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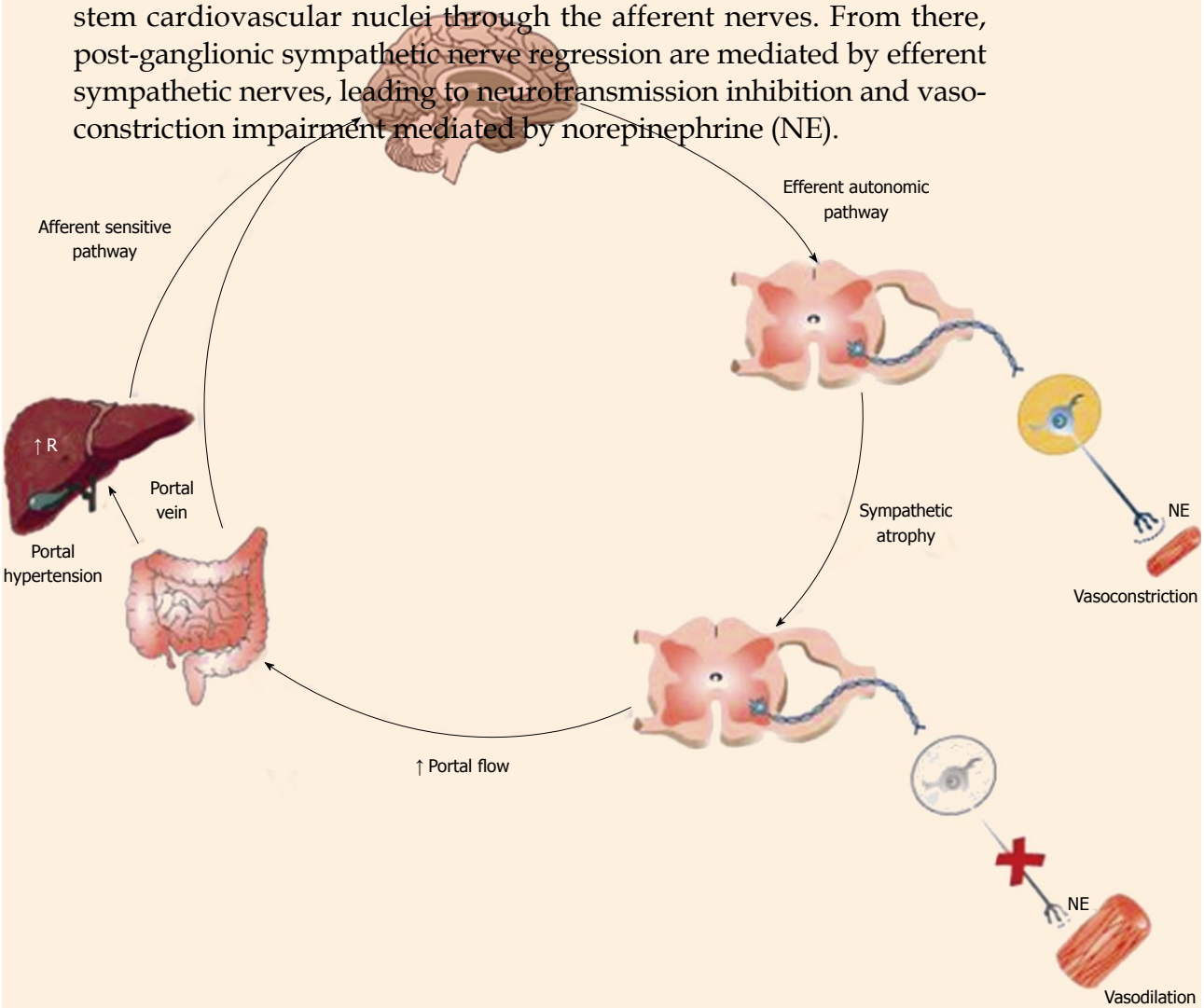
Hypothesis regarding the mechanisms and effects of the sympathetic post-ganglionic atrophy in splanchnic vasodilation. The afferent stimulus of portal hypertension, originating from pressure increases in portal or mesenteric vessels or microvasculature, reaches the brain stem cardiovascular nuclei through the afferent nerves. From there, post-ganglionic sympathetic nerve regression are mediated by efferent sympathetic nerves, leading to neurotransmission inhibition and vasoconstriction impairment mediated by norepinephrine (NE).



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Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

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Telephone: +86-10-5908-0038
Fax: +86-10-8538-1893
E-mail: wjh@wjgnet.com
http://www.wjgnet.com

PUBLISHING
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-8538-1892
Fax: +86-10-8538-1893
E-mail: baishideng@wjgnet.com
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EDITORIAL

Role of unfolded protein response in lipogenesis

Ze Zheng, Chunbin Zhang, Kezhong Zhang

Ze Zheng, Chunbin Zhang, Kezhong Zhang, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, United States
Kezhong Zhang, Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, United States
Author contributions: Zheng Z and Zhang CB were involved in material collection, discussion and the writing of the manuscript; Zhang KZ organized the materials, discussed with Zheng Z and Zhang CB and wrote the manuscript.
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Correspondence to: Kezhong Zhang, PhD, Center for Molecular Medicine and Genetics, 540 E, Canfield Street, Wayne State University School of Medicine, Detroit, MI 48201, United States. kzhang@med.wayne.edu
Telephone: +1-313-5772669 Fax: +1-313-5775218
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Abstract

The signal transduction network in regulating lipid metabolism is a hot topic of biomedical research. Recent research endeavors reveal that intracellular stress signaling from a cellular organelle called endoplasmic reticulum (ER) is critically involved in lipid homeostasis and the development of metabolic disease. The ER is a site where newly-synthesized proteins are folded and assembled into their three-dimensional structures, modified and transported to their precise cellular destinations. A wide range of biochemical, physiological and pathological stimuli can interrupt the protein folding process in the ER and cause accumulation of unfolded or misfolded proteins in the ER lumen, a condition referred to as ER stress. To cope with this stress condition, the ER has evolved highly-specific signaling pathways collectively termed Unfolded Protein Response (UPR) or ER stress response. The UPR regulates transcriptional and translational programs, affecting broad aspects of cellular metabolism and cell fate. Lipogenesis, the metabolic process of *de novo* lipid biosynthesis, occurs primarily in the liver where metabolic signals regulate expression of key enzymes in glycolytic and lipogenic pathways. Recent studies suggest that the UPR plays crucial roles in modulating lipogenesis under metabolic conditions. Here we address some of recent representative evidence regarding the role of the UPR in lipogenesis.

Key words: Endoplasmic reticulum stress; Unfolded protein response; Lipogenesis; Hepatic lipid metabolism; Metabolic disease

Peer reviewer: Nattiya Hirankarn, MD, Associated Professor, Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Rama 4 road, Bangkok 10330, Thailand

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INTRODUCTION

In the liver, the lipid content is regulated by dietary fatty acids or carbohydrates uptake, hepatic fatty acids biosynthesis, esterification, oxidation and export. Metabolic signals such as an excess of fatty acids, glucose or insulin can regulate the activity or abundance of key transcription factors to modulate hepatic lipid metabolism[1]. Many hepatic transcription factors have been identified as prospective targets for *de novo* lipogenesis and fatty acids oxidation including sterol regulatory element binding protein-1c (SREBP-1c), liver X receptor (LXRα), peroxisome proliferator-activated receptors (PPARα, δ, γ1, and γ2) and carbohydrate-responsive element-binding protein

(chREBP)[2-5]. These factors integrate signals from various pathways and coordinate the activity of the metabolic machinery necessary for hepatic lipid metabolism with the supply of energy and fatty acids.

Recently, accumulating evidence suggests that endoplasmic reticulum (ER) stress response is critically involved in hepatic lipid metabolism. The presence of ER stress is evidenced in the liver of high fat diet-induced obese mice[6,7]. The specific type of fat deposited in the liver may directly induce ER stress response and precipitate the development of non-alcoholic fatty liver disease (NAFLD)[8]. The presence of increased circulating and/or hepatic saturated fatty acids but not polyunsaturated fatty acids may exacerbate hepatic steatosis, steatohepatitis and liver cell apoptosis through activating ER stress response[9-11]. These observations implicate a crucial role for the signaling pathways from the ER in the development and progression of hepatic lipid-associated diseases.

UNFOLDED PROTEIN RESPONSE

The ER is the network of interconnected membranous structures within the cytoplasm of eukaryotic cells contiguous with the outer nuclear envelope. The ER has been primarily recognized as a compartment for protein folding and assembly, a pool of free calcium and a site for lipid and sterol biosynthesis[12]. As a protein-folding compartment, the ER provides a high-fidelity quality control system to ensure that only correctly folded proteins can be transported out of the ER while unfolded or misfolded proteins are retained in the ER and eventually degraded. Under stress conditions, such as those that disrupt protein glycosylation, disulfide bond formation, ER and calcium channels, redox/oxidative stress, nutrient deprivation or viral infections, can cause accumulation of abnormally folded proteins or unassembled subunits[13-16]. The ER has evolved a highly specific signaling pathway termed Unfolded Protein Response (UPR) to help relieve the ER from the accumulation of unfolded or misfolded proteins (Figure 1). There are three ER transmembrane proteins that function as UPR transducers: inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). It has been proposed that all three UPR transducers have the ER chaperone glucose regulate protein 78 (GRP78) bound to their ER luminal domains. This interaction allows GRP78 to act as a repressor of activation of the UPR transducers[17]. Under ER stress conditions, dissociation of GRP78 from the ER luminal domains of IRE1α, PERK and/or ATF6 allows them to be activated.

Within minutes to hours after ER stress, the UPR transducer PERK-mediated translation attenuation occurs to prevent newly-synthesized proteins entering into the ER[18,19]. The activated PERK cytosolic domain leads to translational attenuation by phosphorylating α subunit of eukaryotic translation-initiation factor 2α (eIF2α). This procedure also causes cell cycle arrest in G1 phase by translational attenuation of the proteins involved in running the cell cycle. Under ER stress, IRE1α is also activated by homodimerization and autophosphorylation. Activated IRE1α functions as an endoribonuclease to splice the mRNA encoding a basic leucine zipper (bZIP) transcription factor XBP1[20-22]. In addition to its endoribonuclease activity, activated IRE1α can serve as a scaffold protein that recruits tumor-necrosis factor (TNF)-receptor-associated factor 2 (TRAF2) leading to activation of Jun amino-terminal kinases (JNK)-mediated inflammatory stress signaling pathway[23]. Upon UPR activation, ATF6 is released from the ER membrane and transits into the Golgi compartment where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P) to release its functional fragment[24]. This fragment then moves into the nucleus and functions as a bZIP transcription factor to activate expression of UPR target genes. The primary role of the UPR is to protect the cell from ER stress by reducing the amount of proteins translocated into the ER lumen, increasing retrotranslocation and degradation of ER-localized proteins and augmenting the protein-folding capacity of the ER. However, under prolonged ER stress the role of the UPR will change from promoting cellular survival to the pathway of programmed cell death or apoptosis[25]. In recent years, the scope and consequences of ER stress and the UPR have significantly expanded. The UPR is essential for cells to augment ER protein folding capacity and remodel their secretory pathways in response to developmental demands and physiological changes[26]. In particular, the ER stress response can be triggered by metabolic factors and intrinsic feedback effectors. Prolonged or insufficient ER stress response may turn physiological mechanisms into pathological consequences[27,28]. Indeed, the UPR has been identified as fundamental cell signaling that is critical for health and disease.

UPR IN LIPOGENESIS

Recent evidence suggests that ER stress response is closely associated with lipid-associated metabolic disease. Three UPR pathways mediated through IRE1α, PERK and ATF6 were reported to be involved in the regulation of lipid metabolism. The UPR *trans-activator* XBP-1, the downstream target of IRE1α under ER stress, can regulate expression and activities of key enzymes in phospholipid biosynthesis[29]. Under ER stress, the activated form of XBP1 can increase the activity of the cytidine diphosphocholine (CDP-choline) pathway for biosynthesis of phosphatidylcholine and thus induce ER biogenesis[29,30]. Interestingly, a recent study revealed a distinguished role of XBP1 in *de novo* fatty acid synthesis in the liver[31]. The IRE1α/XBP1 UPR branch was activated in the liver of mice under the high-carbohydrate diet and directly controlled the expression of genes involved in fatty acid biosynthesis including the genes encoding acetyl CoA carboxylase 2 (Acc2), diacylglycerol acyltransferase 2 (Dgat2) and stearoyl CoA desaturase

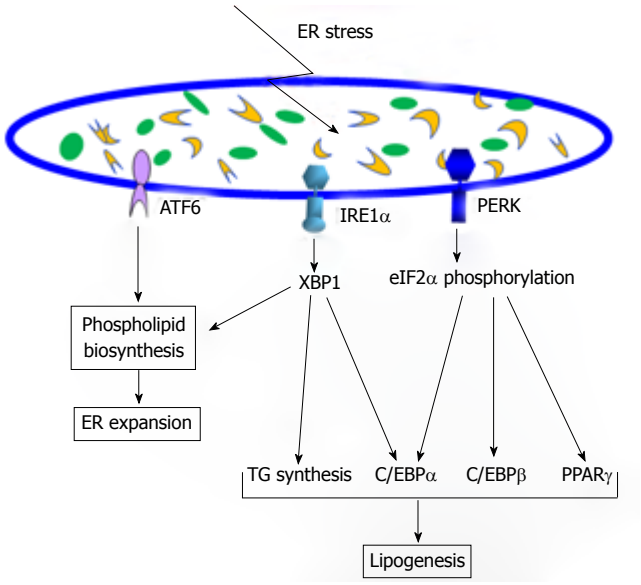

Figure 1 The involvement of the Unfolded Protein Response (UPR) signaling in lipid metabolism. The UPR pathway through inositol-requiring 1α (IRE1α)/XBP1 or activating transcription factor 6 is involved in endoplasmic reticulum (ER) expansion by enhancing phospholipid biosynthesis under ER stress conditions. The IRE1α/XBP1 pathway also regulates lipogenesis by inducing expression of the key enzymes required for triglyceride synthesis under metabolic stress. Additionally, the IRE1α/XBP1 UPR branch can drive C/EBPα expression, facilitating adipogenesis and possibly lipogenesis. The UPR pathway through PERK/eukaryotic translation-initiation factor 2α can stimulate expression of the key lipogenic regulators C/EBPα, C/EBPβ, and PPARγ, promoting lipogenesis under metabolic stress.

1 (Scd1). Deletion of XBP1 in the mouse liver caused profound hypocholesterolemia and hypotriglyceridemia which were primarily due to diminished lipogenesis. Surprisingly, the regulation of lipogenesis by the UPR component IRE1α/XBP1 was unlikely to be related to the ER stress response. The activation of XBP1 by high dietary carbohydrates and its association with other UPR components in regulating lipid metabolism remains to be further elucidated. Additionally, a recent study demonstrated that the IRE1α-XBP1 UPR pathway is indispensable for adipogenesis[32]. XBP1-deficient mouse embryonic fibroblasts and 3T3-L1 cells with XBP1 or IRE1α knockdown exhibit profound defects in adipogenesis. Intriguingly, C/EBPβ, an early adipogenic regulator, induces Xbp1 expression by directly binding to its proximal promoter region. Subsequently, spliced XBP1 binds to the promoter of C/EBPα and activates its gene expression[32]. Since C/EBPα and C/EBPβ are also key regulators of lipogenesis, the interactions between C/EBP family transcription factors and the IRE1α-XBP1 UPR pathway may play a key role in adipocyte differentiation by regulating lipid metabolism and morphological as well as functional transformations during adipogenesis.

In addition to IRE1α/XBP1, the UPR transducer ATF6 was also involved in phospholipid biosynthesis and ER expansion as well as hepatic lipid homeostasis associated with acute ER stress[33,34]. ATF6α knockout mice displayed no obvious phenotype under normal condition but showed profound hepatic steatosis under acute ER stress induced by tunicamycin challenge[34]. Acute ER Stress altered expression of genes involved in maintaining energy and lipid homeostasis. Particularly, the expression of genes encoding microsomal triglyceride transfer protein (MTTP), sterol regulatory element binding protein (SREBP)-1, peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and peroxisome proliferator-activated receptor α (PPARα) was suppressed by ER stress in both wild type and ATF6α-null animals treated with tunicamycin, but this suppression was much greater in ATF6α-null animals. Furthermore, the cytosolic lipid droplet protein marker adipose differentiation-related protein (ADRP) was more significantly up-regulated in ATF6α knockout animals compared to the control animals. The results suggested that the increased lipid accumulation in the livers of ATF6α-deficient animals was partially due to a defect in fatty acid oxidation and possibly augmented by impaired lipoprotein secretion.

The UPR branch mediated through PERK/eIF2α was also implicated in regulating lipogenesis. In the high-fat-fed mice, PERK-mediated eIF2α phosphorylation was crucial for the expression of lipogenic genes and the development of hepatic steatosis by controlling expression of C/EBP family and PPARγ transcription factors[35]. Enforced expression of the eIF2α-specific phosphatase GADD34 can lead to lower liver glycogen levels and susceptibility to fasting hypoglycemia in lean mice and glucose tolerance and diminished hepatosteatosis in the high-fat-fed mice. Attenuated eIF2α phosphorylation resulted in lower expression of PPARγ and its upstream regulators C/EBPα and C/EBPβ suggesting that the translational control through phosphorylation of eIF2α is an important regulatory mechanism for lipogenesis under physiological ER stress. Additionally, the mammary gland lipogenesis was down-regulated in PERK-deficient mammary epithelial cells. SREBP1 expression was significantly down-regulated in the PERK-deficient mammary-gland cells[36]. Therefore, PERK-mediated UPR pathway likely regulates SREBP1-related *de novo* lipid synthesis in mammary gland. Supporting the role of ER stress response in lipogenesis, ER stress has been shown to activate the lipogenic transcription factor SREBP-1 and -2 leading to modulation of the lipogenic pathways[37,38]. Consistent with this observation, over-expression of GRP78/BiP, the master negative regulator of the UPR, in the liver of obese (*ob*/*ob*) mice can inhibit SREBP-1c cleavage and the expression of SREBP-1c and SREBP-2 target genes[39]. Hepatic triglyceride and cholesterol con tents were reduced and insulin sensitivity was improved in GRP78-over-expressed mice. Together, these studies confirmed crucial roles of the UPR pathways in lipogenesis and the pathogenesis of lipid-associated metabolic disease.

CONCLUSION

A growing body of evidence suggested that UPR is critically involved in lipogenesis. The UPR pathways may represent attractive targets for future therapeutic intervention in modulating lipid metabolism associated

with metabolic diseases. Indeed, recent studies demonstrated that small chemical chaperones such as 4-phenylbutyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) can reduce ER stress in liver and adipose tissues and thus enhance insulin sensitivity and glucose tolerance in mouse models of severe obesity and type 2 diabetes (*ob*/*ob*)[7,40]. For future studies it is important to elucidate ER stress-associated mechanisms in regulating lipid metabolism under metabolic conditions. The resultant information will be important for designing novel strategies for the prevention and treatment of metabolic disease.

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REVIEW

Physiopathology of splanchnic vasodilation in portal hypertension

María Martell, Mar Coll, Nahia Ezkurdia, Imma Raurell, Joan Genescà

María Martell, Mar Coll, Nahia Ezkurdia, Imma Raurell, Joan Genescà, Liver Diseases Laboratory, Liver Unit, Department of Internal Medicine, Hospital Universitari Vall d' Hebron, Institut de Recerca, Universitat Autònoma de Barcelona, Barcelona 08035, Spain
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Correspondence to: Maria Martell, PhD, Liver Diseases Laboratory p006, Institut de Recerca Hospital Vall d'Hebron, Pg Vall d'Hebron 119-129, Barcelona 08035, Spain. mmartell@ir.vhebron.net
Telephone: +34-93-4894034 Fax: +34-93-4894032
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Abstract

In liver cirrhosis, the circulatory hemodynamic alterations of portal hypertension significantly contribute to many of the clinical manifestations of the disease. In the physiopathology of this vascular alteration, mesenteric splanchnic vasodilation plays an essential role by initiating the hemodynamic process. Numerous studies performed in cirrhotic patients and animal models have shown that this splanchnic vasodilation is the result of an important increase in local and systemic vasodilators and the presence of a splanchnic vascular hyporesponsiveness to vasoconstrictors. Among the molecules and factors known to be potentially involved in this arterial vasodilation, nitric oxide seems to have a crucial role in the physiopathology of this vascular alteration. However, none of the wide variety of mediators can be described as solely responsible, since this phenomenon is multifactorial in origin. Moreover, angiogenesis and vascular remodeling processes also seem to play a role. Finally, the sympathetic nervous system is thought to be involved in the pathogenesis of the hyperdynamic circulation associated with portal hypertension, although the nature and extent of its role is not completely understood. In this review, we discuss the different mechanisms known to contribute to this complex phenomenon.

Key words: Liver cirrhosis; Portal hypertension; Splanchnic vasodilation; Hyperdynamic circulation; Sympathetic nervous system

Peer reviewer: Radha Krishan Dhiman, MD, DM, FACG, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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INTRODUCTION

Portal hypertension is defined as a pathological increase in portal vein pressure and it is diagnosed when the hepatic venous pressure gradient (HVPG) is above the normal range (1-5 mm Hg). HVPG is assessed by a hepatic hemodynamic study through a suprahepatic vein catheterization and estimates the difference of pressure between the portal vein and the inferior cava vein. Liver cirrhosis is the most frequent cause of portal hypertension in western countries. When HVPG increases to 10 mm Hg or more, portal hypertension of cirrhosis results in severe complications including ascites, hepatorenal syndrome, hepatic encephalopathy and haemorrhage from esophageal varices[1,2]. Two main factors contribute

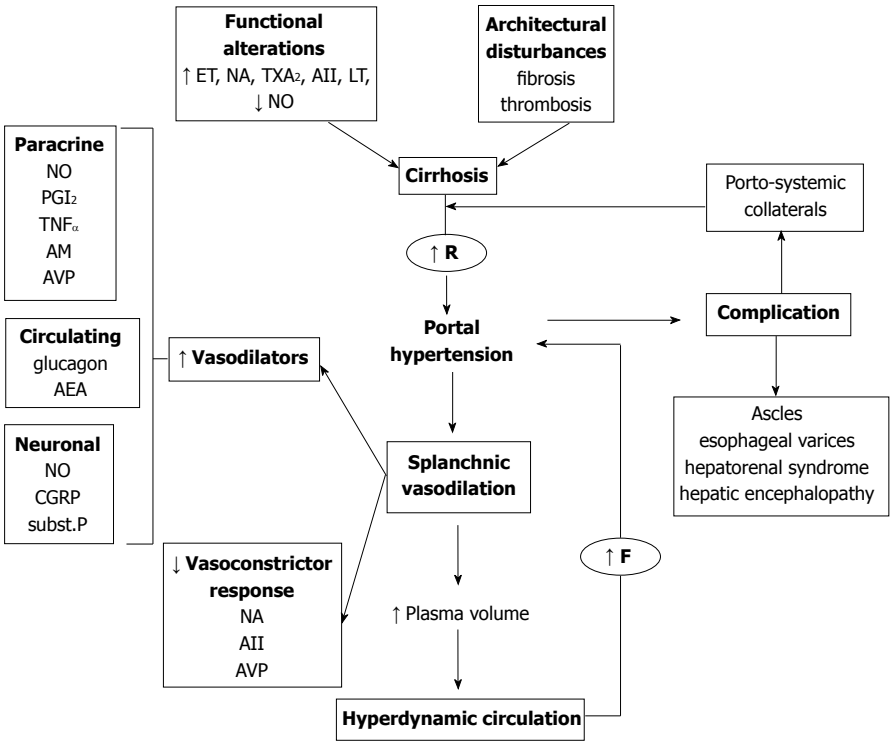

Figure 1 Physiopathology of portal hypertension. In cirrhosis, the initiating factor leading to portal hypertension is an increase in intrahepatic vascular resistance (R), whereas the increase in portal blood flow (F) is a secondary phenomenon that maintains and worsens the increased portal pressure, giving rise to the hyperdynamic circulation syndrome. The different factors implicated in the distinct mechanisms of portal hypertension are shown. AⅡ: angiotensin Ⅱ; AEA: anandamide; AM: adrenomedullin; CGRP: calcitonine gene related peptide; CO: carbon monoxide; ET: endothelin; H_2S: hydrogen sulfide; LT: leukotrienes; NE: norepinephrine; NO: nitric oxide; PGI_2: prostacyclin; SP: substance P; TXA_2: thromboxane A_2.

to establish and maintain portal hypertension: the vascular resistance due to difficult outflow of portal blood to the hepatic veins and the increased splanchnic blood flow (hyperdynamic syndrome). Portal hypertension is also associated with the formation of porto-systemic venous collaterals in an attempt to decompress the portal venous system[3,4]. However, this collateral circulation leads to the generation of varices which contribute to the morbidity and mortality of the disease.

