hydroquinone fumes, not an herbal medicine, uva ursi[13,15]. McGee *et al*[16] associated mate tea with veno-occlusive disease due to pyrroliziding alkaloids detected in small quantity in tea samples. However, they failed to detect pyrroliziding alkaloids in mate tea sold locally[16]. Our patient's liver histopathology was inconsistent with veno-occlusive disease. In addition, the latency period of 12 wk, peak laboratory values and histopathological findings are more compatible with Exolise® and Hydroxycut® hepatotoxicity[6,7].

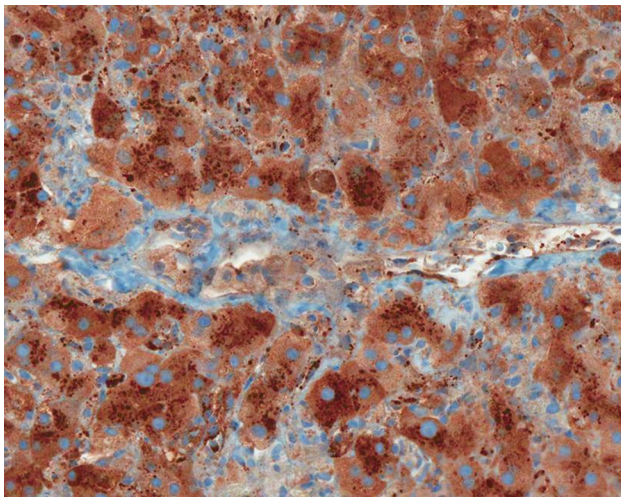

Figure 3 Minute alpha-1 antitrypsin positive globules in periportal hepatocytes; diffuse background staining for alpha-1 antitrypsin is also presented (alpha-1 antitrypsin stain, X200).

Besides taking a hepatotoxic weight-loss supplement, our patient was also heterozygous for alpha-1 antitrypsin deficiency. There remains considerable debate regarding the importance of the alpha-1 antitrypsin MZ phenotype [11, 17-21]. Graziadei *et al*[17] suggested that the alpha-1 antitrypsin MZ phenotype might be a risk factor for chronic liver disease or liver failure. Rakela *et al*[22] showed that subjects with the alpha-1 antitrypsin MZ phenotype might develop liver disease later in life compared to subjects with the alpha-1 antitrypsin ZZ phenotype.

Given our patient's baseline normal liver function, most likely the presence of alpha-1 antitrypsin MZ phenotype increased her vulnerability to severe hepatocellular injury. Tetracycline-induced liver injury was excluded as an offender based on the histopathology, leaving SlimQuick™ as the likely hepatotoxic agent. To our

knowledge, this is the first report of herbal drug-associated DILI in the context of the alpha-1 antitrypsin MZ phenotype. This demonstrates the importance of seeking underlying susceptibility to hepatic injury in previously healthy subjects who develop DILI.

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Events Calendar 2012

January 18, 2012
AHPBA Sponsored Consensus Conference on the Multidisciplinary Treatment of Colorectal Cancer Liver Metastases
San Francisco, CA, United States

January 20-21, 2012
AGA Clinical Congress of Gastroenterology and Hepatology: Practice, Evidence and Quality in 2012
Miami, FL, United States

January 27-28, 2012
28th Annual Meeting of the German Association for the Study of the Liver
Hamburg, Germany

January 30-31, 2012
5th International Conference on the Management of Patients with Viral Hepatitis
Paris, France

February 8-10, 2012
Stockholm Liver Week 2012
Stockholm, Sweden

February 16-19, 2012
22nd Conference of the Asian Pacific Association for the Study of the Liver
Taipei, Taiwan, China

March 16 -17, 2012
Hepatitis Single Topic Conference
Atlanta, GA, United States

March 16-17, 2012
ESGE - Workshop on Advanced Endoscopy with Live Demonstrations
Vienna, Austria

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA, United States

April 18-22, 2012
The International Liver Congress by EASL
Barcelona, Spain

April 27-28, 2012
The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
Stockholm, Sweden

May 16-19, 2012
International Liver Transplant Society 18th Annual International Congress 2012
San Francisco, CA, United States

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 22-23, 2012
EASL Monothematic Conference: Vascular Liver Diseases
Tallin, Estonia

July 1-5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association 2012
Paris, France

September 5-8, 2012
International Congress of Pediatric Hepatology, Gastroenterology and Nutrition
Sharm El-Sheikh, Egypt

September 7-9, 2012
Viral Hepatitis Congress 2012
Macclesfield, United Kingdom

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 14-16, 2012
The International Liver Cancer Association's 6th Annual Conference
Berlin, Germany

September 20-22, 2012
Prague Hepatology Meeting 2012
Prague, Czech Republic

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech Republic

October 18-20, 2012
2nd World Congress on Controversies in the Management of Viral Hepatitis
Berlin, Germany

November 9-13, 2012
AASLD - The Liver Meeting 2012
Boston, MA, United States

November 9-13, 2012
The Liver Meeting - 63rd Annual Meeting and Postgraduate Course of the American Association for the Study of Liver Diseases
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International Conference on Gastroenterology, Hepatology and Nutrition
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Acknowledgments

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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]

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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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).

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