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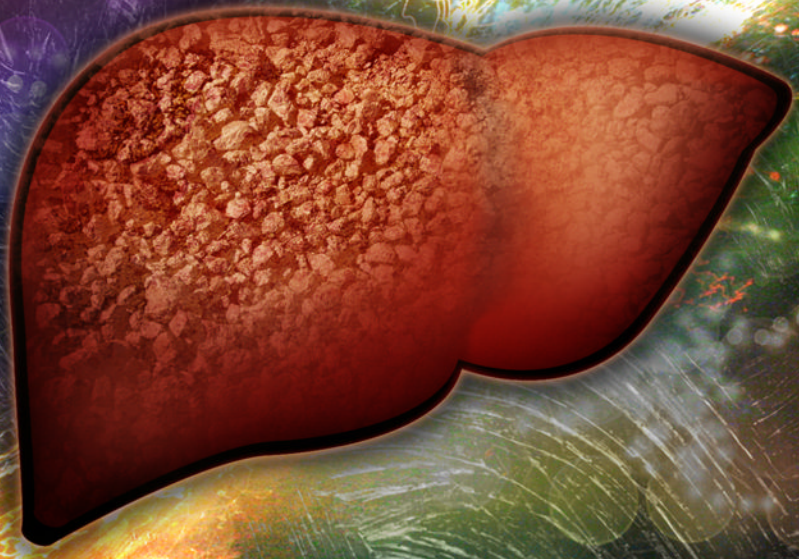
Liver Cirrhosis

Causes, Diagnosis and Treatment



Miranda L. Michelli
Editor

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LIVER CIRRHOSIS: CAUSES, DIAGNOSIS AND TREATMENT

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LIVER CIRRHOSIS: CAUSES, DIAGNOSIS AND TREATMENT

MIRANDA L. MICHELLI
EDITOR

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Contents

Preface

This book presents topical research in the study of liver cirrhosis, including etiologic types of end stage chronic liver disease; hepatocellular carcinoma screening in the cirrhotic patient; hepatic myofibroblasts and their role in liver fibrogenesis; the role of trace elements in the pathogenesis of liver cirrhosis and cell-based therapy for liver cirrhosis.

Chapter 1 – Thalidomide is currently used for treating erythema nodosum leprosum, multiple myeloma, angiogenesis, rheumatoid arthritis, graft-versus-host disease, among others. Thalidomide effects are related to its capacity to inhibit the proinflammatory cytokine tumor necrosis factor-α (TNF-α) and, in consequence, causes immunomodulation on other cytokines. During the establishment of some diseases the balance between proinflammatory and antiinflammatory cytokines is disrupted, promoting a pathological state; thus, elevated levels of proinflammatory cytokines mediate several deleterious processes such as inflammation, necrosis, apoptosis and fibrosis. These events are present in acute and chronic degenerative liver diseases such as hepatitis, cholangitis, cirrhosis and hepatocellular carcinoma (HCC). Then, the immunomodulation on cytokines by drugs seems to be a pharmacological target to ameliorate liver damage and cirrhosis. In fact, there are not sufficient drugs for relief or cure of cirrhosis currently; some of these few are expensive, unstable and palliative or may cause side effects. Novel thalidomide analogs have been synthesized with improved stability and potency as TNF-α inhibitory and immunomodulatory agents, besides low or none teratogenicity. Experimental assessment of thalidomide and its analogs in animal models of liver injury have afforded very hopeful outcomes. Thalidomide and two analogs have evidenced anticholestatic, antinecrotic and antifibrotic activities in bile duct ligation-induced biliary cirrhosis. Another analog protected D-galactosamine/endotoxin-treated mice from liver damage. Thalidomide ameliorated the alcoholic hepatic injury and prevented necrosis, cholestasis and fibrosis induced by CCl_4 in rats. Moreover, this drug salvaged from lethal hepatic necroinflammation and accelerated the recovery from established thioacetamide-provoked cirrhosis in rats. The antiinflammatory, antinecrotic and antifibrotic effects elicited by thalidomide and its analogs are mainly mediated by the inhibition on TNF-α through two different routes, as well as the down-regulation of nuclear factor-κB (NF-κB) signaling pathway and by diminishing adhesion molecules to prevent the progression of liver fibrosis and cirrhosis. Furthermore, thalidomide showed beneficial effects on HCC by decreasing angiogenesis and metastasis in murine models; therefore, diverse clinical phase I/II studies were carried out to evaluate its antitumoral or disease control outcomes. However, thalidomide as a single drug therapy yields very modest benefits, although in most cases this