PHYSIOPATHOLOGY OF PORTAL HYPERTENSION

Applying the Ohm's law in the portal venous system: $\Delta P = Q \times R$, then the portal pressure gradient (ΔP), is the result of the product of the blood flow within the entire portal venous system (Q) and the vascular resistance of the same vascular system (R), including the hepatic vascular bed and the porto-systemic collaterals. Thus, portal hypertension is caused by an increase in blood flow, an increase in resistance or a combination of both. The initial mechanism that leads to portal hypertension in liver cirrhosis is an increase in hepatic resistance, mainly as a result of a mechanical occlusion. In later stages, an increase in splanchnic blood flow leads to the hyperdynamic circulation state, which in turn contributes to the maintenance and aggravation of many of the complications of cirrhosis and portal hypertension (Figure 1)[5].

INCREASED VASCULAR RESISTANCE TO PORTAL BLOOD FLOW

The vascular resistance to portal blood flow is dependent on two factors: the intrahepatic resistance and the resistance generated by the collateral circulation. The increased intrahepatic vasculature resistance (IHVR) to portal blood flow is the main and primary factor of portal hypertension secondary to liver cirrhosis (Figure 1).

Intrahepatic resistance

Classically, structural distortion of the intrahepatic vasculature, as a consequence of fibrosis, scaring and vascular thrombosis, has been considered the only cause of the increased IHVR. The cellular mechanisms involved in fibrosis formation and cirrhosis are well known. In response to hepatocellular injury, hepatic stellate cells are activated and their phenotype changes from a quiescent one to a myofibroblast-like cell. As a result of hepatic stellate cell activation, collagenization (capillarization) of the Space of Disse occurs, and the injured liver becomes cirrhotic[6,7]. The pioneering work by Bathal and Groszmann[8], based on a perfused rat liver model, demonstrated that in addition to the structural changes, a dynamic component, represented by contractile elements of the hepatic vascular bed, might contribute to the increased intrahepatic vascular tone. It has been suggested that this modifiable component represents 40% of the total IHVR[9]. In cirrhosis, an increased production of vasoconstrictors and a deficient release of vasodilators, in combination to an exaggerate response to vasoconstrictors and an impaired vasodilatory response of the hepatic vascular bed, are the mechanisms responsible for the increased dynamic component of IHVR[10].

Among all overexpressed vasoconstrictors[11-14], endothelin (ET) seems to play a particularly important role in the enhanced vascular tone in liver cirrhosis. Patients with liver cirrhosis present elevated ET-1 and ET-3 plas-

ma concentrations[15]. Moreover, not only hepatic ET-1 levels, but also ET receptor density are increased in the cirrhotic rat liver[16]. The ET_A receptor, found on vascular smooth muscle cells, causes vasoconstriction, whereas the ET_B receptor subtype located on endothelial cells induces vasorelaxation by stimulating endothelial nitric oxide synthase (eNOS)[17]. Several studies have been focused on ET blockade therapies. However, in contrast to what might be expected, ET_B receptor stimulation by ET_B agonist administration, resulted in an increased portal pressure in cirrhotic rats[18]. The effect of ET_A antagonists in reducing portal pressure of cirrhotic rats remains controversial[18,19]. In addition to endothelin, other contributing factors to the increased IHVR are the products of S-lipoxygenase (cysteinyl-leukotriene) and the cyclooxygenase pathways (thromboxane A2), angiotensin Ⅱ and the sympathetic system[11,12,14].

In addition to the exaggerated production of vasoconstrictors, the intrahepatic production of vasodilators, mainly nitric oxide (NO), remains insufficient in the cirrhotic liver[20,21]. NO is a potent vasodilator produced from L-arginine by different NOS. Although in the liver both eNOS and inducible NOS (iNOS) isoforms can be active, the insufficient hepatic NO production observed in cirrhosis has been attributed to the endothelial isoform[22,23]. Because mRNA and protein levels of eNOS are found in equal amounts in cirrhotic and normal livers, this NO-deficient production has been attributed to a post-translational dysfunction in eNOS activity[20,24,25]. On one hand, an increased expression of caveolin (an eNOS inhibitory protein)[24], and on the other hand, a decrease in eNOS phosphorilation due to abnormal Akt (protein kinase B) signalling[26], are the mechanisms that might explain the reduced eNOS activity in liver cirrhosis. In addition to a decreased NOS activity, an increased NO degradation has also been suggested to be responsible for the diminished NO bioavailability. Since superoxide (O_2^-) is able to react with NO to generate peroxinitrite ($ONOO^-$), NO bioavailability can be substantially reduced if the O_2^- levels are increased as a consequence of a decrease in superoxide dismutase activity. Indeed, a portal injection of adenovirus containing superoxide dismutase encoding gene reduces portal pressure by increasing NO bioavailability in cirrhotic rats[27].

Collateral circulation

The increase in portal pressure leads to the appearance of direct connections between the portal blood vessels and the general circulation. This attempt to decompress the portal venous system leads to severe complications, such as hepatic encephalopathy and the formation of esophageal varices. Taking into account that porto-systemic shunting diverts a large quantity of portal blood flow away from the liver, the vascular resistance of these vessels might contribute importantly to increasing vascular resistance of the portal venous system. Porto-systemic collaterals formation, which involves both neovascularisation and opening existing vessels[28,29], has been suggested to be angiogenic-dependent. Angiogenesis is mediated mainly by the vascular endothelial growth factor (VEGF). Fernandez *et al* demonstrated that anti-VEGF receptor-2 monoclonal antibody prevented porto-systemic collateral vessel formation in portal hypertensive mice[30,31]. Furthermore, since NAD(P)H is required for VEGF-induced angiogenesis, NAD(P)H oxidase blockade significantly reduced porto-systemic collateral formation[32]. The same authors have demonstrated that portal hypertensive rats treated with signalling inhibitors of VEGF and platelet derived growth factor (PDGF) significantly reduce their porto-systemic collaterization[33]. Also, the use, in experimental rat models of portal hypertension, of Sorafenib, a potent inhibitor of proangiogenic VEGF receptor-2 and PDGF receptor-β, induced an important decrease in splanchnic neovascularisation and in the extent of porto-systemic collaterals, along with a marked attenuation of hyperdynamic splanchnic and systemic circulations[34].

INCREASED SPLANCHNIC BLOOD FLOW

The splanchnic circulation is the main vascular bed responsible for the reduction in vascular resistance in the portal hypertensive state. An increase in splanchnic blood flow in portal hypertension is the result of a marked vasodilation of arterioles in splanchnic organs, which drain blood into the portal venous system[35]. The increase in blood flow in splanchnic organs and the subsequent increase in portal venous inflow, together with an increased resistance to portal inflow, maintains and aggravates the portal hypertensive syndrome[9]. An increased production or activation of vasodilatory mediators and systems, and a decreased vascular reactivity to vasoconstrictors (Figure 1), are probably responsible for this splanchnic hyperaemia (vasodilation). In addition, increased angiogenesis probably collaborates in increasing the splanchnic blood inflow[30,31].

Hyperdynamic circulation

The hyperdynamic circulatory state of portal hypertension is characterized by splanchnic and peripheral vasodilation, increased plasma volume and increased cardiac output[5]. The hyperdynamic splanchnic circulation is mediated in part by arterial vasodilation, but this vasodilation alone is not sufficient to cause the circulation to become hyperdynamic. It is the combination of arterial vasodilation and blood volume expansion that produces optimal conditions for maintaining the hyperdynamic circulatory state in portal hypertension[35,36] (Figure 1). The arterial vasodilation in the peripheral and splanchnic circulation leads to a decrease in central blood volume. This relative arterial hypovolemia leads to the stimulation of cardiopulmonary volume receptors and arterial baroreceptors, activating the sympathetic nervous system, the renin-angiotensin-aldostern system and arginin-vasopresin (antidiuretic hormone). Mediators from these

systems result in sodium and water retention by the kidneys, and consequently, plasma volume expansion. Sodium retention is due to increased tubular reabsorption of sodium, mediated by receptors for aldosterone, angiotensin and alpha-adrenergic stimuli. The decrease in water excretion is due to increased secretion of antidiuretic hormone[37].

The harmful effects of hyperdynamic circulation are not restricted to the hepatosplanchnic circulation. The hyperdynamic circulation also affects the cardiac (increase cardiac output), the pulmonary (hepatopulmonary syndrome) and the cerebral circulation (acute hepatic coma)[38,39]. Other organs such as the kidney and the brain (chronic encephalopathy) appear to be indirectly affected by the vasodilation in the other circulatory beds[5].

Animal models

The development of experimental models to study the hemodynamic alterations of portal hypertension has been of critical importance for the understanding of this syndrome. The pioneering work of Chojkier and Groszmann in establishing the partial portal-vein ligated (PVL) model has been a basic element in understanding portal hypertension pathophysiology[3,40]. In this model, the portal vein is isolated and a stenosis is created by a single ligature around a 20-gauge blunt-tipped needle lying along the portal vein. Subsequent removal of the needle yields a calibrate stenosis of the portal vein.

The PVL model reproduces all systemic and hemodynamic abnormalities detected in portal hypertension and the circulatory hyperdynamic state: portal pressure and portal flow increase, appearance of porto-systemic shunts, splanchnic vasodilation with splanchnic arteriolar resistance reduction and splanchnic flow increase, systemic vasodilation with arterial hypotension, total peripheral resistance reduction and cardiac output increase[40]. This model is extraordinarily homogenous, reproducible and has highly predictable chronobiology that permits the elucidation of the sequence of events involved in the generation of the hyperdynamic syndrome[41,42]. Porto-systemic shunting is detectable at two days after PVL surgery and the percentage of portal blood inflow diverted to collaterals approaches 100% after 1 wk[42]. Circulation becomes hyperdynamic 4-5 d after PVL, and 1 wk after portal vein ligation, rats present the complete range of portal hypertensive alterations with hyperdynamic circulatory syndrome and porto-systemic shunting formation.

Although the PVL model is easy to use and reproducible, the experimental rat models of cirrhosis generated by different mechanisms (basically by carbon tetrachloride administration and bile duct ligation) are probably more similar to human cirrhosis, since in addition to displaying all the hemodynamic alterations of portal hypertension, they present the metabolic, infectious and other complications of advanced liver disease[36,43]. Results obtained in PVL rats are usually tested in these models of cirrhosis.

MECHANISMS OF SPLANCHIC VASODILATION

The arterial vascular tone is determined by the balance between the effects of vasoactive molecules acting on the vascular smooth muscle. As mentioned, an increased concentration of circulatory vasodilators and an enhanced endothelial production of local vasodilators, as well as a decreased vascular responsiveness to endogenous vasoconstrictors have been observed in splanchnic vessels in portal hypertension[36] (Figure 1). Among the molecules and factors known to be potentially involved in this arterial vasodilation, none of them can be described as solely responsible, since this phenomenon is multifactorial in origin[44].

Nitric oxide

NO, an endothelial-derived relaxing factor, has been recognized as the most important vasodilator molecule that mediates the excessive arterial vasodilation observed in portal hypertension[45]. Its involvement, initially suggested by Vallance and Moncada[46], has been confirmed by a number of studies. In cirrhotic patients, increased levels of nitrates and nitrites, degradation products of NO oxidation[47], have been observed. In the splanchnic vascular bed of rats with portal hypertension an overproduction of NO responsible for vasopressor hyposensitivity has been clearly demonstrated[48]. Furthermore, inhibition of NO production reduces porto-systemic shunting and largely prevents the development of the hyperdynamic circulation[49]. NO is produced from L-arginine by the family of NOS enzymes, forming the free radical NO and citrulline as byproducts[50]. NO has a short life and is rapidly oxidized to the stable, inactive end-products, nitrite and nitrate[51]. The mechanism by which NO causes vasodilation is through the stimulation of soluble guanylyl cyclase (sGC) to generate cyclic guanosine monophosphate (cGMP) in vascular smooth muscle[52] (Figure 2). Three isoforms are known to produce NO: constitutively expressed isoforms, eNOS[53] and neuronal NOS (nNOS)[54], and iNOS[55] which, surprisingly, does not appear to be involved in the increased NO production in cirrhosis[56]. The major enzymatic source of the vascular NO overproduction has been shown to be eNOS[57]. In animal models (PVL rats) at least, it has been observed that eNOS upregulation precedes the hyperdynamic circulatory changes[45]. More recent evidence suggests that nNOS is also upregulated in aorta[58] and mesenteric arteries[59], playing a role in the development/maintenance of the hyperdynamic splanchnic circulation in experimental cirrhosis.

In endothelial cells, eNOS is activated by calcium/calmodulin (Ca^{2+}/CaM) in response to an elevation of cytosolic Ca^{2+} and by phosphorilation of eNOS at several sites[60,61]. Initial up-regulation of eNOS starts at the post-translational level by Akt-mediated eNOS phosphorilation[62], which increases its activity at any Ca^{2+} concentration[63]. During early cirrhosis, this pathway is

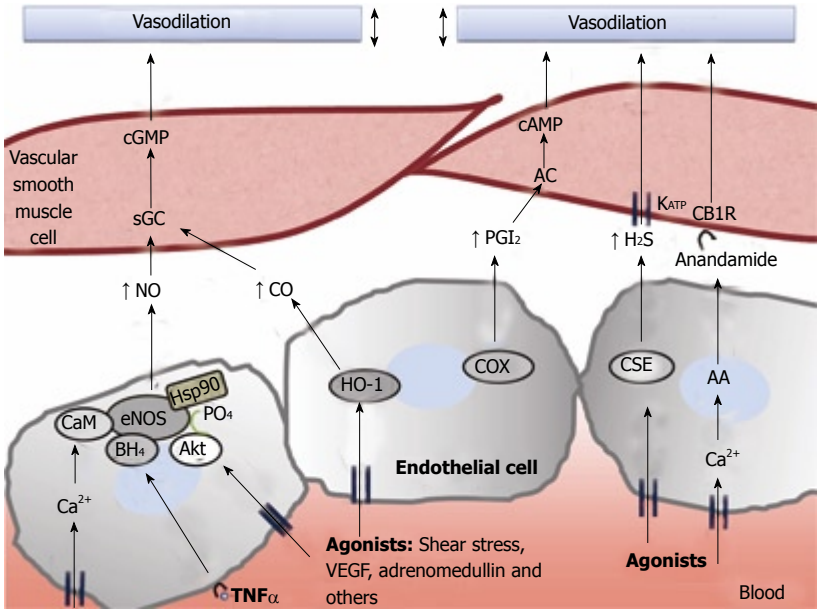

Figure 2 Molecular pathways associated to splanchnic vasodilation. Vasoactive molecules involved in the regulation of vascular tone in the arteries of the splanchnic circulation. Nitric oxide (NO), carbon monoxide (CO), prostacyclin (PGI_2) or hydrogen sulfide (H_2S), generated through different pathways in endothelial cells, cause vasodilation in vascular smooth muscle cells by either activating soluble guanylate cyclase (sGC) to generate cyclic guanosine monophosphate (cGMP), by stimulating adenylate cyclase (AC) and generation of cyclic adenosine monophosphate (cAMP) or through the opening of K_{ATP} channels. Also, anandamide activates endothelial cannabinoid 1 receptors (CB1R) provoking vasodilation. AA: arachidonic acid; AC: adenylyl cyclase; Akt: protein kinase B; BH4: tetrahydrobiopterin; CaM: calmodulin; CSE: cystathionine-γ-lyase; COX: cyclooxygenase; eNOS: endothelial nitric oxide synthase; HSP90: heat shock protein 90; IP3: inositol triphosphate; TNFα: tumor necrosis factor α; VEGF: vascular endothelial growth factor.

stimulated by different forms of stimuli such as vascular endothelial growth factor (VEGF), inflammatory cytokines, and mechanical forces by shear stress[63-65]. This latter mechanism involves an increased interaction of eNOS with the positive regulator molecular chaperone heat shock protein 90 (Hsp90)[66]. Later, in advanced stages of portal hypertension, bacterial translocation activates eNOS through a tumor necrosis factor-α dependent increase in tetrahydrobiopterine, an essential cofactor of eNOS[67,68] (Figure 2). It is worth remarking that, according to several studies, other mechanisms such as changes in subcellular localization of eNOS[69], S-nitrosilation[70,71] or asymmetric dimethylarginine degrading enzyme might be involved in the regulation of eNOS activity[72].

In summary, different mechanisms such as complex protein-protein interactions and posttranslational modifications have been reported to up-regulate eNOS in portal hypertension[73].

Other paracrine vasodilators

In addition to NO, other local paracrine/autocrine vasodilators have been described as possibly being involved in the pathogenesis of the hyperdynamic circulation associated with portal hypertension (Figures 1 and 2).

Carbon monoxide: Carbon monoxide (CO) is a gaseous molecule produced by heme oxygenase (HO) during heme metabolism to biliberdin Ⅸ[74]. CO, in a similar manner to NO in cirrhosis, is believed to relax smooth muscle cells through the activation of NO-dependent sGC, resulting in an increased production of cGMP. Although CO is a far less potent mediator than NO[75], a role in vasodilation of portal hypertension has been suggested[76]. CO-induced vasodilation can also occur *via* Ca^{2+}-activated potassium channels[77]. In portal hypertension, an inducible isoform of HO, HO-1, has been shown to be up-regulated in systemic and splanchnic arterial circulation[78], although the mechanisms of activation remain to be fully understood.

Prostacyclin (PGI_2): Prostacyclin is synthesized by cyclooxygenase and released from the endothelium to promote smooth muscle relaxation by activating adenylyl cyclase and augmenting the intracellular level of cyclic adenosine monophosphate[79]. Increased levels of circulating PGI_2 have been observed in patients with cirrhosis[80] and in portal hypertensive rabbits[81], supporting a role for prostaglandins in the pathogenesis of the hyperdynamic circulatory syndrome.

Hydrogen sulfide (H_2S): Recent evidence has suggested a role for H_2S, a potent vasodilator, in the development of hyperdynamic circulation in cirrhosis[82]. This is based on the observation that in cirrhosis, endotoxaemia leads to upregulation of the enzyme cystathionine-γ-lyase, responsible for H_2S production, which causes vasodilation through the opening of K_{ATP} channels[83].

Circulating vasodilators

Early studies in the physiopathology of portal hypertension focused on the role of circulating vasodilator substances of splanchnic origin accumulated as a consequence of reduced hepatic metabolism and/or increased porto-systemic shunting. The strongest evidence is for glucagon, whereas other substances described here have not been extensively investigated[84,85].

Glucagon: Numerous studies have demonstrated elevated plasma glucagon levels in patients with cirrhosis and in portal hypertensive rat models. Glucagon seems to promote vasodilation by relaxing the vascular smooth muscle and decreasing its sensitivity to endogenous vasoconstrictors, although the exact mechanism remains to be elucidated[86].

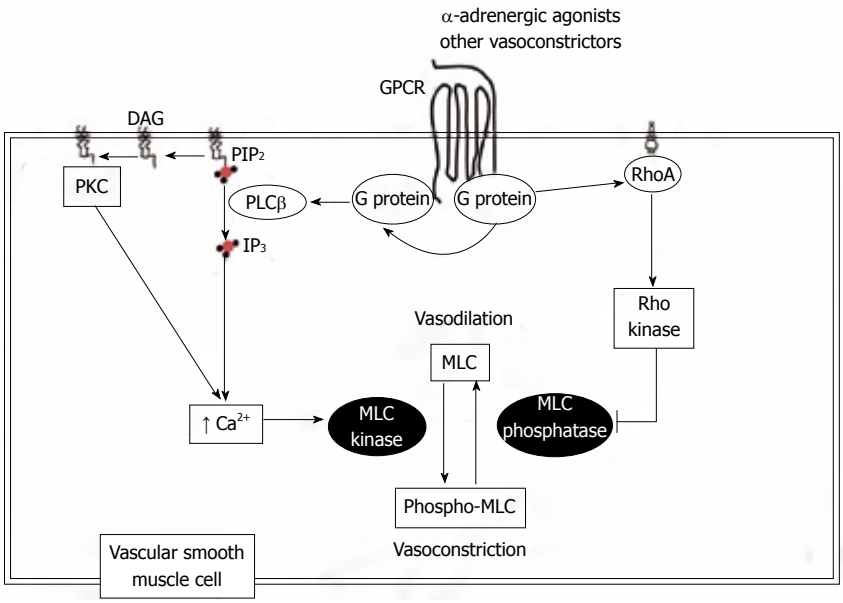

Figure 3 Vasodilation/contractile signaling in vascular smooth muscle cells. The contractile state of vascular smooth muscle depends on the phosphorilation state of myosin light chains (MLCs). Under normal conditions, contractile agonists activate G protein couple receptors (GPCR). These receptors subsequently activate downstream effectors such as phospholipase C (PLC) and GTPase RhoA, leading to the increase of MLC phosphorilation *via* the activation of MLC kinase or the inhibition of MLC phosphatase. DAG: diacylglycerol; IP3: inositol triphosphate; PIP_2: Phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C.

Endocannabinoid: The contribution of the endocannabinoid system in the development of splanchnic vasodilation has been described in several studies proposing various mechanisms. The main endocannabinoid mediator is anandamide, a product of arachinoid acid metabolism. Endocannabinoids activate endothelial cannabinoid 1 receptors and vanilloid receptor 1 causing pronounced vasodilation in BDL rats[87]. Anandamide levels are increased in monocytes in cirrhosis and over-activation of cannabinoid 1 receptors induce mesenteric NO production by eNOS in mesenteric vessels from portal hypertensive rats[87,88].

Adrenomedullin: In a similar way to endocannabinoids, increased peptide adrenomedullin levels have been found in plasma of cirrhotic rats[89] and patients[90]. Adrenomedullin is a vasoactive peptide known to contribute to enhancement of eNOS activity causing vasodilation. This peptide phosphorylates and activates Akt and increases cGMP production in rat aorta, probably promoting vasorelaxation through production of NO[91].

Endothelium-derived hyperpolarizing factor: Endothelium-derived hyperpolarizing factor (EDHF) has been shown to be an important endothelium-dependent vasodilator in resistance vessels of eNOS knockout mice[92]. Its role becomes more significant when the production of NO is inhibited, because NO seems to inhibit the release of EDHF[93].