is well tolerated and offers disease stabilization. Different doses and the combination with other chemotherapeutic agents appear to enhance therapeutic effects; the assessment of the new thalidomide analogs in next clinical trials of HCC healing is strongly suggested. Thalidomide and its analogs may be a feasible option for the treatment of liver diseases and cirrhosis.

Chapter 2 – Cirrhosis is one of the most common causes of mortality worldwide because hepatic dysfunction constitutes a potentially lethal condition. Its etiology is variable being the most important alcoholic liver disease, viral chronic hepatitis and there's no treatment for prevention or regression of this pathology. At the same time, there is an increased risk of sepsis, bacterial peritonitis, variceal bleeding, portal hypertension and development of hepatocelular carcinoma. In this chapter, the authors briefly describe the normal structure and function of the liver including the importance of energy participation, in the normal and cirrhotic liver. Among the liver dysfunctions of this pathology exists a decrease in energy parameters in the blood of alcoholic and nonalcoholic patients with cirrhosis. In experimental cirrhosis induced with CCl_4, a strong correlation between energy parameters of the liver and the red blood cells indicated that blood parameters could be a reflection of liver damage. Their experience regarding the studies of acute and chronic experimental hepatotoxicity using the nucleoside adenosine as an hepatoprotector suggest that the nucleoside effect increasing energy availability by itself, played and important role preventing the mitochondrial dysfunction induced by the hepatotoxics like ethanol, CCl_4 in acute an chronic models. Regardless of the mechanism involved in the effects of adenosine on CCl_4-induced cirrhosis in rats, the maintenance of the energy parameters of the liver cell during a chronic insult could be relevant to preserve liver function and the delay the fibrogenic effect of the toxin. More interesting is the beneficial effect of adenosine and adenosine derivative compound IFC305 on the preestablished CCl_4 cirrhosis associated with an improved liver function. In summary, adenosine and its derivative, through reversed cirrhosis, liver dysfunction and energy parameters of the liver cells could be considered as a tool to study the interrelationship between mitochondrial function and hepatotoxicity. Besides, it could be considered as a potential therapeutic agent for the treatment of chronic hepatic diseases.

Chapter 3 – Cirrhosis and end stage chronic liver disease (CLD), common in adults globally, are largely caused by environmental agents and factors and are prone to develop serious complications including hepatocellular carcinoma (HCC). Till the mid 1960s dietary protein deficiency was considered the cause of cirrhosis in chronic alcoholics and in the malnourished population of developing countries. Soon it was established that alcohol is directly hepatotoxic and that protein-calorie malnutrition does not cause cirrhosis. The major break through on cirrhosis came with the discovery of hepatitis B virus (HBV), and by mid 1970s it was clear that chronic HBV infection caused most CLD and HCC in developing countries and a smaller proportion in developed western countries. From 1990 on, a large segment of CLD not attributable to alcohol abuse or HBV infection was recognized as due to HCV infection. Wide use of blood transfusion and increasing intravenous drug abuse in the post-world war II period led to rapid increase in HCV related CLD and HCC. By early 21st century HBV related diseases decreased significantly in countries that adopted universal control measures for this virus infection including vaccination. In developed countries there was some reduction in HCV related CLD also but in other parts of the world it rose progressively while HBV related CLD showed small or no decline. In recent years alcohol consumption and alcohol related CLD have rapidly increased in developing countries.