Other endogenous humoral vasodilators including atrial-natriuretic peptide, whose levels tend to increase in advanced stages of liver cirrhosis with ascites[94], adenosine, histamine, bile salts, calcitinin gene related protein (CGRP) and substance P, have been proposed to play a role in the arterial vasodilation in portal hypertension[95] (Figure 1).

Contracting signalling alterations

In cirrhosis and portal hypertension, the majority of the vessels are dilated despite systemic activation of vasoconstrictors[96-98]. This splanchnic resistance to vasoconstrictor agents can be attributed to vascular hyporesponsiveness[99,100], explaining why the hyperdynamic circulation increases with progression of the disease despite the stimulation of renin-angiotensin, sympathetic nervous system and vasopressin release. Impaired responsiveness to vasoconstrictors is involved both in the increased vasodilation of splanchnic territories and in vasoconstriction of essential end-organs, triggering the severe complications of cirrhosis.

The contractile state of vascular smooth muscle depends essentially on myosin light chain (MLC) phosphorylation and is regulated via activation of MLC kinase or inhibition of MLC phosphatase[101,102] (Figure 3). In contrast, pathways leading to vasorelaxation decrease MLC phosphorylation *via* deactivation of MLC kinase or activation of MLC phosphatases[103-106]. All vasoconstrictor receptors belong to the superfamily of guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCR). Stimulation of GPCR on the vascular smooth muscle cell activates G proteins and consequently their down stream effectors, phospholipase C β (PLCβ) and the small GTPase, RhoA. PLCβ hydrolyzes phosphatidylinositol 4,5-biphosphate into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses in the cytosol and DAG remains in the plasma membrane activating protein kinase C. Both products cause an increase in intracellular calcium in vascular smooth muscle cells. The released calcium initiates a cascade of intracellular events, causing MLC phosphorilation and resulting in cross-bridging of actin and myosin, leading to contraction[101,102]. In addition, the parallel cascade of G-protein-induced RhoA activation subsequently activates Rho kinase causing inhibition of MLC phosphatase, enhanced MLC phosphorilation and eventually vascular contraction.

In several studies both in animal models and human tissues the diminished contractile response to α_1-ad-

renergic agonists or other vasoconstrictors persisted after removal of the endothelium or pharmacological inhibition of endogenous NO production. It is also known that vascular hyporeactivity is not caused by a down regulation of receptors to most relevant endogenous vasoconstrictors or by a decrease in their affinity. These vasoconstrictor receptors are actually increased in the hepatic artery. Therefore, the defective contractile signalling should be at the subreceptor level[107,108]. Recent evidence suggests that during portal hypertension these contracting signalling pathways are altered early after receptor stimulation, most probably at the level of Gα effectors. In rats with secondary biliary cirrhosis induced by bile duct ligation, it has been observed that impaired response to α-adrenoreceptor stimulation involves a reduced activation of PLCβ and consequently, a diminished formation of inositol phosphates[109], as well as reduced activation of RhoA with subsequently defective Rho kinase activation[110]. Moreover, this impairment in PLCβ and RhoA activation is resistant to endothelium denudation or pharmacological NOS inhibition[109,110], supporting the existence of defects in receptor-mediated activation of contraction.

The impaired response to contractile agonists occurring in portal hypertension has been also explained by desensitization of GPCRs by receptor-desensitising proteins, namely G-protein-coupled receptor kinase 2 (GRK-2) and β-arrestin 2. These receptor-desensitising proteins have been found to be up-regulated in aortas from BDL rats as well as in hepatic arteries from patients with cirrhosis, inducing desensitisation of angiotensin II receptor[111]. Moreover, it is known that the GRK-2/β-arrestin 2 system also induces desensitization of a variety of different receptors and that GRK-2/β-arrestin 2 mediated receptor desensitisation is initiated in response to exaggerated receptor stimulation[112,113]. It seems possible that elevated plasma levels of angiotensin Ⅱ and catecholamines, which are well established in cirrhosis, are responsible for the onset of these processes in hypocontractile vessels[114,115].

Another observation contributing to the understanding of the dysregulation of contractile signalling in portal hypertension has come from the recent studies on increased release and enhanced effect of neuropeptide Y (NPY) on adrenergic mesenteric contraction in PVL rats[116]. By itself, NPY mediates no direct vasoconstriction, but potentiates NE-evoked vasoconstriction in the mesenteric vasculature through the Y1 specific receptor. Enhanced release of NPY may represent a compensatory mechanism to counterbalance arterial vasodilation by restoring the efficacy of endogenous catecholamines, especially in states of high alpha1-adrenergic activity.

Nervous system and portal hypertension

Histological studies have revealed that vascular smooth muscle is innervated by neurons containing NOS immunoreactivity[54], as well as by those containing tyrosine hydroxylase and choline acetyltransferase[117]. These efferent post-ganglionic neurons, identified as nitrergic, noradrenergic and cholinergic, control vasoconstriction of vascular smooth muscle cells from blood vessels. Functionally, nitrergic nerves are more important in vascular tone control than cholinergic nerves, which only play a role in modulating adrenergic and nitrergic nerve functions[118].

In the mesenteric circulation, both in humans and rodents, vasoconstriction induced by the sympathetic nervous system (SNS) is mainly mediated by post-synaptic α_1-adrenoreceptors[119]. Indeed, α_1-adrenoreceptor stimulation is the major mechanism through which the SNS regulates vascular tone. It has been shown that stimulation of perivascular nerves in blood vessels evokes vasoconstriction. This vasoconstriction is blocked by tetrodotoxin (neurotoxin), prazosin (α_1-adrenoceptor antagonist), guanethidine (adrenergic neuron blocker) or 6-hydroxydopamine (neurotoxin that destroys adrenergic neurons)[120,121]. Thus, the vascular tone of peripheral blood vessels might be controlled mainly by sympathetic adrenergic nerves through the release of the neurotransmitter norepinephrine (NE). Moreover, different investigations have also shown that other agents like NPY and adenosine triphosphate are also released in the SNS, acting as co-transmitters of NE and potentiating its action[122].

There are a large number of publications evaluating the role of SNS in human cirrhosis. Increased systemic levels of catecholamines have been found in many studies, tending to increase when liver disease worsens[114,115]. These elevated levels are a result of an increased production of NE (increased plasma levels of NE, spillover of NE from the neuroeffector junctions and muscle sympathetic nervous activity), rather than a decreased clearance[123,124]. However, the origin of this SNS-hyperactivity is not homogeneous, since there are organs or tissues in which increased NE production has not been found. One of the main sites of NE overproduction is the kidney[124,125]. Another important site of NE production is muscle, with many studies showing increased muscle sympathetic nerve traffic[126,127]. There are also regional differences, the upper limb seems to release increased amounts of NE, but the lower limb does not[125,128]. Also, in contrast to the increased sympathetic nerve traffic found in muscles, the skin seems to present a normal level of sympathetic activity[127].

It seems quite clear that the adrenergic system plays a role in the cardiovascular, homeostatic and metabolic dysfunction present in advanced liver disease and that in cirrhosis and portal hypertension there is a global overactivity of this system. What is more questionable is whether this SNS-hyperactivity takes place everywhere and especially in mesenteric vessels. In this regard, our group has recently demonstrated an important down-regulation, both at the transcriptional and translational level, of many proteins implicated in adrenergic neurotransmission in the superior mesenteric artery from PVL and cirrhotic rats[129]. This adrenergic inhibition is accompanied by a remarkable regression/atrophy of the

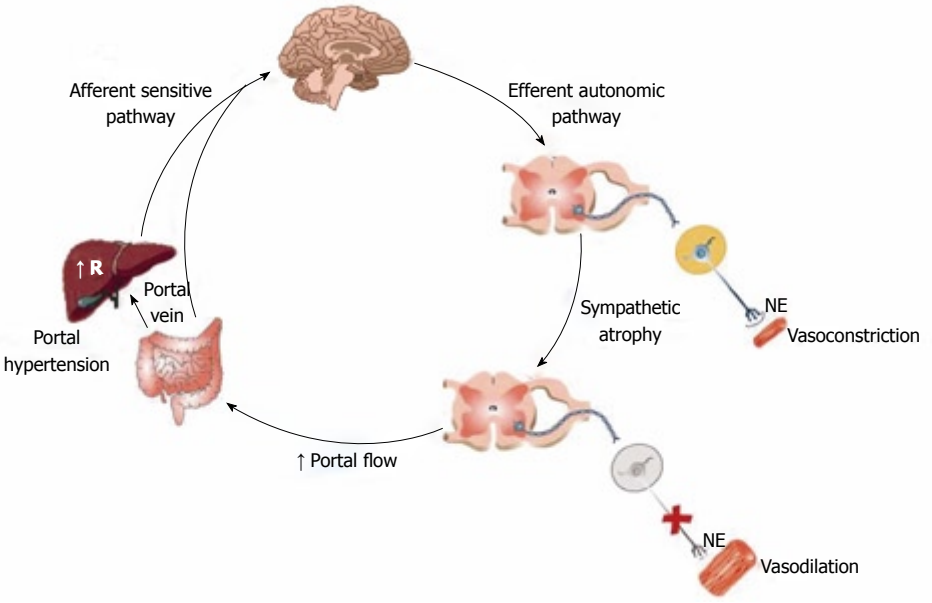

Figure 4 Hypothesis regarding the mechanisms and effects of the sympathetic post-ganglionic atrophy in splanchnic vasodilation. The afferent stimulus of portal hypertension, originating from pressure increases in portal or mesenteric vessels or microvasculature, reaches the brain stem cardiovascular nuclei through the afferent nerves. From there, post-ganglionic sympathetic nerve regression are mediated by efferent sympathetic nerves, leading to neurotransmission inhibition and vasoconstriction impairment mediated by norepinephrine (NE).

sympathetic innervation in the whole mesenteric vascular bed. However, this nervous atrophy is not present in other vascular territories such as the renal arteries[130]. The down-regulation of the mesenteric adrenergic system has been interpreted as a local consequence of portal hypertension that might contribute to aggravating splanchnic vasodilation, which is responsible for a generalized sympathetic overactivity, especially in muscles and kidneys. The observation that alpha-adrenergic agonists, such as norepinephrine and midodrine, are effective in the treatment of hepatorenal syndrome[131,132], the ultimate consequence of arterial vasodilation in cirrhosis, suggests that, at least in some areas, the adrenergic activity rather than overactivated, might be suppressed. The recent observation that NPY restores adrenergic superior mesenteric artery hyporeactivity in PVL rats[116], would also point to a deficient local adrenergic tone in portal hypertension. Also, Joh and co-workers[133] have demonstrated that using antagonists to α-adrenergic receptors, the response to vasoconstrictor blockade in portal hypertensive animals differed drastically from normal rats. Unlike the response of normal rats, α-adrenergic blockade produced essentially no change in intestinal microvascular dimensions, while vasopressin or angiotensin II blockade was associated with arteriolar dilation. These data suggest that loss of adrenergic vascular tone could be a very important functional vasoconstrictor defect in portal hypertension.

The neural pathway controlling the cardiovascular system includes the primary afferent innervation (sensory neurons), the brain stem medullary cardiovascular nuclei, and the effector arm composed of sympathetic and parasympathetic efferent nerves[134,135]. Considering this system, the signal responsible for the post-ganglionic sympathetic nerve regression suggested by our studies probably originates in preganglionic neurons or other neurons with a synaptic connection to post-ganglionic neurons. The afferent stimulus originating from pressure increases in portal or mesenteric vessels or microvasculature would reach the central nuclei through the afferent nerves and from there to the sympathetic ganglia[136,137] (Figure 4). In this context, it is important to mention that several studies suggest that by pharmacologically eliminating the primary afferent nerves by capsaicin administration, the development of hemodynamic alterations is prevented as well as ascites formation, in PVL and cirrhotic rats[138-141]. In addition, these afferent sensory nerves once activated by peripheral stimuli can also release the transmitter content (the vasodilator peptides substance P and CGRP) from their peripheral terminals in innervated tissue to elicit functional responses[142]. It has been described that periarterial nerve stimulation in rat mesenteric resistance arteries produces neurogenic vasodilation mediated by CGRP[143], and that CGRP release suppresses sympathetic nerve mediated vasoconstriction[143]. Finally, different studies showing high levels of substance P and CGRP in patients with cirrhosis and liver failure have suggested that these neuronally generated vasodilators could play a role in splanchnic vasodilation of portal hypertension[144,145].

Perivascular presence of nNOS–containing nerves, so called nitrergic nerves, has been demonstrated in numerous vascular beds and multiple species. These nNOS immunoreactive fibers play an important role in regulating vascular tone, mediating neurogenic vasodilation by releasing NO. Up-regulation of n-NOS has been recently demonstrated in mesenteric arteries of PVL rats[58,146]. This nNOS activation seems to mediate an increased neural NO-mediated vasodilatation and might be an additional pathway for mesenteric smooth muscle relaxation in portal hypertension. Moreover, the non-selective NOS inhibition by L-NAME (N-(G)-nitro-L-arginine methyl ester) and the selective inhibition of nNOS by L-VNIO (vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine), increase the induced adrenergic vasoconstriction in rat mesenteric arteries in response to periarterial

nerve stimulation[147]. These findings strongly suggest that endogenous NO also modulates the neurogenic release of NE from adrenergic nerve terminals.

CONCLONSION

The increase in splanchnic flow that contributes to portal hypertention results from persistent mesenteric vasodilation together with angiogenesis. Studies in animal models and in patients have shown that splanchnic arterial vasodilation is a multifactorial phenomenon. In addition to overproduction of vasodilators (especially nitric oxide), defects in the contractile signalling pathways in smooth muscle cells in response to vasoconstrictor stimulation contribute to vascular hyporesponsiveness to endogenous vasoconstrictors. In addition, sympathetic atrophy seems to participate in the late stages of portal hypertension. It is reasonable to suggest that mesenteric sympathetic atrophy decreases the vascular tone of the mesenteric tree, allowing an increased activity of vasodilatory mediators (humoral and nervous). However, little is known about possible interactions between participating pathways and mechanisms, and further efforts are needed to clarify this essential component of portal hypertension.

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BRIEF ARTICLE

Soluble membrane attack complex in ascites in patients with liver cirrhosis without infections

Mette Bjerre, Peter Holland-Fischer, Henning Grønbæk, Jan Frystyk, Troels K Hansen, Hendrik Vilstrup, Allan Flyvbjerg

Mette Bjerre, Jan Frystyk, Troels K Hansen, Allan Flyvbjerg, The Medical Research Laboratories, Clinical Institute and Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus DK-8000, Denmark
Peter Holland-Fischer, Henning Grønbæk, Hendrik Vilstrup, Department of Medicine V (Hepatology and Gastroentology), Aarhus University Hospital, Aarhus, DK-8000, Denmark
Author contributions: Bjerre M, Frystyk J, Grønbæk H, Vilstrup H and Flyvbjerg A contributed to conception and design of the work; Holland-Fisher P collected clinical data; Bjerre M and Holland-Fisher P analyzed and interpreted the data; and Bjerre M, Grønbæk H, Frystyk J, Krarup-Hansen T, Vilstrup, H and Flyvbjerg A were responsible for drafting, writing and revising of the manuscript.
Supported by grants from the Danish Medical Research Council
Correspondence to: Mette Bjerre, PhD, The Medical Research Laboratories, Clinical Institute and Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Nørrebrogade 44, Building 3, DK-8000 Aarhus, Denmark. mette.bjerre@ki.au.dk
Telephone: +45-8949-2154 Fax: +45-8949-2150
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Abstract

AIM: To study complement activation in 46 patients with alcoholic cirrhosis and ascites but no spontaneous bacterial peritonitis (SBP) and 10 healthy controls.

METHODS: Complement activation was determined by the measurement of soluble membrane attack complex (sMAC) concentrations in ascites and plasma. In patients, metabolic liver function was determined by the galactose elimination capacity and the clinical status assessed by the Model of End-Stage Liver Disease and Child-Pugh scores.

RESULTS: Ascites sMAC levels were markedly higher than in the corresponding plasma sample (median (range): 596 (170 - 1519) *vs* 160 (77 - 848) μg/L; $P < 0.01$). Ascites sMAC levels correlated positively with liver status. There was no relationship between ascites sMAC and leukocyte count. No relationship between ascites sMAC and blood C-reactive protein, albumin or neutrophile count was found. Plasma sMAC concentrations were slightly higher in patients than in controls [130 μg/L (70 - 204); $P = 0.04$]. Neither sMAC in ascites nor plasma was related to mortality.

CONCLUSION: The increased sMAC concentration in ascites and plasma indicate an activation of the complement system in cirrhosis even in the absence of SBP. This was particularly evident in the peritoneal fluid and most marked in patients with preserved liver status. The high ascites sMAC levels may reflect transudation of membrane attack complexes from the liver. Whether this complement activation has any clinical implications remains to be clarified.

Key words: Ascites; Cirrhosis; Complement; sC5b-9; Soluble membrane attack complex

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INTRODUCTION

Bacterial infections are frequent in patients with liver cirrhosis and have major impact on their short and long term morbidity and mortality[1]. Translocation of intestinal bacteria to ascites is among the primary events in the development of spontaneous bacterial peritonitis (SBP), one of the most important infectious complications in patients with advanced cirrhosis and ascites[2,3].

Patients with cirrhosis have immunological dysfunctions which increase their risk of bacterial infections. The complement system is an important mediator of innate and acquired immunity and is activated almost immediately on the appearance of pathogenic microorganisms. Complement proteins, mainly synthesized by the hepatocytes, circulate as inactive forms and are activated by three pathways: the classical, the lectin and the alternative pathway[4-6] (Figure 1). The common final product is the membrane attack complex (MAC), generated by combination of the complement components (C)5b, C6, C7, C8 and C9. MAC forms channels on the surface of cellular membranes leading to cell lysis[7,8]. MAC formed in the absence of target membranes binds to S-protein which inhibits the membrane-damaging effect and creates a stable non-lytic soluble MAC form (sMAC)[9].

Plasma sMAC concentrations may be reduced in patients with cirrhosis as part of their immune incompetence and because of decreased liver function for synthesis of complement proteins. In the light of the frequent occurrence of peritonitis, ascites sMAC might be even lower. Accordingly, low concentrations of complement components in plasma and ascites have been reported to increase the patients' susceptibility to infection[10].

To further study this hypothesis, we measured corresponding concentrations of sMAC in plasma and ascites in a group of patients with decompensated alcoholic cirrhosis with ascites but without SBP.

MATERIALS AND METHODS

Study design

We consecutively included non-infected patients (n = 46) with liver cirrhosis and ascites admitted for a diagnostic or therapeutic paracentesis between August 2002 and April 2008. The diagnosis of cirrhosis was established by a combination of biochemical, clinical and ultrasonographic findings and none of the patients had a liver biopsy. We only included patients with no signs of SBP as all patients had ascites neutrophil counts < 250 × 10^6/L. Etiologies of cirrhosis were alcoholic cirrhosis (n = 39) and autoimmune or cryptogenic cirrhosis (n = 7). None of the patients were diagnosed with chronic viral hepatitis. Fasting EDTA-plasma (all) and ascites (n = 44) were collected concomitantly and stored at -80℃. Evaluation of the severity of hepatic encephalopathy (HE-grade)[11] was Grade 0 (n = 38) and Grade 1 (n = 8). Five patients had previous episodes of SBP. During the period of follow-up (median follow-up 53 mo), 61% (n = 28) of the patients died. Blood samples from ten age and sex-matched healthy subjects served as controls. Informed consent was obtained according to the declaration of Helsinki and the local authorities of ethics approved the study.

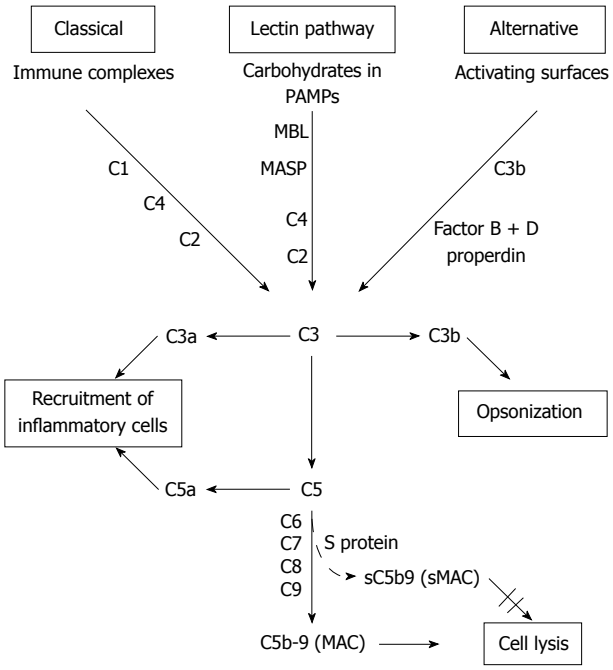

Figure 1 Activation of the complement system through three pathways results in different effecter mechanisms. Binding of S-protein to the MAC complex lead to the formation of soluble MAC (sMAC). Complement component 1 (C1), mannan-binding lectin (MBL), MBL associated serine protease (MASP), pathogen-associated molecular pattern (PAMP), membrane attack complex (MAC). The figure is modified from reference [12].

Paraclinical analyses

Bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, C-reactive protein (CRP) concentrations, coagulation time and total protein concentration in ascites were determined by standard laboratory methods at the Hospital Department of Clinical Biochemistry. Leukocyte counts in blood and ascites were measured by an automated technique followed by manual counting and separation into mono- and polymorphnuclear leucocytes by a total count > 200 cells/mL.

sMAC in plasma and ascites

sMAC concentration was measured by a sensitive time-resolved immunofluorometric assay (TRIFMA) described in details elsewhere[12]. The limit of detection for the assay was 1 μg/L. The intra- and inter-assay coefficients of variance were below 5% and 12% respectively.

Liver function

Clinical status was assessed according to the Model for End-stage Liver Disease (MELD) Score[13] and the Child-Pugh Score[14]. The Galactose Elimination Capacity (GEC) was used to quantitatively measure metabolic liver function. The GEC was determined from blood galactose concentration-decay curves after iv administration of

galactose corrected for urinary excretion as described by Tygstrup[15].

Statistical analyses

Comparisons between groups were performed by one-way ANOVA or by Mann-Whitney tests according to the data distribution. sMAC concentrations showed a skewed distribution and values are given as median (range). Spearman correlation with two-tailed probability values was used to estimate the strength of association between variables. Mortality and sMAC was evaluated by ROC analysis and the connection between time to death during the follow-up period was estimated by the Kaplan-Meier method, grouped by sMAC concentrations above or below the median in either plasma or ascites. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Group characteristics

There was no difference in gender or age between the patient group and the control group (Table 1). All patients had increased CRP and decreased albumin ($P < 0.0001$). The patients had slightly increased ALT, ALP (compared with normal range) and bilirubin ($P < 0.0001$). No patient had an ascites neutrophil count above 250 × 10^6/L (i.e. none had SBP). However, five patients had a history of SBP.

Increased sMAC in ascites

The patients had markedly increased sMAC concentrations in ascites compared with plasma ($P < 0.001$) (Figure 2). The average ascites/plasma ratio was 3.5. There was only a weak and borderline significant correlation between sMAC in plasma and ascites ($R^2 = 0.04$, $P = 0.05$). Furthermore, plasma sMAC was slightly higher in the patients than in controls ($P = 0.04$) (Table 1).

sMAC in ascites correlates positively with liver function parameters

There was a positive correlation between ascites sMAC and the GEC ($R^2 = 0.28$, $P < 0.01$) (Figure 3). Furthermore, we observed a negative correlation to MELD scores ($R^2 = -0.13$, $P = 0.02$) and Child-Pugh scores ($R^2 = -0.07$, $P = 0.09$), i.e. an increase in sMAC levels equals a better liver status. There was a positive correlation between ascites total protein concentration and ascites sMAC ($R^2 = 0.18$, $P = 0.007$) and between ascites sMAC and P-CRP ($R^2 = 0.16$, $P = 0.03$). Ascites leukocyte count did not correlate with sMAC concentrations either in ascites or plasma. A negative correlation was found between ascites sMAC and ALT ($R^2 = -0.07$, $P = 0.03$). None of the correlations were found for plasma sMAC concentration. The mortality rate of patients was not associated with either plasma or ascites sMAC. ROC curves showed no association between sMAC concentration and death; area under the ROC curve for sMAC levels in ascites and in plasma was 0.55 and 0.56 respectively.

Table 1 Clinical characteristics of cirrhosis patients and controls (means ± SD, or range)

	Patients (n = 46)	Controls (n = 10)	P value
Sex (% men)	65	63	NS
Age (years)	54 ± 9	55 ± 7	NS
Plasma analysis			
Leukocyte counts (10^9 cells/L)	9.9 ± 6.3	ND	
CRP (mg/L)	233 ± 183	NR < 10	
Albumin (μmol/L)	359 ± 78	641 ± 36	< 0.0001
Creatinine (μmol/L)	81.2 ± 44.6	74.7 ± 11	NS
sMAC (μg/L)	160 (77 - 848)	130 (70 - 204)	0.04
Ascitic fluid analysis			
Leukocyte counts (10^6 cells/L)	130 (0 - 213)	-	
Total protein (g/L)	11.9 ± 6.6	-	
sMAC (μg/L)	596 (170 - 1519)	-	
Liver function			
ALT (U/L)	51 ± 75	NR 7 - 56	
Bilirubin (μmol/L)	99.4 ± 89.8	11.5 ± 4.9	< 0.0001
ALP (U/L)	312 ± 230	NR 38 - 126	
Coagulation time (ratio of expected)	0.44 ± 0.18	1.11 ± 0.15	< 0.0001
INR	1.56 ± 0.35	0.86 ± 0.05	< 0.0001
MELD score	14.1 ± 7.1	0.9 ± 2.3	< 0.0001
Child Pugh score	10.5 ± 1.8	ND	
GEC (mmol/min)	1.54 ± 0.4	ND	
GEC (ratio of expected)	0.56 ± 0.1	ND	

ND: Not determined; NS: Not significant; NR: Normal range; CRP: C-reactive protein; sMAC: Soluble membrane attack complex; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio for prothrombin time; MELD: Model of end-stage liver disease; GEC: Galactose elimination capacity.

DISCUSSION

The main findings of this study were that ascites sMAC concentrations were markedly higher than the corresponding plasma values in non-infected patients with decompensated alcoholic cirrhosis. Furthermore, sMAC levels in ascites but not in plasma paralleled measures of liver status. Finally, in patients the plasma values were slightly higher than in controls. These findings are new and contrasted our stated *a priori* assumptions.

Increased sMAC reflects increased complement activation. Therefore, the increased plasma values in the patients is likely to represent a discreet complement activation as part of such patients' frequent systemic low-grade inflammation[16], confirmed by elevated CRP levels in all patients.

The increased ascites sMAC correlated with better liver status assessed by the Child-Pugh and MELD scores as well as by GEC. In a study of patients, newly-diagnosed cirrhosis GEC was found to be a strong predictor of short and long term all-cause and cirrhosis-related mortality[17]. These observations support the positive relationship of ascites sMAC to each of them and the possibility of preserved liver status being causally related to increased levels of ascites sMAC. None of the patients had SBP (or other infections) at inclusion (ascites leukocyte count, < 250 × 10^6/L), signifying that the increased ascites complement activation was not due

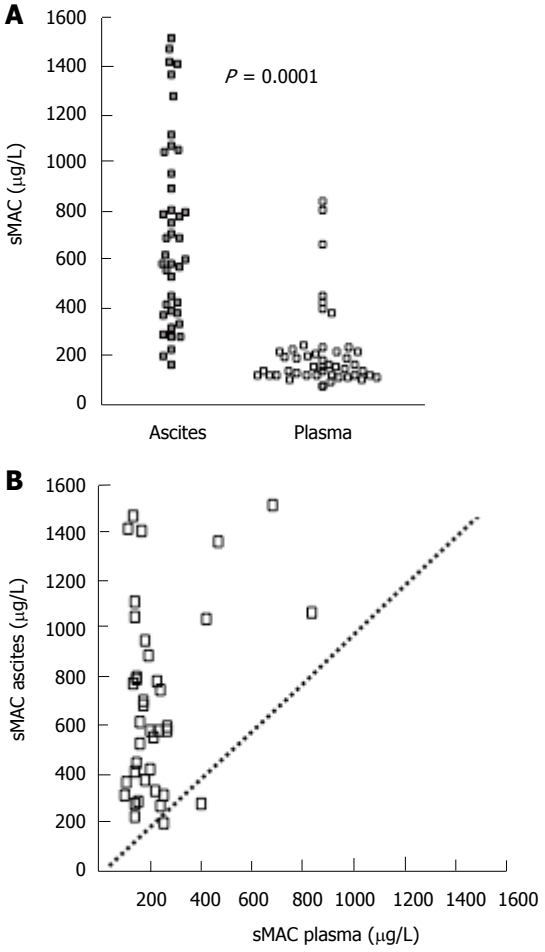

Figure 2 sMAC levels in cirrhotic patients. A: Levels of sMAC in ascites (Grey Square) compared with plasma (White Square); B: Ascitic sMAC *vs* plasma sMAC. Each dot represents an individual and the dashed line indicates line of identity.

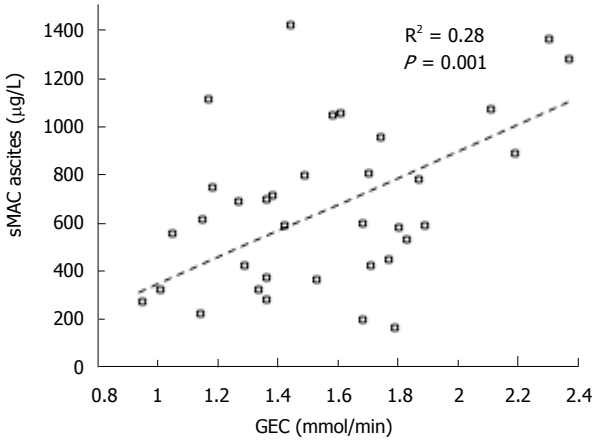

Figure 3 Association between Galactose elimination capacity and sMAC levels in ascites. Linear regression lines show the correlation. Each dot represents an individual.

to local bacterial invasion. However, the presence of increased sMAC showed that the complement system in ascites had become activated *in vivo*. Without leukocyte infiltration a possible explanation for this observation may be complement activation caused by an increased cell-turnover or cell death due to chronic low-grade inflammation in the cirrhotic liver. This notion may be supported by the correlation between ascites sMAC and ALT.

In cirrhosis, there is sinusoidal portal hypertension and protein rich 'ultrafiltrate' from Disse's space passes between hepatocytes and is transsudated across the peritoneal endothelium and into the abdominal cavity[18]. The positive correlation found between ascites total protein and sMAC supports that both were due to that mechanism. The mechanistic link between high ascites sMAC in relationship to good liver status is not clear. The most likely explanation is that a well-preserved liver function is more able to respond to infections with a sufficient activation of the complement components. This may protect against bacterial growth in the opsonin-poor ascites fluid and thus explain why patients with good liver function are less prone to peritonitis. In support, the only patient in our cirrhosis group with an ascites/plasma ratio of sMAC below 1 and a very poor liver status was readmitted with SBP 3 wk after discharge.

Considering this, it may be surprising that ascites sMAC was not related to long-term mortality with the emergence of SBP being associated with markedly increased mortality. Duration of cirrhosis in this prospective study may have an influence. In biopsies from cirrhotic livers, Polihronis *et al*[19] found variable amounts of MAC and sMAC deposited in the cirrhotic connective tissue. The amount of deposited sMAC did not correlate with the cause of cirrhosis and they found no indication that sMAC was involved in the pathogenesis of hepatic injury.

A study of bacterial translocation in patients with liver cirrhosis without SBP showed that ascites sMAC was increased but only in those ascites which contained an increased bacterial DNA content[20]. Since the presence of bacDNA is not available in our samples a direct comparison is not possible. Five patients were previously diagnosed with SBP but no association to sMAC levels were found and thus other factors are likely to be involved. This is supported by elevated sMAC in ascites reported in other patient groups characterised by cell changes or degeneration (ovarian cancer and patients with non-malignant endometriosis)[21,22]. The high ascites sMAC levels may reflect transudation of membrane attack complexes from the liver. Thus it may be of interest in order to analyse patients with other causes of transudative ascitis.

In conclusion, the high sMAC concentrations in ascites indicate an increased activation of the complement system in cirrhotic patients caused by systemic low-grade inflammation and hepatic activation. The increased sMAC levels correlated with liver function but had no association with survival. The role of sMAC in liver cirrhosis is still unclear and studies in cirrhotic patients with SBP and other infections may help to elucidate this.

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COMMENTS

Background

Patients with cirrhosis have immunological dysfunctions which increase their risk of bacterial infections. The complement system is an important mediator of innate and acquired immunity and is activated almost immediately on the appearance of pathogenic microorganisms. The activation generates the membrane attack complex (MAC) and in the absence of target membranes a stable non-lytic soluble MAC (sMAC) form. Low concentrations of complement components in plasma and ascites have been reported to increase patients' susceptibility to infection.

Research frontiers

Low concentrations of complement components in plasma and ascites have been reported to increase patients' susceptibility to infection. However, it is unknown whether liver cirrhosis *per se* is able to initiate complement activation and, if so, how this affects the clinical outcome of patients.

Innovations and breakthroughs

The main findings of this study were that ascites sMAC concentrations were markedly higher than the corresponding plasma values in non-infected patients with decompensated alcoholic cirrhosis. Furthermore, sMAC levels in ascites but not in plasma paralleled measures of liver status.

Application

Increased sMAC may protect against bacterial growth in the opsonine-poor ascites fluid and thus explain why patients with good liver function are less prone to peritonitis

Peer review

This manuscript is interesting, original and well designed. The presentation is good and readable, and the statistical methods used are appropriate. It may be accepted for publication with modification.

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BRIEF ARTICLE

Twenty four-week peginterferon plus ribavirin after interferon-β induction for genotype 1b chronic hepatitis C

Hiroaki Okushin, Kazuhiko Morii, Koichi Uesaka, Shiro Yuasa

Hiroaki Okushin, Kazuhiko Morii, Koichi Uesaka, Shiro Yuasa, Department of Internal Medicine, Himeji Red Cross Hospital, 1-12-1 Shimoteno, Himeji-shi, Hyogo 670-8540, Japan
Author contributions: Okushin H designed the study; Okushin H, Morii K, Uesaka K and Yuasa S performed the research; and Okushin H wrote the paper.
Correspondence to: Hiroaki Okushin, MD, Department of Internal Medicine, Himeji Red Cross Hospital, 1-12-1 Shimoteno, Himeji-shi, Hyogo 670-8540, Japan. hiroaki_okushin@hotmail.co.jp
Telephone: +81-79-2942251 Fax: +81-79-2964050
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Abstract

AIM: To investigate the possibility of shortening the duration of peginterferon (Peg-IFN) plus ribavirin (RBV) combination therapy by incorporating interferon-β (IFN-β) induction therapy.

METHODS: A one treatment arm, cohort prospective study was conducted on seventy one patients. The patients were Japanese adults with genotype 1b chronic hepatitis C, HCV-RNA levels of ≥ 5.0 Log IU/mL or 100 KIU/mL, and platelet counts of ≥ 90 000/μL. The treatment regimen consisted of a 2 wk course of twice-daily administration of IFN-β followed by 24 wk Peg-IFN plus RBV combination therapy. We prolonged the duration of the Peg-IFN plus RBV therapy to 48 wk if the patient requested it.

RESULTS: The patients, including 44% males, were characterized by an median age of 63 years (range: 32-78 years), an median platelet count of 13.9 (range: 9.1-30.6) × 10^4/μL, 62% IFN-naïve, and median HCV-RNA of 6.1 (range: 5.1-7.2) Log IU/mL. The sustained virologic response (SVR) rates were 34% (Peg-IFN: 1-24 wk, *n* = 61, 95% confidence interval (CI): 24%-47%) and 55% (Peg-IFN: 20-24 wk, *n* = 31, 95% CI: 38%-71%, *P* < 0.001; *vs* Peg-IFN: 1-19 wk). The SVR rate when the administration was discontinued early was 13% (Peg-IFN: 1-19 wk, *n* = 30, 95% CI: 5%-30%), and that when the administration was prolonged was 50% (Peg-IFN: 25-48 wk, *n* = 10, 95% CI: 24%-76%, *P* < 0.05; *vs* Peg-IFN: 1-19 wk). In the patients who received 20-24 wk of Peg-IFN plus RBV, only the higher platelet count (≥ 130 000/μL) was significantly correlated with the SVR (odds ratio: 11.680, 95% CI: 2.3064-79.474, *P* = 0.0024). In 45% (14/31) of the patients with a higher platelet count (≥ 130 000/μL) before therapy, the HCV-RNA level decreased to below 3.3 Log IU/mL at the completion of IFN-β, and their SVR rate was 93% (13/14) after 20-24 wk administration of Peg-IFN plus RBV.

CONCLUSION: These results suggest the possibilities of shortening the duration of Peg-IFN plus RBV combination therapy by actively reducing HCV-RNA levels using the IFN-β induction regimen.

Key words: Peginterferon; Ribavirin; Interferon-β; Induction therapy; Short-term therapy; Chronic hepatitis C; Genotype 1b

Peer reviewer: Ruben Ciria, PhD, Uniersity Hospital Reina Sofia, Departmento De Cirugia Hepatobiliar Y Trasplante HEPÁTICO, Avennida Menendez PidalI s/n, Cordoba 14004, Spain

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INTRODUCTION

The administration of peginterferon (Peg-IFN) and

ribavirin (RBV) for 48 wk is currently the standard treatment for genotype 1b chronic hepatitis C. Interferon (IFN) plus RBV combination therapy is more effective than IFN monotherapy[1,2], and, unlike conventional IFN, Peg-IFN needs to be administered only once a week. However, in some cases, subjective side effects such as general fatigue, rashes, stomatitis, anorexia, taste abnormalities, and anemic symptoms develop, which, along with laboratory abnormalities such as decreased hemoglobin levels and reduced neutrophil and platelet counts, necessitates the reduction of RBV or discontinuation of RBV and/or IFN. When the total dose of IFN or RBV is insufficient, the efficacy of therapy is also unsatisfactory[3,4].

Recent studies have reported that only when HCV-RNA has become negative after 4 wk of treatment (rapid virologic response: RVR) in patients with a low viral level, high sustained virologic response (SVR) rate can be expected even if the duration of administration is shortened to 24 wk[5-9]. Although, in general, RVR rate is only 15%-20% in genotype 1b chronic hepatitis C patients[10], it is important to conduct studies on the possibility of reducing the duration of the patient's physical pain by avoiding excessive administration.

In Japan, interferon-β (IFN-β) can is approved for the treatment of chronic hepatitis C. A characteristic of IFN-β is that dividing its administration between equal morning and evening doses increases the HCV-RNA reduction rate[11-15]. Studies have reported that the administration of 3 million units each of IFN-β in the morning and evening for 2 wk reduces HCV-RNA levels by 3 Log IU/mL in many cases, whereas the co-administration of Peg-IFN and RBV decreases them by 1-2 Log IU/mL[16,17], clearly indicating that the former regimen results in a higher rate of HCV-RNA decrease in the early phase of treatment.

We speculated that an enforced reduction in HCV-RNA levels would shorten the duration of treatment in more patients, including those with a high viral load. In this study, chronic hepatitis C patients with a high viral load of genotype-1b hepatitis C virus were administered IFN-β for 2 wk as induction therapy, followed by 24 wk Peg-IFN plus RBV combination therapy, and its therapeutic effect was evaluated.

MATERIALS AND METHODS

Patients

We studied Japanese adult chronic hepatitis C patients who had visited our hospital between October 2006 and January 2009, and received IFN-β followed by Peg-IFN plus RBV combination therapy. They had HCV genotype 1b, with an HCV-RNA level of ≥ 100 KIU/mL or ≥ 5.0 Log IU/mL. Those with any of the following were excluded: other concomitant viral hepatitis, autoimmune liver disease, drug-induced liver injury, cirrhosis, renal failure, hypersensitivity to IFN or RBV, concomitant HCC, a PLT count of < 90 000/μL, or a serum albumin level of < 3.5 g/dL. The study was conducted in accordance with the institutional ethical procedures and the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients before enrollment in this study. Seventy-one patients enrolled in this study.

Treatment regimen

A one arm, cohort prospective study was conducted, in a pilot fashion. Patients were initially administered IFN-β for 2 wk, followed by Peg-IFN-α2a or Peg-IFN-α2b for 24 wk, which was extended to 48 wk if the patient wished at the completion of Peg-IFN. During administration of Peg-IFN, RBV was concomitantly used.

Three million IU of IFN-β (FERON®, TORAY Industries, Inc.) was dissolved in 100 mL of physiological saline or 5% glucose for injection , and the solution was administered twice daily by intravenous drip infusion over 30 min in the morning (9:00-10:00) and evening (19:00-20:00). To prevent IFN-β-induced fever, 60 mg of loxoprofen sodium was administered 30 min before the start of the drip infusion. Subsequent to IFN-β, 180 μg of Peg-IFN-α2a (Pegasys®, Chugai Pharmaceutical Co.) or 1.5 μg/kg of Peg-IFN-α2b (Pegintron®, Schering-Plough Co.) was administered once weekly. RBV (Copegus®, Chugai Pharmaceutical Co., when Pegasys® was used, or REBETOL®, Schering-Plough Co., when Pegintron® was used) was administered at a dose of 600 mg/d (200 mg after breakfast and 400 mg after dinner) to patients weighing < 60 kg, 800 mg/d (400 mg after breakfast and 400 mg after dinner) to those weighing 60-80 kg, and 1 000 mg/d (400 mg after breakfast and 600 mg after dinner) to those weighing > 80 kg. The daily dose of RBV was reduced by 200 mg when the hemoglobin level decreased to < 10 g/dL, and RBV was discontinued when the hemoglobin level fell to < 8.5 g/dL. When side effects made the continued administration difficult, or when HCV-RNA levels did not decrease, IFN was discontinued at the discretion of the physician.

Laboratory methods

Patients were screened for the study by HCV-RNA quantification, HCV genotyping using RT-PCR, and biochemical and hematological tests. During and after IFN administration, patients were followed-up by employing biochemical and hematological tests, HCV-RNA quantification, quantitative and qualitative HCV-RNA assays, and urinalysis. Serum HCV-RNA was measured using the Cobas Amplicor HCV Monitor Test version 2.0 (Roche Diagnostics, detection range: 5-5 000 KIU/mL), Cobas Amplicor HCV Monitor Test version 1.0 (Roche Diagnostics, detection range: 0.5-500 KIU/mL), and Cobas Taqman HCV Test (Roche Diagnostics, detection range: 1.2-7.8 Log IU/mL). Qualitative serum HCV-RNA determination was performed using the Amplicor assay (Roche Diagnostics, detection sensitivity: 50 IU/mL). Urinary protein was measured by the pyrogallol red method.

Assay of efficacy

An SVR was defined as the persistent absence of detec-

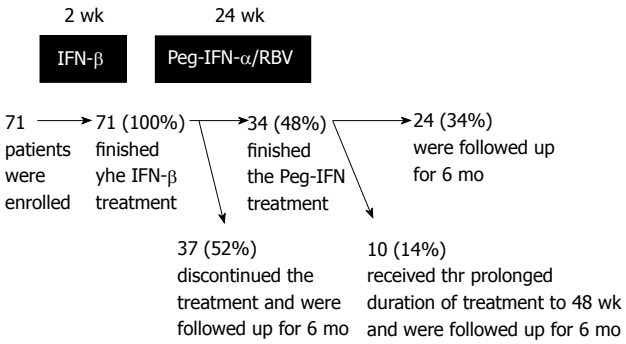

Figure 1 Duration of interferon-β (IFN-β) therapy and peginterferon-α (Peg-IFN-α) plus ribavirin (RBV) combination therapy, and the numbers of patients.

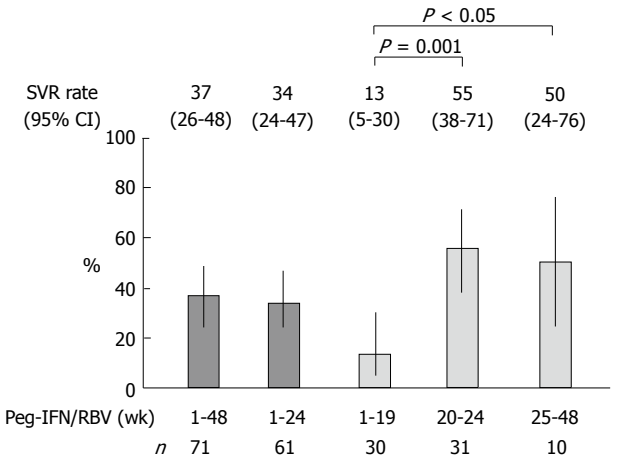

Figure 2 The SVR rate and 95% confidence interval (95% CI) by dosing duration of peginterferon (Peg-IFN) plus ribavirin (RBV) after interferon-β induction therapy. The significance of the difference was evaluated employing Fisher's exact test.

table HCV-RNA (by the Amplicor or Taqman HCV Test) for 6 mo after the cessation of treatment. In this study, a rapid virologic response (RVR) was defined as a serum HCV-RNA level of < 50 copies/mL (qualitatively HCV-RNA-negative or < 1.7 Log IU/mL by the Taqman HCV Test) at 4 wk after the start of IFN (2 wk after the start of Peg-IFN). A complete early virologic response was defined as the absence of HCV-RNA (by the Amplicor or Taqman HCV Test) at 12 wk after the start of IFN (10 wk after the start of Peg-IFN). The end of the treatment response was defined as the absence of HCV-RNA (by the Amplicor or Taqman HCV Test) at the cessation of Peg-IFN.

Statistical analysis

The significance of differences was evaluated by the χ^2 test, paired *t*-test, Fisher's exact test, Student's *t*-test, or analysis of variance. Nominal logistic regression analysis was used for the analysis of SVR-related variables. A *P* value of less than 0.05 was considered significant. Statistical analyses were performed using JMP® software version 5.0 (SAS Institute Inc.).

Table 1 Clinical background of patients

	Background
n of patients	71
Male [*n* (%)]	31 (44)
Age (yr)	63 (32-78)
Body weight > 60 kg [*n* (%)]	33 (46)
HCV-RNA (Log IU/mL)	6.1 (5.1-7.2)
Alanine aminotransferase (U/L)	97 (27-513)
Platelet count [$\times 10^4/\mu L$]	13.9 (9.1-30.6)
9.0 to 13.0 [*n* (%)]	28 (39)
⩾ 13.0 [*n* (%)]	43 (61)
Albumin (g/dL)	3.9 (3.5-4.5)
Hemoglobin (g/dL)	13.6 (10.5-16.9)
Interferon-naïve [*n* (%)]	44 (62)
Peginterferon-α2a/-α2b (*n*)	34/37
Initial dose of ribavirin 600/800/1 000 (mg/d) (*n*)	38/30/3

Continuous variables: Median (range).