Intrahepatic biliary tract diseases and autoimmune liver disease have all through constituted a small proportion of CLD. On the other hand, a sizeable number of end stage CLD currently evolve from nonalcoholic fatty liver disease (NAFLD), most of which clinically present as cryptogenic. Also, cases of noncirrhotic portal fibrosis (NCPF), a generally benign liver disease known by several other names, have presented as end stage cryptogenic cirrhosis needing liver transplantation.

Etiologic types of end stage CLD are changing over time. HBV and HCV related CLD are preventable and even curable if detected early. HBV related CLD, once highly prevalent is steadily declining and is expected to become minimal by mid twenty first century. HCV related ones are likely to come down in time though more slowly, while alcoholic CLD is probably going to persist or even increase during the next few decades. End stage CLD is rare in children, caused mainly by genetic or developmental anomalies. Unique forms of non-Wilsonian copper overload CLD are rare and ill understood.

Chapter 4 – Hepatocellular carcinoma (HCC) accounts for 85– 90% of all primary liver cancers. It is the sixth most common cancer overall, and the third most common etiology of cancer-related deaths worldwide, accounting for nearly 600,000 new diagnoses annually and approximately 600,000 related deaths. There is wide geographic variation of prevalence of HCC, with Asia and Africa having 40 times more cases than other parts of the world. However, the incidence of HCC has been rising throughout the world, with particularly large increases seen in industrialized nations such as Japan, the United States, and Denmark. In the United States, this rise in incidence, has led to increasing mortality due to HCC from 1.54 to 2.58 per 100,000 between 1980 and 1990.

Both incidence and mortality rates are higher in male subjects with a mean male to female ratio of 2–4:1. The possibility of a hormonal cause of HCC has been raised due to male predominance, but experimental data and the lack of efficacy of hormonal manipulation suggest that other factors are likely involved. Ethnicity may also be a risk factor in HCC development. In the United States, substantial variation has been noted among different ethnic groups, with the highest rates among those of Asian, Hispanic, and African Americans. In one study utilizing the SEER registry, an increased occurrence of HCC has been documented among African-Americans compared to Caucasians, with prevalence of 25% for white males (6.7% females) and 40% for black males (13% females). Similarly, the incidence rate in the United States is lowest for whites (male 2.3 and female 1.1 per 100,000).

Chapter 5 – The definition of hepatic myofibroblasts is currently attributed to a rather heterogenous population of cells that sustain liver fibrogenesis and then fibrotic progression of chronic liver diseases of different aetiology to the common advanced-stage of cirrhosis. These highly proliferative and contractile myofibroblasts actively participate to the progression of the chronic disease by means of their multiple phenotypic responses, including excess deposition of extracellular matrix components and its altered remodelling as well as the synthesis and the release in a paracrine/autocrine way of a number of critical growth factor which sustain and perpetuate fibrogenesis, chronic inflammatory response and neoangiogenesis. According to current literature hepatic myofibroblasts, which are mostly α-smooth muscle actin (α-SMA) - positive cells, mainly originate from hepatic stellate cells or from fibroblasts of portal areas through a process of activation and trans-differentiation. Hepatic myofibroblasts have been reported to originate also from bone marrow – derived stem cells, including mesenchymal stem cells or circulating fibrocytes, able to engraft chronically injured liver. It is currently debated whether myofibroblasts may also originate

from hepatocytes and cholangiocytes through a process of epithelial to mesenchymal transition. Hepatic myofibroblasts have been reported to play additional crucial roles, including modulation of immune responses in the chronically injured liver and the cross talk with hepatic progenitor (stem) cells as well as with malignant cells of either primary hepatocellular carcinomas or of metastatic cancers.

Chapter 6 – Liver cirrhosis (LC) is not a single disease. It is the outcome of different diseases that are associated with chronic loss of hepatocytes and its replacement by fibrosis and formation of regenerative nodules. The process distorts the architecture of liver parenchyma resulting in development of porto-systemic shunting of blood and impairment of hepatic function. Chronic alcohol abuse, chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infections are major causes of liver cirrhosis in the world. There are other minor causes of LC. Prevalence of the type of LC in any country or community is determined by geo-cultural factors. Whereas HBV infection is the major cause cirrhosis in Asia and Africa, HCV infection is the predominant cause in Japan. Alcohol and now emerging HCV infection are important causes of cirrhosis in the West. Among minor causes of cirrhosis hepatic vena cava disease (HVD) is reported only from developing countries. The reported incidence of LC in HVD varied from 71 to 100%.