RESULTS

Patient background

The durations of IFN treatment and the numbers of patients are shown in Figure 1. During administration of Peg-IFN, 37 (52%) patients discontinued the treatment: one patient due to work commitments, 11 (15%) patients whose HCV-RNA levels did not decrease, and 25 (35%) patients due to side effects.

The patients' backgrounds are shown in Table 1. When HCV-RNA levels were determined using the Amplicor HCV monitor, the results (in KIU/mL) were converted to Log IU/mL.

Efficacy

Figure 2 shows the SVR rate and 95% confidence interval (95% CI). Next, the patient background variables by virologic response were analyzed univariately (Table 2). When HCV-RNA levels were determined using the Amplicor HCV monitor, the results (in KIU/mL) were converted to Log IU/mL. Since the age, body weight, platelet count, hemoglobin level, and dosing duration were SVR-related variables in the patients who received Peg-IFN for 1-24 wk, and the age and platelet count were SVR-related variables in the patients who received that for 20-24 wk, nominal logistic regression analysis was performed regarding these variables (Table 3).

HCV-RNA response

The HCV-RNA reduction and SVR rate were evaluated (Table 4). The relapse rate was 46% (18/39) and 29% (7/24) in the patients who received Peg-IFN for 1-24 wk and those for 20-24 wk, respectively. In Table 4, when HCV-RNA levels were determined using the Amplicor HCV monitor, the results (in KIU/mL) were converted to Log IU/mL. The SVR rates associated with the platelet count (identified as an independent factor by multivariate analysis) in patients whose HCV-RNA levels decreased at the end of IFN-β treatment are shown in Figure 3.

Table 2 Clinical background by therapeutic response

	Dosing duration of peginterferon plus ribavirin			
	1-24 wk		20-24 wk	
	SVR non-SVR	*P* value	SVR non-SVR	*P* value
n of patients	21 40		17 14	
Male [*n* (%)]	12 (57) 13 (33)	0.0636[b]	10 (59) 4 (29)	0.1493[c]
Age (yr)	58 (35-74) 65 (37-78)	0.0030[a]	58 (35-74) 63 (58-71)	0.0134[a]
Body weight > 60 kg [*n* (%)]	13 (62) 14 (35)	0.0441[b]	10 (59) 3 (21)	0.0669[c]
HCV-RNA (Log IU/mL)	5.8 (5.1-6.9) 6.1 (5.1-7.2)	0.1685[a]	6.0 (5.2-6.9) 6.1 (5.2-6.6)	0.9967[a]
Alanine amino-transferase (U/L)	97 (52-269) 93 (27-513)	0.7699[a]	86 (57-138) 80 (28-260)	0.6410[a]
Platelet count ($\times 10^4$/μL)	16.8 (10.9-30.6) 13.3 (9.1-24.0)	0.0020[a]	17.0 (10.9-30.6) 11.4 (9.1-15.4)	0.0007[a]
Albumin (g/dL)	3.9 (3.5-4.4) 3.9 (3.5-4.5)	0.7410[a]	3.9 (3.5-4.2) 3.9 (3.5-4.5)	0.9211[a]
Hemoglobin (g/dL)	14.4 (11.4-15.4) 13.1 (10.5-15.4)	0.0126[a]	14.5 (11.4-15.4) 13.5 (10.7-15.4)	0.1379[a]
Interferon-naïve [*n* (%)]	12 (57) 25 (63)	0.6847[b]	9 (53) 8 (57)	0.8150[b]
Peginterferon-α2a/-α2b (*n*)	12/9 16/24	0.2016[b]	9/8 10/4	0.4607[c]
Dosing duration of peginterferon (wk)	24 (4-24) 15 (2-24)	0.0009[a]	24 (21-24) 24 (21-24)	0.5082[a]

Continuous variables: Median (range); Statistical analysis: [a]Analysis of variance; [b]χ^2 test; [c]Fisher's exact test; SVR: Sustained virologic response.

Table 3 Nominal logistic regression analysis of clinical background variables related to an sustained virologic response (SVR)

Variable	Odds ratio (95% CI)	*P* value[1]
Dosing duration of peginterferon: 1-24 wk (*n* = 61)		
Age < 65 yr	1.3210 (0.2140-8.1481)	0.7586
Body weight > 60 kg	3.7283 (0.6986-25.865)	0.1257
Platelet count ≥ 130 000/μL	15.301 (2.5378-155.22)	0.0018
Hemoglobin ≥ 14.0 g/dL	4.7957 (0.9337-30.599)	0.0606
Dosing duration of Peg-IFN 20-24 wk (*vs* 1-19 wk)	33.551 (5.5858-352.77)	< 0.0001
Dosing duration of peginterferon: 20-24 wk (*n* = 31)		
Age < 65 yr	1.1028 (0.1670-6.6558)	0.9149
Platelet count ≥ 130 000/μL	11.680 (2.3064-79.474)	0.0024

CI: Confidence interval; [1]Statistical analysis: Likelihood ratio test.

Table 4 HCV-RNA response during treatment and outcome

Response outcome	IFN-β[1] *n* (%)[1]	HCV-RNA[1]	RVR *n* (%)	cEVR *n* (%)	ETR *n* (%)
Dosing duration of peginterferon: 1-24 wk (*n* = 61)					
Yes	43 (70)	6.0 (5.1-7.1)[b]	15 (25)	36 (59)	39 (64)
SVR	21 (49)[a]	5.8 (5.1-6.9)	7 (47)	20 (56)[c]	21 (54)[d]
Non-SVR	22 (51)[a]	6.0 (5.1-7.1)	8 (53)	16 (44)[c]	18 (46)[d]
No	18 (30)	6.2 (5.5-7.2)[b]	46 (75)	25 (41)	22 (36)
SVR	0 (0)[a]	-	14 (30)	1 (4)[c]	0 (0)[d]
Non-SVR	18 (100)[a]	6.2 (5.5-7.2)	32 (70)	24 (96)[c]	22 (100)[d]
Dosing duration of peginterferon: 20-24 wk (*n* = 31)					
Yes	25 (81)	6.0 (5.2-6.9)	11 (35)	24 (77)	24 (77)
SVR	17 (68)[e]	6.0 (5.2-6.9)	7 (64)	17 (71)[f]	17 (71)[g]
Non-SVR	8 (32)[e]	5.7 (5.2-6.6)	4 (36)	7 (29)[f]	7 (29)[g]
No	6 (19)	6.4 (5.5-6.6)	20 (65)	7 (23)	7 (23)
SVR	0 (0)[e]	-	10 (50)	0 (0)[f]	0 (0)[g]
Non-SVR	6 (100)[e]	6.4 (5.5-6.6)	10 (50)	7 (100)[f]	7 (100)[g]

[1]Number (%) of patients that HCV-RNA decreased to below 3.3 Log IU/mL or turned negative at the end of IFN-β treatment, and median (range) of the HCV-RNA (Log IU/mL) before treatment among those patients. RVR: Rapid virologic response; cEVR: Complete early virologic response; ETR: End-of-treatment response. [a]$P < 0.0001$; [b]$P < 0.05$; [c]$P < 0.001$; [d]$P < 0.0001$; [e]$P < 0.01$; [f]$P < 0.01$; [g]$P < 0.01$; Statistical analysis:[a, c, d, e, f, g]; Fisher's exact test:[b]; Analysis of variance.

In the patients who received Peg-IFN for 25-48 wk, the SVR rate of patients whose HCV-RNA levels were lower than 3.3 Log IU/mL at the end of IFN-β treatment was 5/7. Among these patients, the SVR rate of those whose preoperative platelet counts were higher than 130 000 was 3/4, and that of those with lower platelet counts was 2/3. The three other patients whose HCV-RNA levels were not decreased at the end of IFN-β treatment were non-SVR.

Adverse events

No patients discontinued IFN-β treatment because of side effects. The highest body temperature after administration of IFN-β was lower than 37.0℃ (98.6°F) in 85% (60/71) of patients. Of 11 patients who had a fever above 37.0℃ (98.6°F), 4 (6%) developed a fever above

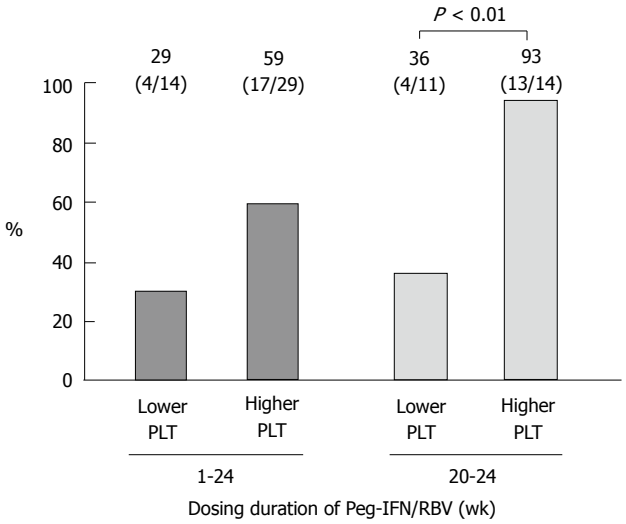

Figure 3 The SVR rate (%, *n*) by the baseline platelet count in patients whose HCV-RNA level decreased to below 3.3 Log IU/mL at the end of interferon-β treatment. Higher PLT indicates a platelet (PLT) count of ⩾ 130 000/mL at the start of therapy. The significance of the difference was evaluated using Fisher's exact test. Peg-IFN: Peginterferon; RBV: Ribavirin.

38℃ (100.4°F), which was resolved within two days. Twenty percent (14/71) of patients developed proteinuria during IFN-β treatment, and showed a urinary protein level of ⩾ 300 g/mL at the end of IFN-β treatment (at 2 wk). Subsequently, urinary protein became negative by 2 wk after Peg-IFN treatment. The mean platelet count before IFN-β treatment was 14.7 ± 4.1(× 10^4/μL), it decreased to 7.3 ± 3.2(× 10^4/μL) ($P < 0.0001$) at the end of IFN-β treatment (2 wk), and returned toward the pretreatment level (10.5 ± 4.8 × 10^4/μL, $P < 0.0001$) at 4 wk (at 2 wk of Peg-IFN treatment), although it remained lower than the pretreatment level (paired *t*-test).

Peg-IFN side effects leading to treatment discontinuation were nephrotoxicity, decreased hemoglobin, and physical symptoms (fatigue, anorexia, and anemia) in 1 (1%), 3 (4%), and 21 (30%) patients, respectively. In 30 (42%) of the 71 patients, the RBV dose was reduced because the hemoglobin level fell below 10 g/dL during Peg-IFN plus RBV combination therapy. The hemoglobin level before IFN-β treatment was 13.6 ± 1.3 g/dL, which rose to 14.1 ± 1.4 g/dL at the end of IFN-β (2 wk) ($P < 0.0001$, paired *t*-test). The hemoglobin level at the end (or discontinuation) of Peg-IFN treatment was 11.0 ± 1.5 g/dL, which was lower than that at the end of IFN-β treatment ($P < 0.0001$, paired *t*-test). The lowest hemoglobin level during Peg-IFN treatment was 10.5 ± 1.5 g/dL.

DISCUSSION

The aim of this study was to investigate the possibility of shortening the duration of Peg-IFN plus RBV combination therapy for chronic hepatitis C with a high viral load of genotype 1b by previously administering a 2 wk course of a twice-daily IFN-β regimen, which has been reported to be highly effective in reducing HCV-RNA, as described below.

A clinical trial of 48 wk Peg-IFN-α2a plus RBV combination therapy in 199 chronic hepatitis C Japanese patients, with a mean age of 52.0 years and a platelet count of more than 90 000/μL, including genotype 1b (92.0%), high viral load (44.2%, > 850 KIU/mL), women (31.7%) and IFN-naïve patients (49.7%), reported an SVR rate of 56.3% (103/183, genotype 1b)[18]. The EVR rate of IFN-naïve patients in genotype 1b was 75% (73/97) and 77% (56/73) of them achieved an SVR. Treatment was discontinued in 16.6% (33/199), being due to side effects in 13.6% (27/199). In another open study of 48-wk Peg-IFN-α2b plus RBV combination therapy in 586 genotype 1b chronic hepatitis C Japanese patients, with a mean age of 57.8 ± 10.3 years and a mean platelet count of (161 ± 52) × 10^3/μL, including women (45.2%), and 48.9% IFN-naïve patients, Furusyo *et al*[19] noted that they achieved an overall SVR rate of 42.4% (249/586), with those treated for less than 24, 24-47, and 48 wk achieving SVR rates of 4.7 (5/105), 36.4 (12/33), and 51.8% (232/448), respectively. Treatment was discontinued in 23.5% (138/586), being due to side effects in 14.1% (83/586) and failure of the HCV-RNA level to decrease in 3.8% (22/586).

In the present study, although the duration of treatment was shortened by one half, the SVR rate in the patients who received Peg-IFN treatment for 20-24 wk was 55%, and was comparatively higher than those studies cited above. However, presumably due to the high treatment discontinuation rates (52 *vs* 16.6 and 23.5%, respectively; 35 *vs* 13.6 and 14.1%, respectively, due to side effects) the SVR rate in the patients who received Peg-IFN treatment for 1-24 wk was low (34 *vs* 56.3 and 42.4%, respectively). We speculate that the treatment discontinuation rates were high because, in the present study: (1) the patients were less tolerant of side effects such as general fatigue, because their age was high, at 63 (median, range: 32-78) *vs* 52.0 (median) and 57.8 ± 10.3 (mean, standard deviation) years in other studies, or the percentage of women was high, at 56 *vs* 31.7 and 45.2%, respectively; and (2) treatment was discontinued early in more patients (15% *vs* none or 3.8%, respectively) because HCV-RNA did not decrease during the administration of IFN-β or Peg-IFN plus RBV.

Next, we performed subgroup analysis to sample a group of patients showing a high SVR rate. Since other studies have reported the importance of the RVR as an indicator for shortened treatment duration[5-9], we also evaluated it in the present study. Although, generally, the RVR rate of patients with genotype 1 chronic hepatitis C is only 15%-20%[10], a meta-analysis of studies on the shortening of the treatment duration reported that among patients who achieved RVR, 84%-96% and 83%-100% of patients with an baseline HCV-RNA level of < 400 000 IU/mL achieved an SVR by 24 and 48 wk of treatment, respectively, showing no significant difference in the SVR rate between these two groups of patients[9]. Although only patients with a high viral load were included in the present study, good RVR rates of 25 and 35% were achieved in the patients who received

Peg-IFN treatment for 1-24 wk and those for 20-24 wk, respectively, by actively reducing HCV-RNA levels through the use of IFN-β induction therapy (Table 4). However, the SVR rate among the patients who achieved an RVR in the present study was only 47% to 64%, and was clearly lower compared with the above-cited reports. These results suggest that an RVR is not necessarily an appropriate predictor of an SVR when administering IFN-β induction therapy.

When the platelet count (closely correlated with an SVR), among the patient background variables, was combined with the HCV-RNA level at the end of IFN-β treatment, we were able to predict a higher SVR rate. That is, as shown in Figure 3, 45% (14/31) of the patients who received Peg-IFN treatment for 20-24 wk, with a pretreatment platelet count of ≥ 130 000/μL and a HCV-RNA level of < 3.3 Log IU/mL at the end of IFN-β treatment, achieved an SVR rate of 93% (13/14). Thus, we were able to identify an important indicator for determining the duration of treatment when administering Peg-IFN plus RBV combination therapy after the IFN-β induction regimen.

In Japan, the use of IFN-β aiming to enhance viral clearance in the early phase of treatment when administering induction therapy has been reported[20-23]. A characteristic of IFN-β is that the administration in equally divided doses in the morning and evening increases the HCV-RNA reduction rate[11-15], as we first reported (in Japanese) in 1995[24]. In a subsequent study, 18 (13 genotype 1b, 4 genotype 2a, and 1 genotype 2b) chronic hepatitis C patients with an HCV-RNA level of less than 100 Kcopies/mL (Amplicor Monitor) received 3 million units of IFN-β for 4 wk twice daily in the morning and evening, followed by 10 million units of IFN-α2b three times weekly for 14 wk. As a result, all of them became negative for HCV-RNA during treatment, and achieved an SVR rate of 82.4% or 14/17 (in Japanese)[25]. Asahina *et al*[13] measured HCV-RNA levels by real-time PCR for up to fourteen days of IFN-β treatment, and reported that HCV-RNA levels, except in a small number of null responders, declined in a biphasic manner, consisting of a first phase of several days followed by a second phase. The exponential decay slopes (Log_{10}/d) of the first and second phases were 1.62 ± 0.81 and 0.02 ± 0.09*, respectively, in the case of a once-daily regimen, and 1.91 ± 0.57 and 0.16 ± 0.09*, respectively, in the case of a twice-daily regimen (*$P = 0.0003$). On the other hand, it was reported that the HCV-RNA levels (Log_{10} IU/mL, median, range) before and fourteen days after the initiation of Peg-IFN plus RBV combination therapy were 5.9 (5.37-6.34) and 4.93 (< 2.78-5.95), respectively, a decrease of approximately 1 Log IU/mL during the early phase of treatment[16], which clearly indicates that IFN-β is highly effective in reducing HCV-RNA levels.

Since the main subjective symptoms of side effects of IFN-β were fever and associated influenza-like symptoms, it was important to control fever. In contrast to persistently high titers of Peg-IFN in the blood, the T1/2 of IFN-β in the blood was short, at 15-43 min[26], and fever tended to occur within the first several hours after administration of IFN-β. Perhaps it was effective that the timing of IFN administration was arranged so that the blood level of loxoprofen became elevated during this time period. Laboratory abnormalities such as low platelet counts and proteinuria were noted, but they subsided or disappeared during Peg-IFN plus RBV combination therapy, presenting no problems.

The results of this study suggest the possibility of shortening the duration of Peg-IFN plus RBV combination therapy for chronic hepatitis C patients with a high viral load of genotype 1b virus by using an IFN-β induction regimen. Further clinical studies involving many more patients are needed to test this.

COMMENTS

Background

The standard treatment for chronic hepatitis C patients with a high viral load of the genotype-1b hepatitis C virus is 48 wk Peg-IFN plus RBV combination therapy. This therapy is more effective than the conventional IFN therapy, but side effects such as fever and general fatigue are problematical. Since early treatment discontinuation is associated with poor sustained virologic response (SVR) rates, physicians should encourage patients to complete the full course of therapy, and to endure these side effects.

Research frontiers

The SVR rate of Peg-IFN plus RBV combination therapy for 24 wk is usually about 50%. Only when patients with an HCV-RNA level of < 400 000 IU/mL become negative for HCV-RNA after 4 wk of treatment (RVR), and they achieve an SVR rate of 84%-96% after 24 wk of treatment, are they eligible for the shortened treatment option. However, since only 15%-20% of the total patients, mainly consisting of those with a low viral load, achieve an RVR, the number of patients eligible for the shortened treatment schedule is limited.

Related publications

It has been reported that the twice-daily administration of IFN-β in the morning and evening for 2 wk reduces HCV-RNA levels by an average of 3 Log IU/mL, except in a small number of null responders.

Innovations and breakthroughs

The SVR rate of chronic hepatitis C patients with a high viral load of genotype-1 hepatitis C virus receiving 2 wk IFN-β induction therapy followed by 20 to 24 wk Peg-IFN plus RBV combination therapy was 55%. Of these patients, 45% patients, with a pretreatment platelet count of ≥ 130 000/μL and a HCV-RNA level of < 3.3 Log IU/mL at the end of IFN-β treatment, achieved an SVR rate of 93%.

Applications

The duration of Peg-IFN plus RBV combination therapy can be shortened by actively reducing HCV-RNA levels through prior induction therapy.

Peer review

The manuscript is somewhat interesting and the design is acceptable. Some changes must be performed before It can be accepted for publication after some changes are performed.

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BRIEF ARTICLE

Significance of MD-2 and MD-2B expression in rat liver during acute cholangitis

Hui-Lai Miao, Zhi-Dong Qiu, Fu-Long Hao, Yong-Hui Bi, Ming-Yi Li, Ming Chen, Nian-Ping Chen, Fei Zhou

Hui-Lai Miao, Zhi-Dong Qiu, Ming-Yi Li, Ming Chen, Nian-Ping Chen, Department of Hepatobiliary Surgery, the Affiliated Hospital of Guangdong Medical College, Zhanjing 524001, Guangdong Province, China
Fu-Long Hao, Yong-Hui Bi, Fei Zhou, Department of Hepatobiliary Surgery, Research Institute of Guangdong Medical College, Zhanjiang 524001, Guangdong Province, China
Author contributions: Miao HL was responsible for making the plan and design of work, interpreting and discussing the results, writing and revising the manuscript and financial support; Qiu ZD helped in the performance of laboratory investigations, collection of data, writing and revising the manuscript; Hao FL and Bi YH interpreted and discussed the results of fluorescence quantitative PCR, closely supervised the different sets of fluorescence quantitative PCR, wrote and revised the text; Li MY provided scientific supervision of the work; Chen M helped in the interpretation and discussion of the results and performance of laboratory investigations; Chen NP gave scientific interpretation of the results of liver biopsies; and Zhou F established the animal model, analyzed and processed data.
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Correspondence to: Hui-Lai Miao, MD, Professor of Surgery, Department of Hepatobiliary Surgery, the Affiliated Hospital of Guangdong Medical College, No. 57 Renmin Road, Xiashan 524001, Guangdong Province, China. miaohuilai@tom.com
Telephone: +86-759-2387417 Fax: +86-759-2231754
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Abstract

AIM: To investigate the expression of myeloid differentiation protein-2 (MD-2), MD-2B (a splicing isoform of MD-2 that can block Toll-like receptor 4 (TLR4)/MD-2 LPS-mediated signal transduction) and TLR4 in the liver of acute cholangitis rats.

METHODS: Male Sprague-Dawley rats (SPF level) were randomly divided into four groups: (A) sham-operated group; (B) simple common bile duct ligation group; (C) acute cholangitis group; and (D) acute cholangitis anti-TLR4 intervention group (n = 25 per group). Rat liver tissue samples were used to detect TLR4, MD-2 and MD-2B mRNA expression by fluorescence quantitative PCR in parallel with pathological changes.

RESULTS: In acute cholangitis, liver TLR4 and MD-2 mRNA expression levels at 6, 12, 24, 48 and 72 h were gradually up-regulated but MD-2B mRNA expression gradually down-regulated (P < 0.05). After TLR4 antibody treatment, TLR4 and MD-2 mRNA expression were lower compared with the acute cholangitis group (P < 0.05). However, MD-2B mRNA expression was higher than in the acute cholangitis group (P < 0.05). MD-2 and TLR4 mRNA expressions were positively correlated (r = 0.94981, P < 0.05) and MD-2B mRNA expression was negatively correlated with MD-2 and TLR4 mRNA (r = -0.89031, -0.88997, P< 0.05).

CONCLUSION: In acute cholangitis, MD-2 plays an important role in the process of TLR4- mediated inflammatory response to liver injury while MD-2B plays a negative regulatory role.

Key words: Acute cholangitis; Toll-like receptor 4; Myeloid differentiation protein-2; Myeloid differentiation protein-2 splicing isoform

Peer reviewers: Xun-Di Xu, MD, PhD, Department of Gastroenterological Surgery, Xiangya Second Hospital, Central South University, Renmin Zhong Road 139, Changsha 410011, Hunan Province, China; Pierce KH Chow, MD, PhD, Professor, Department of General Surgery, Singapore General Hospital, Singapore 169608, Singapore

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INTRODUCTION

In acute cholangitis (AC), a large number of breeding intestinal flora gain access to the bile duct, releasing large amounts of toxins and directly or indirectly triggering excessive inflammation and causing liver damage[1-3]. In recent years, Toll-like receptor 4 (TLR4) has been identified as an important transmembrane signaling system involved in endotoxin-induced inflammation[4-5]. TLR4, as the main receptor for bacterial lipopolysaccharide (LPS), mediates a signal transduction pathway that includes formation of the LPS-LBP-CD14-secreted protein MD-2-TLR4 receptor complex, combination with myeloid differentiation factor 88, phosphorylation of IRAK and activation of a series of cell kinases, collectively controlling the activation of the transcription factor nuclear factor κB (NF-κB) and its biological effects[6-9].

Myeloid differentiation protein-2 (MD-2) is a recently described 160 amino-acid secreted glycoprotein and an important regulatory element in natural immune recognition[10]. Tamai confirmed that MD-2 is involved in synergistic effects of IL-8 production[11]. Research shows that following mutation of MD-2 glycosylation sites, MD-2 can still be expressed at the cell surface and combination with TLR4 is maintained but that TLR4-mediated activation of NF-κB weakens. When two glycosylation sites are simultaneously mutated, MD-2 can no longer promote LPS-induced IL-8 secretion[12]. MD-2 serves as the bridge between TLRs and LPS, playing an important role in the LPS signal transduction[10].

Using RT-PCR, Ohta *et al*[13] amplified MD-2B, an MD-2 splicing isoform, from mouse spleen and bone marrow cells. NF-κB instruction analysis suggests that HEK 293 cells cotransfected with equal amounts of MD-2 and MD-2B have a slower NF-κB up-regulation and weakened activity. When 8 fold more MD-2B than MD-2 was transfected, NF-κB activity was reduced to similar levels observed in cells transfected with the TLR4 and MD-2 without LPS stimulation. These results suggest that MD-2B can dose-dependently block TLR4/MD-2/LPS-mediated NF-κB signal transduction.

In the present study, real-time quantitative PCR was used to quantitatively detect TLR4, MD-2 and MD-2B mRNA expression in the liver during acute cholangitis. Data were used to study the interrelationship between MD-2, its splicing isoform (MD-2B) and TLR4 and establish the role of MD-2 and MD-2B in the TLR4-mediated inflammatory signaling pathway.

MATERIALS AND METHODS

Experimental strain

E. coli type Oll1B4 was provided by the Chinese drugs biological preparations examination institute. Bacteria were vaccinated on blood plates, incubated at 37℃ for 24 h before experiment, eluted with the aseptic physiological saline to a density of 5 × 10^5 CFU/mL and preserved at 4℃ until use.

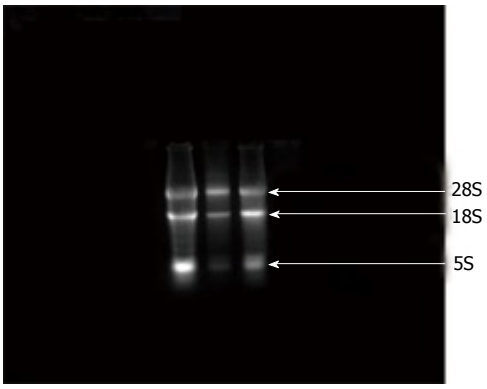

Figure 1 RNA electrophoretograms. There are three visible bands with 28s and 18s, 5s, showing no pollution in the DNA, no degradation with RNA which indicates the purity of RNA is higher.

Animal grouping and model preparation

The Guangdong Medical College hospital ethical committee approved the research protocol and the use of animals in this study was in accordance with the statement.

100 SPF level SD male rats weighing between 220-250 g were obtained from the institutional animal experimentation center (production license SCXK Guangdong 2004-0008, animal quality certification 2007A034) and were stochastically divided into 4 groups (n = 25 per group): a sham-operation (Group A), a control group (Group B), an acute cholangitis group (Group C) and an acute cholangitis anti-TLR4 intervention group (Group D).

The sham group (Group A) received operations simply to free the common bile duct and the control group had only a simple choledoch ligation (Group B). Rat choledoch dissociation was performed using a surgery microscope with the dissociated choledoch dual ligated with No. 1 silk thread at a length of approximately 1 cm. The acute cholangitis (Group C) was induced by injection of 0.2 mL E. coli suspension into the end near the ligation with a No. 4 silk scalp acupuncture needle followed by 1 mL physiological saline. The acute cholangitis anti-TLR4 intervention group (Group D) received an injection of anti-TLR4 monoclonal antibody 1 mL (2.5 g, diluted 1:200). Each group of rats received abdominal wall sutures layer by layer. After surgery rats were individually housed and had unconfined drinking and eating. Rats from each group were serially killed at 6, 12, 24, 48 and 72 h after surgery and then liver tissue was collected for pathological examination and real-time quantitative PCR.

Detection of TLR4, MD-2 and MD-2B mRNA expression

TLR4, MD-2 and MD-2B mRNA expression was detected in rat liver tissue using real-time PCR at various time points. The purity of isolated total RNA was verified by electrophoresis on 1% agarose gels (Figure 1). Templates from RNA reverse transcription were amplified in 10 μL 5 × quantitative PCR buffer, 1 μL upstream primer F (10 pmol/μL), 1 μL downstream

Table 1 Primers used for real-time PCR

	Forward	Reverse
MD-2 (130 bp)	5'-AAATCCCTATTTCAATTAGTTCTGAACC-3'	5'-GAGTTGATATTGATGAACAGGTTGAAAT-3'
MD-2B (93 bp)	5'-AAATCCCTATTTCAATTAGTTCTGAACC-3'	5'-GCTTCGGCAATTTTGGAATG-3'
TLR4 (117 bp)	5'-GATTGCTCAGACATGGCAGTTTC-3'	5'-CTGCTAAGAAGGCGATACAATTCG-3'
GAPDH (97 bp)	5'-CCGAGGGCCCACTAAAGG-3'	5'-GCTGTTGAAGTCACAGGAGACAA-3'

Table 2 TLR4 expression in rat liver tissue (mean ± SE) (copy ratio 10^4)

	6 h	12 h	24 h	48 h	72 h
Group A	4.32 ± 0.721	4.17 ± 0.708	4.40 ± 0.435	4.36 ± 0.396	4.48 ± 0.504
Group B	3.96 ± 0.051	4.90 ± 0.075[a]	8.52 ± 0.023[a]	11.11 ± 0.029[a]	12.90 ± 0.042[a]
Group C	3.87 ± 0.031	5.23 ± 0.033[a,c]	9.87 ± 0.025[a,c]	13.70 ± 0.115[a,c]	19.70 ± 0.123[a,c]
Group D	0.16 ± 0.003[e]	0.17 ± 0.002[e]	0.22 ± 0.002[e]	0.58 ± 0.001[e]	0.77 ± 0.006[e]

[a]$P < 0.05$ *vs* the same group at different time points; [c]$P < 0.05$ *vs* group B at the same time; [e]$P < 0.05$ *vs* group C at the same time.

primer R (10 pmol/μL), 1 μL probe (10 pmol/μL), 1 μL Taq enzyme (5 U/μL), 1 μL dNTPs (10 mmol), 5 μL, cDNA and 32 μL ddH2O using an ABI7000 completely automatic fluorescence quota PCR meter.

After amplification, data were adjusted according to the start value, stop value and threshold value of the baseline, the standard curves under the Std curve window was adjusted to optimal (the correlation value to be situated between 0.97 and -1, with the correlation value $|r|$) and finally, unknown specimen values B were obtained under the Reporter window by auto-calculating analysis (Qty). Considering the difference of total RNA density from each sample, the final computed results were calculated according to the following formula: A (copy counts/ug total RNA) = [B (copy counts/μL cDNA)]/(OD260 × 5/6). Masculine specimen RNA was reverse transcribed as templet.

PCR amplification was performed using a PE9600PCR meter and the primers used for PCR are shown in Table 1.

The amplification conditions were 2 min at 93℃, 40 cycles of 1 min at 93℃, 1 min at 55℃, 1 min at 72℃ and finally 7 min at 72℃ for extension. PCR products were electrophoresed in 2% low melting point agarose gel and under UVA objective zones were removed, recycled and purified with the recycling reagent box (QIA quick Gel Extraction Kit). Determination of OD 260/280 > 1.8 indicated the purity of the samples. Concentration (copy/μL) was calculated using the OD260 value and fragment length and then samples were gradient diluted to a positive quantitative standard gradient sample. Dual-distilled sterile water was employed as fluorescence-negative control standard sample.

Analysis and data processing

MD-2, MD-2B, TLR4 RNA copy number ratios were calculated using the formula: copy number ratio = copies/ug total RNA/GAPDH (internal parameters) copies/ug total RNA.

The data obtained were analyzed using SPSS 10.0 to perform completely stochastic variance comparison analyses among groups. Results were considered significant when $P < 0.05$. A q test was used for individual comparisons. The relevance of the various indexes was calculated using Pearson correlation analyses with statistical significance accepted when $P < 0.05$.

RESULTS

Change in liver tissue expression of TLR4 mRNA

In Group A, liver TLR4 mRNA expression was detected at all time points examined. TLR4 mRNA expression in Group B rats started to increase 12 h after surgery and by 72 h had increased significantly ($P < 0.05$). In Group C, TLR4 mRNA had increased by 12 h after surgery and this increased expression extended through 72 h. At the 12 h time point, TLR4 mRNA levels were significantly higher in Group C than in Group B ($P < 0.05$). In Group D, TLR4 mRNA expression declined 6 h after surgery with levels that were significantly different from those in Group C at corresponding time points ($P < 0.05$) (Table 2).

Change in liver tissue expression of MD-2 mRNA

In Group A, MD-2 mRNA expression was detected in liver tissue at different time points. MD-2 mRNA expression of Group B rats started to rise at 12 h after surgery and by 72 h had risen significantly ($P < 0.05$). In Group C, MD-2 mRNA expression was also present within 12 h and expression continued through 72 h. At 12 h, expression in Group C was significantly different than that of Group B ($P < 0.05$). MD-2 mRNA expression declined in Group D rats from 6 h after surgery onwards and was significantly different from Group C at the 6 h time point ($P < 0.05$) (Table 3).

Change in liver tissue expression of MD-2B mRNA

In Group A, liver MD-2mB RNA expression was detected at all time points. MD-2 mRNA expression in Group B rats started to decline at 12 h after surgery and

Table 3 MD-2 expression in rat liver tissue (mean ± SE) (copy ratio 10^3)

	6h	12h	24h	48h	72h
Group A	1.16 ± 0.042	1.17 ± 0.057	1.14 ± 0.016	1.16 ± 0.028	1.17 ± 0.048
Group B	1.15 ± 0.015	2.11 ± 0.042[a]	3.29 ± 0.034[a]	15.41 ± 0.267[a]	21.97 ± 0.271[a]
Group C	1.17 ± 0.028	4.10 ± 0.054[a,c]	12.27 ± 0.078[a,c]	21.158 ± 0.185[a,c]	30.21 ± 0.342[a,c]
Group D	0.33 ± 0.017[e]	0.40 ± 0.061[e]	1.15 ± 0.025[e]	3.61 ± 0.089[e]	4.58 ± 0.034[e]

[a]$P < 0.05$ *vs* the same group at different time points; [c]$P < 0.05$ *vs* group B at the same time; [e]$P < 0.05$ *vs* group C at the same time.

Table 4 MD-2B expression in rat liver tissue (mean ± SE) (copy ratio 10^2)

	6h	12h	24h	48h	72h
Group A	2.35 ± 0.013	2.38 ± 0.013	2.35 ± 0.012	2.35 ± 0.012	2.35 ± 0.010
Group B	2.47 ± 0.021	1.32 ± 0.035[a]	0.98 ± 0.006[a]	0.71 ± 0.006[a]	0.48 ± 0.002[a]
Group C	2.47 ± 0.021	1.42 ± 0.015[a,c]	0.26 ± 0.002[a,c]	0.09 ± 0.001[a,c]	0.07 ± 0.003[a,c]
Group D	6.03 ± 0.015[e]	4.50 ± 0.017[e]	3.85 ± 0.015[e]	3.62 ± 0.015[e]	2.39 ± 0.022[e]

[a]$P < 0.05$ *vs* the same group at different time points; [c]$P < 0.05$ *vs* group B at the same time; [e]$P < 0.05$ *vs* group C at the same time.

continued a gradual decline through 72 h. By 72 h, MD-2mB RNA expression had significantly declined ($P < 0.05$). Group C MD-2 mRNA expression also declined starting at 12 h and was significantly different from Group B at the 12 h time point ($P < 0.05$). In Group D, liver MD-2 mRNA expression increased starting at the 6 h time point compared with corresponding time point in Group C and was significantly different ($P < 0.05$) (Table 4).

Correlation analysis between TLR4 mRNA and MD-2, MD-2B mRNA

In rats with acute cholangitis, expression of MD-2 mRNA and TLR4 mRNA was positively correlated ($r = 0.94981$, $P < 0.05$) while MD-2 mRNA and MD-2B mRNA were negatively correlated ($r = -0.89031$, $P < 0.05$). TLR4 mRNA and MD-2B mRNA were also negatively correlated ($r = -0.88997$, $P < 0.05$).

DISCUSSION

In acute cholangitis, bacterial translocation from the intestinal tract and enterogenic endotoxemia may occur. Miyaso[14] investigated the relationship between LPS, the signaling molecule TLR4 and liver damage during obstructive icterus endotoxemia. These observations revealed that LPS-induced liver damage was time dependent and involved TLR4 signaling. The present results show that in the livers of rats with acute cholangitis, TLR4 mRNA expression increased significantly 12 h after induction, continued to increase even higher as inflammation developed in the first 72 h and showed a clear positive correlation with the degree of inflammation. This high level of TLR4 expression suggests that it is closely related to liver damage in acute cholangitis. As TLR4 is an essential component of inflammatory pathways, over expression of TLR4 mRNA during acute cholangitis may result in an excessive inflammatory response leading to liver damage.

Some research has indicated that synergistic up-regulation of other proteins may be necessary for enhancement of TLR-mediated immune responses[15]. MD-2 is a newly discovered, soluble secretary protein which binds to the extracellular region of TLR4. Upon binding, MD-2 enhances TLR4 reactivity to LPS, strengthens the subsequent signal transduction intensity and facilitates LPS-mediating signaling through the NF-κB pathway. Other reports showing that treatment with LPS causes a dose-dependent increase in MD-2 expression[16] and that MD-2 gene knockout mice do not respond to LPS and can survive LPS-induced shock[17], support the concept that extracellular MD-2 directly binds to LPS and unites it with the TLR4 external functional region. Thus MD-2 is indispensable in TLR4-mediated LPS signal transduction[18-19].

MD-2 mRNA expression was up-regulated in the livers of rats with acute cholangitis and its expression increased as inflammation developed in 72 h, suggesting that MD-2 was also an essential media element in the associated inflammatory pathways. The positive correlation between MD-2 and TLR4 during inflammation medium may result from their roles as essential components of LPS complexes[10] and is critical during the occurrence and development of acute cholangitis. Anti-TLR4 group rats showed lower expression of both MD-2 and TLR4 mRNA compared with rats in the sham-operation group, control group and acute cholangitis group at corresponding time points ($P < 0.05$). These observations were mainly due to the inhibition of normal TLR4 expression by anti-TLR4 and the resultant inhibition of MD-2. When anti-TLR4 bound to TLR4, it is likely that it also suppressed MD-2 that was bound to TLR4 and inhibited MD-2 expression and function. These observations suggest that in acute cholangitis, MD-2 mediates the LPS inflammatory pathway collaboratively with TLR4 through protein-protein interaction[20].

Selective slicing has been the subject of intensive

research in the recent years, particularly since the human genome project was completed and may potentially contribute to the discovery and development of new medical treatments, drug targets and diagnosis markers[21-23]. Many key signaling molecules and downstream effector molecules in the TLR4 signaling pathway including MD-2, TRL4, MyD88, IRAK and IκB produce selective splicing isoforms[24-29]. Interestingly, most of these isoforms play a negative role in regulating TLR4 pathway. In acute cholangitis, liver MD-2B decreases to levels that are lower than that in the normal state which is opposite to the observed up-regulation of the inflammatory mediators MD-2 and TLR4. These data suggest that MD-2B plays an inhibitory role in the inflammation process and that MD-2B expression increases as the expression of MD-2 and TLR4 are reduced. Thus, in acute cholangitis, MD-2B is also involved in the inflammatory process by inhibiting MD-2, TLR4 or MD-2 and TLR4 complex. However, the underlying inhibitory mechanism and the exact functioning location of MD-2B need further investigation.

Ohta *et al*[13] identified MD-2B as an MD-2 splicing isoform and showed that it had a negatively regulatory function during TLR4-mediated LPS signal transduction, reducing cell and tissue damage caused by inflammatory reactions[30-32]. The experiments reported here show that in rats, the liver normally expresses only a small but consistent amount of MD-2B mRNA. In rats with induced, acute cholangitis, MD-2 mRNA expression started to decline 12 h after surgery ($P < 0.05$). However, in rats treated with anti-TLR4, MD-2 mRNA expression rose after 6 h as compared with its expression in other groups at the same time point ($P < 0.05$). This up-regulation may be due to the combination of anti-TLR4 with the TLR4 extracellular region which partially blocks the combination of CD14 and MD-2 and subsequently leads to the enhanced expression of the MD-2B, the splicing isoforms of MD-2.

COMMENTS

Background

TLR4 as the main receptor for lipopolysaccharide (LPS) activates transcription factor NF-κB which controls gene expression in various ways to produce biological effects. Myeloid differentiation protein-2 (MD-2) is the bridge between the Toll-like receptor (TLR) and LPS, playing an important role in the lipopolysaccharide signal transduction. MD-2B is an editing isomer of MD-2 extracted from the mouse spleen cells and bone marrow cells. It can block dose-dependent toll-like receptor 4 (TLR4)/MD-2 LPS-mediated signal transduction. The study focused on the expressions of MD-2 and its editing isomers (MD-2B) in acute cholangitis and its mutual relations to TLR4.

Research frontiers

TLR4 has been identified as an important transmembrane signaling system involved in endotoxin-induced inflammation. MD-2 serves as the bridge between TLRs and LPS, playing an important role in the LPS signal transduction. MD-2B can dose-dependently block TLR4/MD-2/LPS-mediated NF-κB signal transduction. In the present study, data were used to study the interrelationship between MD-2, its editing isomers (MD-2B) and TLR4 and establish the role of MD-2 and MD-2B in the TLR4-mediated inflammatory signaling pathway.

Innovations and breakthroughs

In acute cholangitis, MD-2 plays an important role in the process of TLR4-mediated inflammatory response to liver injury while MD-2B plays a negative regulatory role.

Applications

This may potentially contribute to the discovery and development of new medical treatments, drug targets and diagnosis markers. They may offer early diagnosis for severe acute cholangitis.

Peer review

This paper describes an interesting and novel experiment that provides confirmatory evidence of the role of MD-2 in the process of TLR4- mediated inflammatory response to liver injury, while MD-2B plays a negative regulatory role.

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CASE REPORT

Long-term survival of a HCC-patient with severe liver dysfunction treated with sorafenib

Christoph Roderburg, Jhenee Bubenzer, Michael Spannbauer, Nicole do O, Andreas Mahnken, Tom Luedde, Christian Trautwein, Jens JW Tischendorf

Christoph Roderburg, Jhenee Bubenzer, Michael Spannbauer, Nicole do O, Tom Luedde, Christian Trautwein, Jens JW Tischendorf, Department of Medicine Ⅲ, University of Aachen (RWTH), Pauwelstraße 30, Aachen 52074, Germany
Andreas Mahnken, Department of Radiology, University of Aachen (RWTH), Pauwelstraße 30, Aachen 52074, Germany
Author contributions: Roderburg C, Bubenzer J, Spannbauer M, do O N, Mahnken A, Luedde T, Trautwein C, and Tischendorf JJW designed research; Roderburg C, Mahnken A, and Tischendorf JJW performed research; Roderburg C, Mahnken A, Luedde T, and Tischendorf JJW analyzed data; and Roderburg C, Bubenzer J, Spannbauer M, do O N, Mahnken A, Luedde T, Trautwein C, and Tischendorf JJW wrote the paper.
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Correspondence to: Christoph Roderburg, MD, Department of Medicine Ⅲ, University of Aachen, Pauwelstraße 30, Aachen 52074, Germany. croderburg@ukaachen.de
Telephone: +49-241-8035367 Fax: +49-241-8082107
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Abstract

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver. Prognosis and treatment options are stage dependent. In general, prognosis of patients with unresectable HCC is poor, especially for those patients with impaired liver function. Whereas treatment with the novel molecular tyrosine kinase inhibitor sorafenib (Nexavar) was shown to result in prolonged survival in patients with preserved liver function, its' possible application in HCC-patients with strongly impaired liver function has not been clearly assessed. Here, we report on a 47-year-old male patient who presented with Child-Pugh class C liver cirrhosis and multifocal, non-resectable HCC. The patient was treated for 27 mo with Sorafenib, which was not associated with major drug-related side effects. During treatment, a reduction in tumour size of 24% was achieved, as assessed by regular CT scan. Moreover, within the 27 mo interval of stable tumour disease, liver function improved from Child-Pugh class C to class A.

Key words: Hepatocellular carcinoma; Sorafenib; Liver cirrhosis; Child-Pugh score; Long-term survival

Peer reviewers: Norberto C Chavez-Tapia, MD, PhD, Departments of Biomedical Research, Gastroenterology and Liver Unit, Medica Sur Clinic and Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan 14050, Mexico City, Mexico; Cheng-Shyong Chang, MD, Assistant Professor, Attending physician, Division of Hemato-oncology, Department of Internal Medicine, Changhua Christian Hospital, 135, Nan-Hsiao St. Changhua, 500, Taiwan, China

Roderburg C, Bubenzer J, Spannbauer M, do O N, Mahnken A, Luedde T, Trautwein C, Tischendorf JJW. Long-term survival of a HCC-patient with severe liver dysfunction treated with sorafenib. *World J Hepatol* 2010; 2(6): 239-242 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i6/239.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i6.239

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer-related death[1-5]. Therapeutic options and prognosis are stage dependent. In the case of localized disease, prognosis has been significantly improved in the last decade due to progress in diagnostic techniques and introduction of combined modality therapy[1-3]. In the case of unresectable or metastatic disease, however, HCC is still associated with a poor prognosis, and systemic therapy with cytotoxic agents provides only marginal benefit[4-6]. The introduction of targeted therapies such as receptor tyrosine kinase inhibitors represents a breakthrough in the management of HCC[3,6,7].

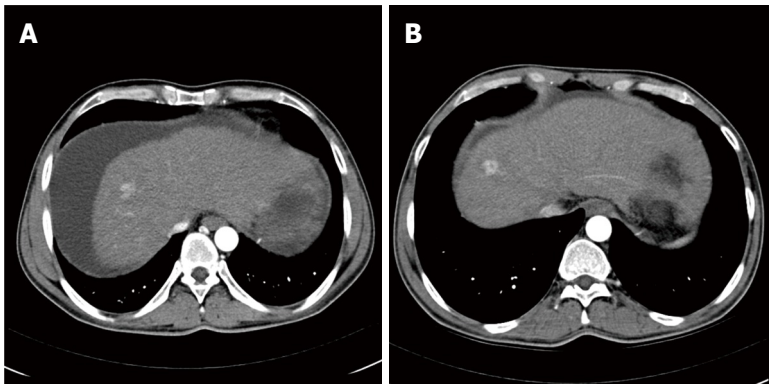

Figure 1 Computed tomography (CT)-scans: Axial Ⅳ contrast enhanced CT. Arterial phase image showing the reference lesion at the indicated time points. Size of this lesion remained stable between 2007 and 2009. A: CT liver November, 2007; B: CT liver December, 2009.