Chapter 7 – This chapter will present the critical interplay between liver cirrhosis and surgery. First, the question of patients with cirrhosis needing to undergo a surgical procedure will be explored. Specifically, the different ways that the level and severity of cirrhosis can be estimated will be examined, and how these could determine the safety of proceeding with a surgery, as well as the prognosis of these patients, according to the different surgical procedures. The second issue to be examined is the role of surgery as therapy for portal hypertension, one of the more life-threatening complications of cirrhosis. This will be achieved by analyzing the pathophysiology of portal hypertension in cirrhosis and presenting the different surgical procedures to treat portal hypertension and its complications, with special emphasis on the indications and contraindications for each. Finally, an established danger of cirrhosis is the development of hepatocellular carcinoma. This chapter will present the pathophysiology that leads to this progression, as well as the role of surgery as treatment of hepatocellular carcinoma in a cirrhotic patient with the two main alternatives being surgical resection versus transplantation. The aim would be to identify the groups of patients that would be best served by each alternative.

Chapter 8 – Primary Biliary Cirrhosis (PBC) is a chronic cholestatic liver disease that usually progresses to liver failure and death unless liver transplantation is performed. Its pathophysiological hallmarks include the destruction of small bile ducts by T cells and the production of autoantibodies to mitochondrial antigens that bind the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). Although a combination of environmental factors and genetic predisposition likely triggers PBC in susceptible individuals as suggested similarly for the etiology of a majority of autoimmune diseases, this devastating liver disorder is considered to be rather immune - mediated than autoimmune due to its (relative) unresponsiveness to immunosuppressive agents.

One frequent cause of immune activation under pathologic conditions is represented by overt or covert bacterial or viral infections, but revealing microbial involvement and understanding the cellular and molecular mechanisms leading to immune - mediated tissue damage generally remain a challenge. Recent clinical studies strongly suggest that infection with the ubiquitous alphaproteobacterium *Novosphingobium aromaticivorans* is specifically

associated with susceptibility to PBC. Notably, there is compelling evidence that patients with Primary Biliary Cirrhosis (PBC) express antibodies against various lipoylated enzymes of *Novosphingobium* including the conserved bacterial homolog of the mitochondrial PDC-E2 enzyme, the major PBC antigen. This seropositivity for *Novosphingobium* is highly specific of PBC and not found in healthy subjects and other diseases.

Infection alone is likely not sufficient to confer disease, genetic susceptibility plays an important role. Naturally occurring allelic polymorphisms determine the susceptibility to autoimmunity. Underlying single nucleotide polymorphisms (SNPs) likely evolved due to microbial pressure and reveal a consequence of natural selection for altered susceptibility to certain pathogens. Notably, the authors have not only developed a mouse model that recapitulates key features of the human disease upon infection with *Novosphingobium*, but also identified genetic susceptibility loci that drive susceptibility to PBC. Given the emerging role of genetic factors, defining their control and regulation will improve the understanding of pathogenic mechanisms in PBC. The translation of findings from the mouse model into human disease will help to define these genes as novel disease risk factors and should subsequently help guide the development of diagnostic tests and/or effective therapies next to ursodeoxychloic acid. These are needed as current immunosuppressive drugs are often not target specific, ineffective and elicit severe side effects.

Chapter 9 – Portal hypertension is a major complication of liver cirrhosis and may lead to life-threatening hemorrhage of esophageal varices, ascites, hepatic encephalopathy and/or renal failure.

From the pathophysiological point of view, this hypertension is caused by an increased intrahepatic resistance due to the liver cirrhosis and is aggravated by a decreased sensitivity to endogenous vasopressors and an increased local concentration of vasodilators.