At present, the multi-tyrosine kinase inhibitor Sorafenib is the first and only molecular targeted drug that has been approved for treatment of patients with advanced HCC[8]. Encouraging preclinical results in several human tumours and a large phase Ⅱ trial including 137 patients with advanced HCC led to a multi-center trial with a randomized, placebo-controlled design[9]. The phase Ⅲ Sorafenib HCC Assesment Randomized Protocol (SHARP) trial demonstrated a 31% decrease in risk of death with a median survival of 10.7 mo for the sorafenib arm versus 7.9 mo for placebo[10]. Similar results were acquired by the ASIAN-trial. However both trials were restricted to patients with non-impaired liver function (Child-Pugh class A). Whether patients with impaired liver function can safely be treated with sorafenib and how this treatment might influence liver function and tumour progression is presently unclear.

CASE REPORT

A 47-year-old Egyptian patient was referred to our outpatient unit with clinical signs of chronic liver insufficiency. The patient was diagnosed with liver cirrhosis in 1996, caused by long-term ethanol abuse (stopped in 2001) and a hepatitis C infection after vaccination in Egypt in 1993. Between October 1997 and 2002 different interferon- and interferon/ribavirin-based therapies were conducted and finally discontinued due to non-response and at the request of the patient. Between 2002 and May 2007 no clinical visit was documented. In May 2007 laboratory testing and a sonography of the abdomen was performed during an external clinical visit. No hepatic lesions suspect for HCC were observed at this time-point. Liver function was severely reduced with a Child-Pugh score of 12 (Child-Pugh class C).

On admission to our outpatient unit in September 2007, blood tests showed a severe impairment of liver function (Bilirubin 5.6 mg/dL, Albumin 28 g/L, Quick 48%) and a mild rise in alpha-fetoprotein (AFP) level (17 μg/L). No ethanol was detected in the serum. Furthermore serum gamma-glutamyl transferase (GGT) as well as serum triglycerides were within the normal limits, indicating long-term alcohol abstinence. Clinically the patient showed jaundice of skin and sclera and stage 1 encephalopathy. Taken together, these data resulted in a Child-Pugh score of 12 (Child-Pugh class C). Ultrasound examination of the abdomen demonstrated a hepatic mass in liver-segment Ⅶ accompanied by portal hypertension, massive ascites and severe meteorism, limiting the sensitivity of this examination to detect smaller lesions. Arterial phase contrast-enhanced multislice spatial computed tomography (MSCT) confirmed this lesion and further lesions [lobus caudatus (7 mm × 8 mm); segment Ⅱ (5 mm × 3 mm); segment Ⅵ/Ⅶ (12 mm × 17 mm) and segment Ⅷ (17 mm × 18 mm and 23 mm × 14 mm)] became apparent (Figure 1). Furthermore, two lesions smaller than 5 mm in largest diameter (segment Ⅱb and Ⅷ) were described. In order to further discriminate between HCC and benign lesions (such as hyperplastic nodules) magnetic resonance imaging (MRI) with a liver specific contrast agent was performed. Here five lesions suspect for HCC became apparent, strongly suggesting the presence of a multifocal HCC. After evacuation of 3.5 L ascites, a CT-guided puncture of the hepatic lesion in segment Ⅷ was performed. Pathological analysis confirmed the presence of a well-differentiated hepatocellular carcinoma (G2). Neither on MSCT scan nor in MRI was extrahepatic tumour spread detected.

Overall, the patient was classified as BCLC stage D, suggesting treatment focussed on palliation as the remaining therapeutic option. However, considering the young age of the patient, the presence of a hepatitis C infection and the lack of extrahepatic tumour spread, the potential risks and benefits of a treatment with 800 mg sorafenib daily were discussed with the patient. This therapy was initiated in October 2007 and has continued until now. Regular clinical evaluations have included physical examination and laboratory testing (complete blood count, creatinine, ALT, AST, AP, GGT and Quick) every 4 wk. CT scans with intravenous and oral contrast of the abdomen have been performed every three months. As depicted in Figure 2, on admission the patient presented with a Child-Pugh score of 12 (MELD score: 19). The lesion in segment Ⅷ with a diameter of 22.8 mm was chosen as a reference for evaluation of tumour growth. After the onset of sorafenib treatment a 24% reduction in tumour size was achieved as the best radiological response, corresponding to long-term (27 mo) tumour control. Interestingly evaluation of tumour mass by serum AFP measurements and MSCT were in perfect correlation (Figure 2B). In the meantime an intensified

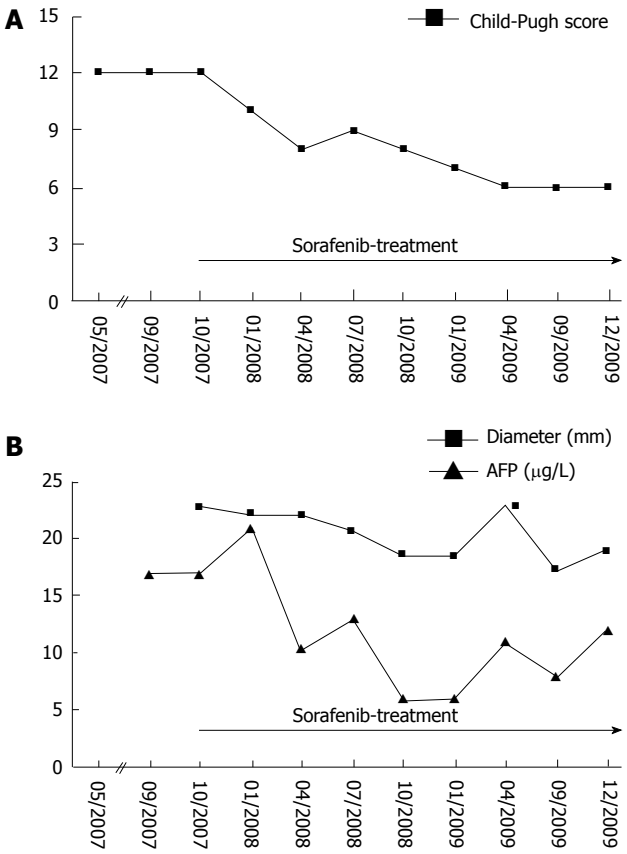

Figure 2 Clinical course of the patient. Therapy evaluation was performed by physical examination, complete blood count and creatinine, ALT, AST, AP, GGT, INR and assessment of adverse events as well as CT scans of the abdomen. A: Child-Pugh score; B: Serum AFP levels and the diameter of the reference lesion at the time-points indicated. ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; GGT: gamma glutamyltransferase; INR: international normalized ratio; AFP: alpha-fetoprotein.

diuretic treatment with spironolactone and torasemide was introduced, leading to continuous improvement of liver function (no signs of encephalopathy, mild ascites, Bilirubin 1.2 mg/dL, Albumin 35.1 g/L, Quick 82%; MELD score: 9) and a down-staging of impairment of liver function according to the Child-Pugh score from Child-Pugh class C to class A (Figure 2A). Currently the patient is stable under a combination-therapy with spironolactone (100 mg daily) and torasemide (20 mg daily). Given the improvement of liver function, local ablative therapy was proposed but is currently rejected by the patient. The option of liver transplantation was rejected due to excessive tumour load according to the Milano criteria (> 3 lesions) and lack of a living liver donor.

Sorafenib-treatment was well tolerated over the whole time; only one event of grade 3 diarrhoea was reported.

DISCUSSION

Advanced or non-resectable hepatocellular carcinoma has a poor therapeutic outcome. For many patients, therapeutic options are limited to palliation[3-5]. Only recently, molecular targeted therapy such as sorafenib has become an option for treatment of patients with advanced HCC[6,7,11]. Sorafenib is one of the new molecular targeted agents that inhibits both proangiogenic (VEGFR-1, -2, -3; PDGFR-β) and tumorigenic (RET, Flt-3, c-Kit) receptor tyrosine kinases. It also inhibits the serine/threonine kinase Raf-1. Raf-1 has been shown to be activated in a wide range of human malignancies and is therefore recognized as a strategic target for therapeutic drug development. Thus, sorafenib has been proven to be effective in a wide range of solid tumours comprising renal cell carcinoma, melanoma and hepatocellular carcinoma by inhibiting proangiogenic and pro-apoptotic pathways.

Sorafenib demonstrated a significant survival benefit in patients with non-resectable or advanced HCC in the SHARP- and the ASIAN- trial[10] and has become a treatment standard for HCC-patients in advanced stages of disease. A subgroup analysis of both studies showed that those patients lacking extrahepatic tumor spread and chronic hepatitis C infection benefit particularly from treatment with sorafenib. This may be due to the fact that chronic hepatitis C induces signalling *via* Ras/Raf-pathway, which is one of the main targets of sorafenib. However, the positive outcome of these studies applied only to Child-Pugh class A and a few of Child-Pugh class B patients. The question of the efficacy and safety of sorafenib treatment of patients with impaired liver function had therefore remained unanswered. In a large phase Ⅱ trial including 137 patients with advanced HCC, 28% of patients were classified as Child-Pugh class B. Pharmacokinetic parameters showed no difference in patients with cirrhosis Child-Pugh class A and B, and common adverse events associated with sorafenib were similar[9]. However, cirrhosis worsened more frequently in Child-Pugh class B patients[9]. It remained unclear whether this was a drug-related effect or was caused by disease progression. While differences in sorafenib pharmacokinetics between Child-Pugh class A and B patients were not clinically significant, study-based safety data are not available for patients with Child-Pugh class C cirrhosis[9]. To our knowledge there are only two reports on sorafenib treatment of HCC patients with Child-Pugh class C cirrhosis. Pinter et al. and Wörns et al. report on ten and four patients, respectively, treated with sorafenib. Based on their data, they concluded that sorafenib had no significant benefit in patients with high grade impaired liver function because of their limited life expectancy (OS < 3 mo) and lack of improvement in clinical parameters[12,13].

In sharp contrast to these data, we here report the case of a HCC-patient with Child-Pugh C liver cirrhosis who has experienced a long-term (27 mo) phase of stable tumour disease under treatment with sorafenib. On admission the patient displayed a Child-Pugh score of 12 [at this time since at least 5 months lasting (May 2007-Octobre 2007)] and seven hepatic HCC-lesions, as shown by MRI/ MSCT scan. The AFP level was only slightly enhanced, which is consistent with the fact that up to 20% of HCC patients do not produce AFP during the

course of the disease[14,15]. During the period of treatment with sorafenib we observed a reduction in tumour size of 24%, corresponding to stable disease according to RECIST criteria.

In addition hepatological treatment such as optimization of nutrition and lifestyle as well as optimization of medication (e.g. diuretics) was implemented and resulted in an improvement of Child-Pugh score from class C to class A (score 12 to score 6). Given the improved liver function, the patient became suitable for treatment concepts such as TACE which are currently rejected by the patient due to the risk of worsening of liver function. Furthermore liver transplantation was considered as an option for this patient. Liver transplantation represents a curative treatment modality for a selected patient population as defined by tumour burden. For HCC, liver transplantation only yields good results for patients whose tumour masses do not exceed the Milano-criteria (1 lesion $\leqslant$ 5 cm, or 2 to 3 lesions $\leqslant$ 3 cm)[16]. In recent years living donor liver transplantation has been discussed for patients exceeding these criteria[17]. However due to tumour load and lack of a liver donor neither cadaveric nor living donor liver transplantation were an option for the patient.

In summary these data suggest that for a highly selected population of HCC- patients (e.g. young age, lack of extrahepatic tumour spread, chronic HCV infection) sorafenib-treatment might be a well tolerated option even in cases of detoriated liver function.

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CASE REPORT

Spontaneous splenic rupture during Pringle maneuver in liver surgery

Jesse M van Buijtenen, Bas Lamme, Erik J Hesselink

Jesse M van Buijtenen, Department of Surgery, Westfriesgasthuis, PO Box 600, AR Hoorn Hoorn 1620, The Netherlands
Bas Lamme, Erik J Hesselink, Department of Surgery, Gelre Hospitals, Lukas, Apeldoorn 7300 DS, The Netherlands
Author contributions: van Buijtenen JM, Lamme B and Hesselink EJ contributed equally to this work.
Correspondence to: Erik J Hesselink, MD, PhD, Department of Surgery, Gelre Hospitals, Lukas, PO Box 9014 DS, Apeldoorn, The Netherlands. e.hesselink@gelre.nl
Telephone: +31-55-5818181 Fax: +31-55-5818131
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Abstract

During liver resection clamping of the hepato-duodenal ligament (the Pringle maneuver) is performed to reduce intraoperative blood-loss. During this maneuver acute portal hypertension may lead to spontaneous splenic rupture requiring rapid splenectomy in order to control blood loss. We present 2 case of patients with hemorrhage from the spleen during clamping for liver surgery. A review of the literature with an emphasis on the pathophysiology of splenic hemorrhage is presented.

Key words: Spleen; Rupture; Pringle maneuver; Liver surgery

Peer reviewers: Paolo Feltracco, MD, Assistant Professor of Anaesthesia and Intensive Care, Istituto di Anestesiologia e Rianimazione, Università di Padova, Via Cesare Battisti 267, Padova 35100, Italy; Ruben Ciria, PhD, Department of Hepatobiliary Surgery and Liver Transplantation, Uniersity Hospital Reina Sofia, Avennida Menendez PidalI s/n, Cordoba 14004, Spain

van Buijtenen JM, Lamme B, Hesselink EJ. Spontaneous splenic rupture during Pringle maneuver in liver surgery. *World J Hepatol* 2010; 2(6): 243-245 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i6/243.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i6.243

INTRODUCTION

Previously, only a few cases of spontaneous splenic rupture during liver surgery have been described in the literature[1-4]. This rare complication may lead to acute life-threatening situations requiring immediate splenectomy in order to control massive blood loss. We present 2 cases of patients with splenic hemorrhage during portal vein and hepatic artery clamping (Pringle maneuver) for liver surgery as well as a review of the relevant literature.

CASE REPORTS

Patient 1

A 68-year-old man with a previous history of diabetes mellitus and a coronary artery bypass graft presented with ongoing abdominal complaints after a sigmoid-resection for colon carcinoma (pT3N0Mx) 2 mo previously. His family history was negative for coagulopathic disorders. Computed tomography (CT) and positron emission tomography (PET) scans revealed 3 liver metastases although no other abnormalities to the internal organs were detected.

After cessation of calcium carbasalate (100 mg/d) treatment 7 d preoperatively, the patient underwent a laparotomy with intraoperative ultrasound of the liver. This revealed 3 liver metastases in the liver, one located close to the left hepatic vein and two located on the right side of the liver. Metastasectomy with an ultrasonic dissector (Ethicon Inc., Johnson and Johnson Medical) was performed. The two right-sided metastases were resected easily. In order to resect the left-sided metastasis a Pringle maneuver was performed by clamping of the hepatoduodenal ligament. Initially during this third metastasectomy the patient remained stable, however after only 20 min of clamping the operation field was rapidly filled with blood pouring from the left upper quadrant. Upon close inspection, a splenic rupture was detected and an emergency splenectomy was performed by quickly ligating the splenic artery and vein as well as the short

gastric arteries. After the splenectomy the patient remained hemodynamically stable and the metastasectomy of the liver could be continued without any other complications. To compensate for the intraoperative blood-loss, 2 liters of packed-cells were administered.

On pathological examination the removed spleen showed a ruptured capsule and partial ablation with hematoma. The spleen weighed 95 grams and had a maximum diameter of nine centimeters. Microscopically, the radically removed liver metastases showed moderate differentiated adenocarcinoma of the intestinal type surrounded by normal liver parenchyma.

Postoperatively, the patient developed pneumonia which was treated successfully with antibiotics and was discharged after Pneumovax vaccination 14 d postoperatively. At 4 mo follow-up, multiple, diffuse liver metastases and a lung metastasis were detected on CT-scan and chemotherapy was initiated.

Patient 2

A 61-year-old-female treated for osteoporosis and rheumatoid arthritis was presented with rectal blood-loss. She underwent colonoscopy which revealed a poorly differentiated colonic carcinoma 25 cm from the anal rim. The carcinoembryonic antigen (CEA) level was 3.5 ng/mL. An uncomplicated sigmoid resection was performed with radical resection of the tumor (pT2N0). After 4 years of uneventful follow up, serum CEA-markers increased to 5.3 ng/mL and CT-scan disclosed an intrahepatic lesion of 3 cm diameter highly suspicious for a metastasis. Furthermore, this scan showed no other metastases and no signs of portal hypertension. The patient underwent a laparotomy for metastasectomy *via* bilateral subcostal incisions. After opening of the abdomen the retractors were closely positioned. After dissection of the liver hilum, the hepatoduodenal ligament was clamped according to the Pringle maneuver, as well as the right hepatic vein. The metastasis located at the right top of the liver was resected by ultracision. Sudden major blood-loss originating from the spleen was noted only 15 min after initiating the Pringle maneuver. An immediate splenectomy was performed. After the splenectomy blood-loss stopped and the metastectomy was continued uneventful. The tumor was resected with a margin of 1-2 centimeters. Total blood-loss was approximately 2.5 liters and the patient was therefore given 6 Units of packed-cells.

On pathological examination the removed spleen measured 10 cm × 8 cm × 2 cm and weighed 90 grams. A ruptured splenic capsule was noted. The contralateral side showed an isolated hematoma and ablation of the capsule. The resected metastasis was identified histologically as poorly differentiated adenocarcinoma and the encircling liver parenchyma appeared normal. The patient recovered uneventfully and was released from hospital 8 d postoperatively. To date, no evidence for recurrent disease was found during follow-up.

DISCUSSION

Only 5 cases of spontaneous splenic rupture during Pringle maneuver in liver surgery have been described previously[2-4]. Spontaneous splenic rupture can result from either intrasplenic or extrasplenic causes. Intrasplenic causes are of infectious, neoplastic, vascular or congenital etiology, whereas extrasplenic causes are altered blood pressure in the portal system, coagulopathy disorders, pregnancy or auto-immune anomalies (Figure 1). Spontaneous splenic rupture caused by malignancy, infection, congenital disorders or auto-immune disorders, has been documented frequently in case reports.

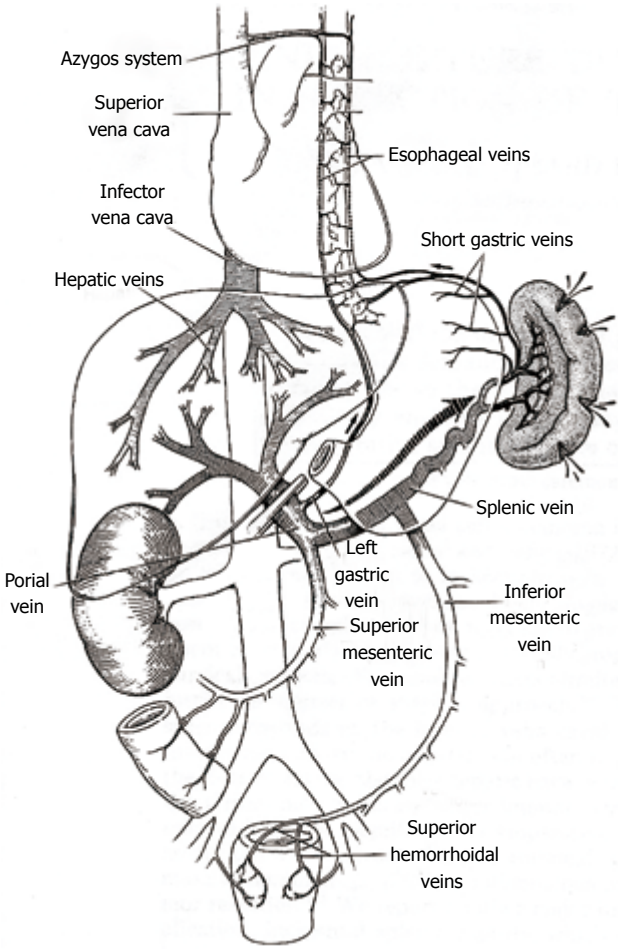

Figure 1 Intrasplenic or extrasplenic causes result in spontaneous splenic rupture.

The Pringle maneuver can be employed during hepatic resections and consists of clamping of the portal vein, hepatic artery and common bile duct. This 'inflow occlusion' technique significantly reduces intraoperative blood loss and improves both short- and long-term outcomes[5,6]. Although used by surgeons for decennia, it was not until 1997 that a randomized trial proved that the Pringle maneuver resulted in less blood loss and in preservation of liver function in the early postoperative period due to retrograde flow from the hepatic veins[6]. Described adverse effects of the Pringle maneuver are ischaemic-reperfusion injury, leading to suppressed DNA synthesis, hepatocyte proliferation and delayed hepatic regeneration[7,8]. In 1999 a prospective randomized study concluded that intermittent clamping was favorable over

continuous clamping, especially in patients with abnormal liver parenchyma such as in steatosis and cirrhosis and in patients particularly sensitive to ischaemia[8].

However, a recent randomized trial showed that hepatic resection without any form of hepatic pedicle clamping can be performed safely through novel surgical devices used for transection of the liver parenchyma and anaesthesiological techniques for controlling parenchymal bleeding[9]. In this way ischaemic-reperfusion injuries are avoided.

In performing the Pringle maneuver, intermittent clamping is carried out, at present only in case of excessive blood loss during liver transection. Portosystemic collaterals, which are collapsed normal under physiological circumstances, can be opened by a temporary increase in portal pressure and thus assist in lowering portal hypertension. Contrary to the situation in our patients, patients with cirrhosis and portal hypertension have well-developed portosystemic collateral channels that compensate for the sudden increase in portal venous pressure[3].

The Pringle maneuver leads to a small decrease in cardiac output, a decrease in ventricular filling pressure, an increase in arterial pressure and a marked increase in systemic vascular resistance. The latter counteracts the decrease in cardiac output and thus arterial pressures tend to remain at or higher than preclamping levels. Activation of baroreceptors in the portal venous system and spleen may elicit a reflex increase in systemic arterial pressures. Clamping of the portal vein leads to a moderate increase in portal venous pressure by shunting through portosystemic collateral channels. Delayed adaption in splangic bed capacity contributes to further pooling of venous blood[5,10]. The combination of these factors can ultimately lead to a spontaneous splenic rupture, as happened in our patients (Figure 1).

In conclusion, resection without any form of hepatic pedicle clamping can be performed safely and avoids ischemic injury as well as portal hypertension. In case of excessive blood-loss, the Pringle maneuver can be performed, preferably intermittently. However, using the Pringle maneuver, the risk of a spontaneous splenic rupture should be taken into account.

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CASE REPORT

Simultaneous occurrence of a hepatocellular carcinoma and a hepatic non-Hodgkin's lymphoma infiltration

Sabine Heidecke, Dirk L Stippel, Arnulf H Hoelscher, Inga Wedemeyer, Hans-Peter Dienes, Uta Drebber

Sabine Heidecke, Dirk L Stippel, Arnulf H Hoelscher, Department of General-, Visceral- and Cancer Surgery, University Hospital of Cologne, Cologne 50937, Germany
Inga Wedemeyer, Hans-Peter Dienes, Uta Drebber, Department of Pathology, University Hospital of Cologne, Cologne 50937, Germany
Author contributions: Heidecke S did the research of this project and wrote the case report; Stippel DL did the laparoscopic segmentectomy; Hoelscher AH is the head of department of surgery; and Wedemeyer I, Dienes HP and Drebber U did the pathological examinations.
Correspondence to: Sabine Heidecke, MD, Department of General-, Visceral- and Cancer Surgery, University Hospital of Cologne, Kerpener Str. 62, Cologne 50937, Germany. sabine.heidecke@uk-koeln.de
Telephone: +49-176-21906869
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Abstract

To investigate the simultaneous occurrence of hepatocellular carcinoma and non-Hodgkin's lymphoma, we report the case of a 70 year old patient with a primary diagnosis of non-Hodgkin's lymphoma in 2002. In a routine follow up investigation of his chronic lymphocytic leukemia a newly detected mass in the Couinaud's segments 2 and 3 was found. No hepatitis C virus or hepatitis B virus infection or cirrhosis was evident. After laparoscopic segmentectomy the histological examination revealed a hepatocellular carcinoma. While the relation between liver parenchyma damages and hepatocellular carcinoma or non-Hodgkin's lymphoma is well known, only a few publications have focused on the coexistence of hepatocellular carcinoma and non-Hodgkin's lymphoma. With this case we demonstrate the coexistence of these diseases without having a predamaged liver parenchyma.

Key words: Hepatocellular carcinoma; Non-Hodgkin's lymphoma; Etiology; Carcinogenesis; Histology

Peer reviewers: Luca Vigano, MD, Department of HPB and Digestive Surgery, Ospedale Mauriziano Umberto I, Largo Turati 62, Torino 10128, Italy; Derek Anthony O'Reilly, PhD, FRCS, Department of Surgery, North Manchester General Hospital, Delaunays Road, Manchester, M8 5RB, United Kingdom

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common type of primary liver cancer and is well investigated concerning its development and risk factors. In most of the cases HCC arises from chronic liver disease and cirrhosis. As a main risk factor the chronic infection by hepatitis C virus (HCV) or hepatitis B virus (HBV) is known[1-2].

The invasion of the liver by non-Hodgkin's lymphoma (NHL) has also been part of several clinical investigations. Hepatitis infection has also been reported frequently as a risk factor for NHL[3-5].

Only a few publications have focused on the coexistence of HCC and NHL. For both conditions the risk factors of cirrhosis and hepatitis infection have been described, which lead to a damage of liver parenchyma. HCV has been suggested to be lymphotrophic as well as hepatotrophic and has therefore attracted speculation about a causative role in some cases of lymphoma. With this case we demonstrate the coexistence of HCC and NHL without having a predamaged liver parenchyma.

CASE REPORT

A 70 year old patient with a primary diagnosis of NHL

in 2002 was assigned to our hospital in October 2008 for further treatment of a newly detected mass in the left lateral liver lobe. Prior to this he underwent routine investigations once a year to follow up the current status of his chronic lymphocytic leukemia (CLL). There was no need for any intervention concerning the CLL given stable blood parameters and hardly any clinical complaints. In the last routine check-up an unknown mass in the left lateral liver lobe was found.

The physical examination on admission showed a slightly overweight patient (170 cm tall and 91 kg bodyweight) in good health. Premedication included amlodipine against arterial hypertension and acetylcysteine and betahistidine relating to therapy for Menière's Disease. Neither the medical history nor the outer appearance gave any hint of alcoholic or nicotine abuse.

No upper abdominal pain was expressed and the patient did not report any other complaints in the previous months.

Diagnostic

Viral serological tests were negative for anti-HCV antibodies, anti-HB core antibodies and HB surface antigen. The patient's previous medical history included hepatitis A virus (HAV) infection revealed by a positive Anti-HAV (IgG/IgM). Alphafetoprotein (AFP) was negative, white blood cells were elevated at 84.000/μL due to the CLL. Further laboratory investigation revealed Glutamic-oxaloacetic transaminase (GOT) 25 U/L, Glutamic-pyruvic transaminase (GPT) 37 U/L, γ-Glutamyltransferase (γ-GT) 29U/L. Furthermore Albumin, Bilirubin and the parameters of coagulation were normal. So there was no indication of any liver parenchyma disorder, which would be reflected in a higher Child-Pugh-Classification.

Diagnostic imaging was done by computed tomographic (CT) scan and magnetic resonance imaging. Contrast-CT scan (Figure 1) showed a hyperdense (hypervascularised) lesion in Couinaud's segments 2 and 3, which appeared hyperattenuating in the early arterial phase of contrast enhanced CT.

Magnetic resonance imaging of the abdomen (Figure 2) confirmed an early enhancing mass in Couinaud's segments 2 and 3.

The radiological findings suggested that the newly detected mass was not a manifestation of the NHL. In CT scan done with contrast media an arterial enhancement was seen, followed by a venous washout and a late ring enhancement. This characteristic vascular profile led to the differential diagnosis of hepatocellular carcinoma. Imaging indicated no signs of cirrhosis.

Additionally, splenomegaly and huge masses in paraaortic lymph nodes reaching down to the femoral vessels were observed, related to the patient's already known lymphatic disease. There was no biopsy taken preoperative.

Treatment

On the sixth of January 2009 the patient underwent a laparoscopic liver resection of Couinaud's segments 2 and 3. No complications occurred after the operation and the further hospital stay was uneventful. He underwent a new check-up to establish the current status of the NHL and was then released 8 d after admission.

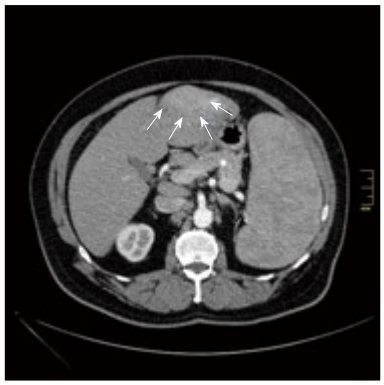

Figure 1 Contrast-computed tomographic (CT) scan shows a hyperdense (hypervascularised) lesion in the left lateral liver section, appearing hyperattenuating in the early arterial phase of contrast enhanced CT.

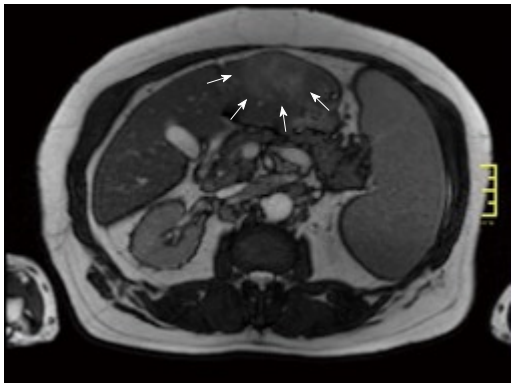

Figure 2 Magnetic resonance imaging shows an early enhancing mass in the left lateral segments of the liver.

Pathological findings

Histological examination of the resected specimen showed that there was no significant parenchymal fibrosis. Pathological findings suggested neither hepatitis, cirrhosis nor any noticeable hepatosteatosis. One could see a 4.8 cm, predominantly trabecular configured HCC with a histopathological grading of G2 and with a tumor staging of pT2, pNx, pMx. No lymphatic vessel invasion (L0) or blood vessel invasion (V0) was found.

Furthermore, the diffuse infiltration of NHL in the liver parenchyma was confirmed. Portal tracts appeared densely infiltrated by an atypical small lymphocytic population, typically seen in B-CLL[6]. Immunohistochemical examination with positive reactions for the B-cell-marker CD20 and for CD5, CD23 and CD43 confirmed the diagnosis of B-CLL (Figure 3).

Macroscopically, the liver parenchyma showed prominent enhanced greyish portal tracts and a tumorous nodular lesion measuring 4.8 cm at its widest point (Figure 4).

Microscopically, a border of non-neoplastic liver

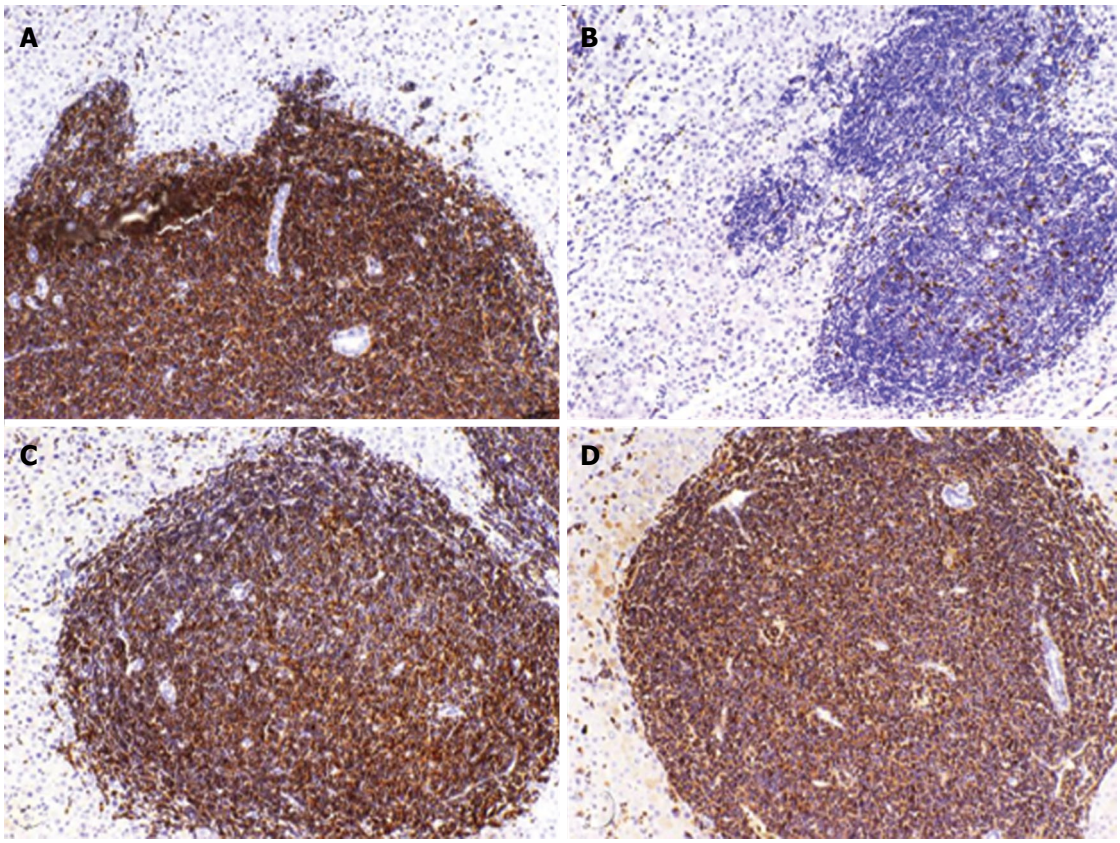

Figure 3 Immunohistochemical examination of the atypical small lymphocytic infiltrate in portal tracts. A: CD20; B: CD3; C: CD5; D: CD23.

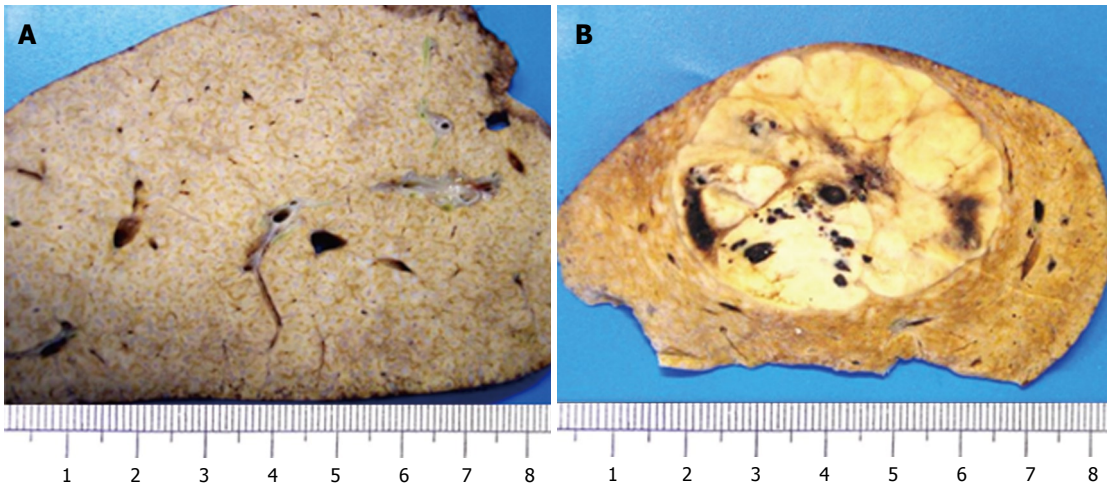

Figure 4 Macroscopical view of the liver parenchyma and the tumor mass. A: Macroscopically liver parenchyma shows prominent enhanced greyish portal tracts; B: An at most 4.8 cm measuring tumorous nodular lesion.

parenchyma and hepatocellular carcinoma could be seen. Furthermore, a trabecular configured HCC with atypical lymphocytic infiltration by B-CLL was detected. Portal tracts were rounded and showed an atypical dense lymphocytic infiltration by B-CLL. There was neither significant fibrosis, cirrhosis nor steatosis in the liver parenchyma (Figure 5).

The case of this patient was discussed in the hospital oncological conference. The haemato-oncologist re-evaluated the data and confirmed the diagnosis of the NHL. Cytology showed a lymphocytosis with typical smudge cells. Furthermore flow cytometric immunophenotyping of peripheral blood confirmed the diagnosis of CLL, whilst ZAP70 and CD38 were negative. The results indicated a good prognosis and lead to the conclusion that even with infiltration of CLL and simultaneous occurrence of HCC in the liver there would be no indication for chemotherapy.

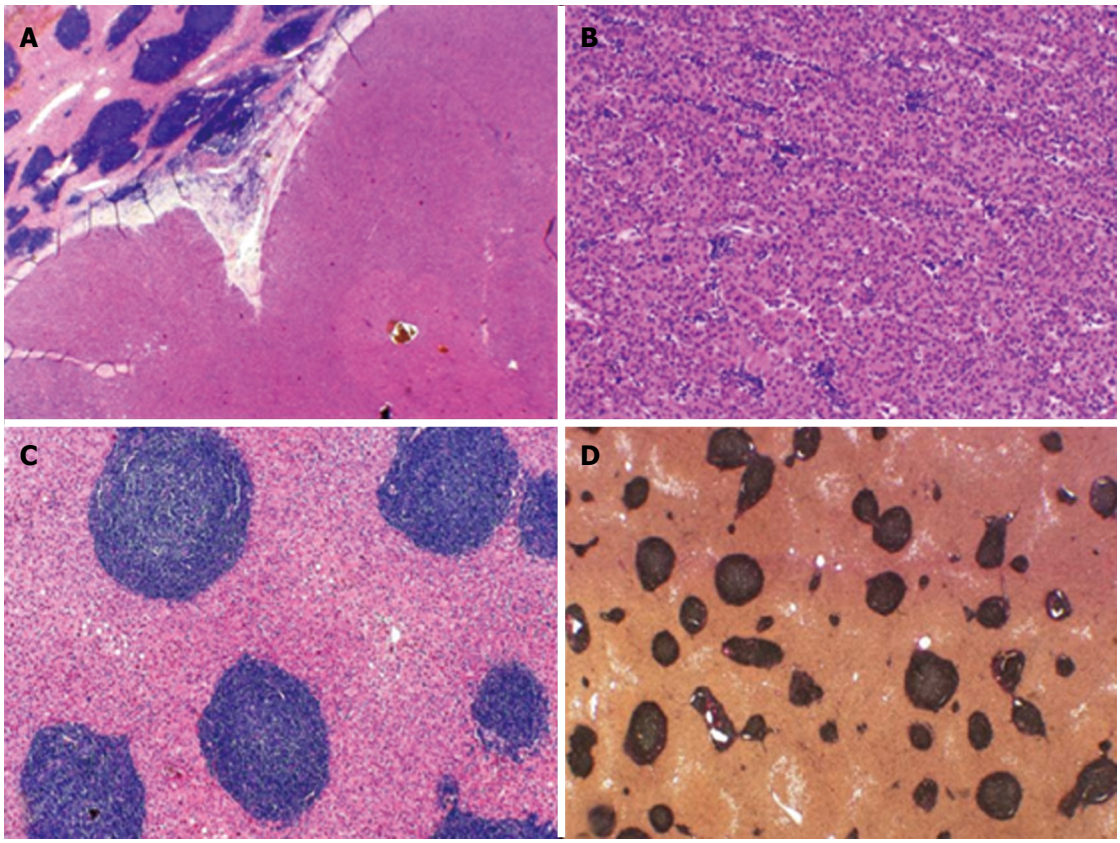

Figure 5 Histological specimen. A: Border of non-neoplastic liver parenchyma (upper left) and hepatocellular carcinoma (HCC) (lower right); B: Trabecular configured HCC with atypical lymphocytic infiltration by the B-cell lineage (B-CLL); C: Portal tracts are rounded and show an atypical dense lymphocytic infiltration by B-CLL; D: There is neither significant fibrosis nor cirrhosis in liver parenchyma (van Gieson stain).

Table 1 A summary of the relevant reports found in PubMed concerning the simultaneous occurrence of HCC and NHL

Author	Year	Type of cancer(s)	Predamaged parenchyma
Cavanna *et al*	1994	HCC and NHL	Chemotherapy
Tanaka *et al*	1997	HCC and NHL	Chemotherapy
Kataoka *et al*	2006	HCC and NHL	Nodular regenerative hyperplasia
Ohtsubo *et al*	2006	HCC and NHL	Hepatitis cinfection
Xiong *et al*	2008	HCC and NHL	
Utsunomiya *et al*	2009	HCC and NHL	Hepatitis c infection

HCC: hepatocellular carcinoma; NHL: non-Hodgkin's lymphoma.

The patient underwent a follow-up examination every four months for both the CLL and HCC.

To date (17 mo after surgery) the patient is in good health, and no recurrence has been detected by follow-up imaging. His CLL has not required treatment until now.

DISCUSSION

The end stage of chronic liver disease may be the onset of hepatocellular carcinoma. Therefore, liver cirrhosis could be considered to be a pre-neoplastic lesion. The question arises in this patient of why a HCC occured in the absence of cirrhosis or hepatitis infection. Which other risk factor have to be considered? In this case the HCC was discovered during a routine follow up without clinical complaints. There have been recent investigations concerning the possible relationship between metabolic syndrome and the occurrence of HCC. Although our patient was obese with a Body mass index of 31 kg/m^2 and was affected by mild arterial hypertension, there were no other signs of metabolic syndrome such as dyslipidemia or diabetes. One has to consider that metabolic syndrome has been identified as a risk factor for the occurrence of HCC. As mentioned above, there was no alcohol or nicotine abuse with this patient. In addition, the pathological findings did not show any sign of cirrhosis, hepatitis or steatosis which might have resulted in to a predamaged liver parenchyma.

Chronic lymphocytic leukemia of the B-cell-lineage (B-CLL) is the most common form of adult leukemia and predominantly a disease of older individuals. Due to the strong heterogeneity in the clinical course of B-CLL with survival ranging from months to decades, treatment regimens are strongly based upon clinical staging. This includes clinical presentation, laboratory values and lymphocyte doubling time. Until now the CLL in our patient has not been symptomatic and laboratory data have been stable since primary diagnosis.

One might speculate that the occurrence of a HCC in this patient was promoted by the NHL as a cofactor.

In PubMed research one can find 19 positive findings for "NHL and HCC", and 6 relevant reports for the discussion of our case were found (Table 1).

In 1994 Cavanna *et al* reported a patient diagnosed with NHL in the liver, who received chemotherapy. Six years later a needle aspiration was carried out. The histopathological findings showed a HCC, which was completely resected[7]. Three years after this operation a NHL was detected again and the patient died a short time after due to gastrointestinal bleeding. As Cavanna *et al* stated at that time, this was the first case report concerning the coincidence of NHL and HCC.

In 1997 Tanaka *et al*[8] demonstrated a higher risk for the development of a primary liver cancer (PLC), in all cases a HCC, if a preexistent NHL was found in the liver. Furthermore, patients who received chemotherapy as NHL treatment had a significantly increased risk of HCC.

In 2006 there was a report about a case of NHL and HCC in the absence of hepatitis infection or any signs of liver cirrhosis, but with nodular regenerative hyperplasia, which is a premalignant lesion[9]. The diagnosis was focused on a CT scan, which showed a simultaneous infiltration by NHL and HCC. After a short clinical period the patient died.

In 2006 Othsubo *et al*[10] reported on a 66 year old man with HCV-related liver cirrhosis and simultaneous hepatic relapse of NHL and HCC.

In 2009 Xiong *et al*[11] discussed the influence of the Cdc6 promoter, which plays an important role in DNA replication and carcinogenesis. They reported on the Cdc6 G1321A polymorphism, which lowers the risk for the development of both NHL and HCC.

Last year a case report was published about the co-existence of HCC and NHL, although this was again combined with an HCV infection[12].

In our case report the patient did not undergo chemotherapy, thereby excluding any possible influence of chemotherapy on outcomes. Furthermore, we have no evidence of any chronic hepatitis infection, cirrhosis or alcohol abuse. So this case report is in fact the first one to describe the simultaneous occurrence of HCC and NHL without having any other risk factors such as hepatitis infection or other premalignant lesions. Our pathological findings support the clinical history which indicated the absence of cirrhosis or any other liver parenchyma disorders.

The publications indicating a coincidence of NHL and HCC in the liver showed no proper evidence of the pathogenesis.

Future studies are required to establish firm evidence of the relationship between the occurrence of NHL and HCC.

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Cheng-Shyong Chang, MD, Assistant Professor, Attending physician, Division of Hemato-oncology, Department of Internal Medicine, Changhua Christian Hospital, 135, Nan-Hsiao St. Changhua, 500, Taiwan, China

Norberto C Chavez-Tapia, MD, PhD, Departments of Biomedical Research, Gastroenterology and Liver Unit, Medica Sur Clinic and Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan 14050, Mexico City, Mexico

Pierce KH Chow, MD, PhD, Professor, Department of General Surgery, Singapore General Hospital, Singapore 169608, Singapore

Ruben Ciria, PhD, Department of Hepatobiliary Surgery and Liver Transplantation, Uniersity Hospital Reina Sofia, Avennida Menendez PidalI s/n, Cordoba 14004, Spain

Stephen Lam Chan, MD, Department of Clinical Oncology, The Chinese University of Hong Kong, Prince of Wales Hospital, 30-32 Ngan Street, Shatin, New Territories, Hong Kong, China

Radha Krishan Dhiman, MD, DM, FACG, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Paolo Feltracco, MD, Assistant Professor of Anaesthesia and Intensive Care, Istituto di Anestesiologia e Rianimazione, Università di Padova, Via Cesare Battisti 267, Padova 35100, Italy

Nattiya Hirankarn, MD, Associated Professor, Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Rama 4 road, Bangkok 10330, Thailand

Neil Louis Julie, MD, Gastroenterology and Hepatology, 7609 Exeter Rd, Bethesda, MD 20814, United States

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Derek Anthony O'Reilly, PhD, FRCS, Department of Surgery, North Manchester General Hospital, Delaunays Road, Manchester, M8 5RB, United Kingdom

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Xun-Di Xu, MD, PhD, Department of Gastroenterological Surgery, Xiangya Second Hospital, Central South University, Renmin Zhong Road 139, Changsha 410011, Hunan Province, China

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Events Calendar 2010

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Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 25-28
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The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

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American Society of Clinical Oncologists Annual Meeting

June 16-19
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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