The therapeutic options range from symptomatic medical treatment to endoscopic treatment (band ligation) to radiological interventions (placement of a transjugular intrahepatic portosystemic shunt) to surgical procedures.

In this review, the authors present different treatment regimes for the treatment of portal hypertension focusing on the different shunt procedures for surgical treatment of portal hypertension. The operative options include side-to-side shunts as well end-to-side anastomoses between the portal vein and the inferior vena cava, distal spleno-renal shunts (Warren-shunts) and mesocaval shunt procedures (Drapanas-shunts). Furthermore, there are several rarely performed shunts (e.g. Linton-shunts) and devascularization procedures.

While liver transplantation is an option for curative treatment of liver cirrhosis, the different shunt procedures are important for the symptomatic treatment of portal hypertension, especially in CHILD-PUGH A patients. Furthermore, due to the persistent shortage of donor organs the shunt procedures are important interventions prior to transplantation to prevent potential life-threatening complications of portal hypertension, such as massive hemorrhage of esophageal varices, as a bridging therapy to liver transplantation.

Chapter 10 – Hepatic cirrhosis is the end-stage of chronic liver diseases. The majority of patients with hepatic cirrhosis die from life-threatening complications at early age. Liver transplantation has been the most effective treatment for patients with hepatic cirrhosis. Since liver transplantation is critically limited by the shortage of available donor livers, searching for an effective alternative therapy has attracted great interest in preclinical studies. The encouraging advances in stem cell research have paved the way towards the treatment of the end-stage of chronic liver diseases. In view of the pathogenic fundamentals of hepatic

cirrhosis, cell-based treatment should be aimed to complement or replace damaged liver cells and to correct the imbalanced extracellular matrix regeneration/degradation. Understanding the transition of hepatocyte regeneration to hepatic fibrogenesis during chronic liver injury could guide the appropriate utilization of cell-based therapy. This chapter is intended to describe the characteristics and therapeutic potential of various stem cells, including hepatocytes, liver progenitor cells, hematopoietic stem cells, mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells. Since autologous adult stem cells have the least obstacles for clinical application, their potential interventions on cirrhosis are especially illustrated in terms of the cellular and molecular mechanisms of hepatic fibrogenesis.

Chapter 11 – The role of trace elements in the pathogenesis of liver cirrhosis and its complications is still not clearly understood.

Zinc, copper, manganese and magnesium are essential trace elements whose role in liver cirrhosis and its complications is still a matter of research.

Zinc is associated with more than 300 enzymatic systems. Zinc is structured part of Cu-Zn superoxide dismutase, important antioxidative enzyme. Zinc acts as an antioxidant, a membrane and cytosceletal stabilizator, an anti-apoptotic agent, an important co-factor in DNA synthesis, an anti-inflammatory agent, etc. Copper is an essential trace element which participates in many enzymatic reactions. Its most important role is in redox processes. Reactive copper can participate in liver damage directly or indirectly, through Kupffer cell's stimulation. Scientists agree that copper's toxic effects are related to oxidative stress. Manganese is a structural part of arginase, which is an important enzyme in the urea metabolism. Manganese acts as an activator of numerous enzymes in Krebs cycle, particularly in the decarboxilation process.

Magnesium is important for the protein synthesis, enzyme activation, oxidative phosphorilation, renal potassium and hydrogen exchange etc.

Since zinc, copper, manganese and magnesium have a possible role in the pathogenesis of liver cirrhosis and cirrhotic complications, the aim of their study was to investigate the serum concentrations of mentioned trace elements in patients with liver cirrhosis and compare them with concentrations in controls.

Serum concentrations of zinc, copper, manganese and magnesium were determined in 105 patients with alcoholic liver cirrhosis and 50 healthy subjects by means of plasma sequential spectrophotometer. Serum concentrations of zinc were significantly lower (median 0.82 vs. 11.22 μmol/L, $p<0.001$) in patients with liver cirrhosis in comparison to controls. Serum concentrations of copper were significantly higher in patients with liver cirrhosis (median 21.56 vs. 13.09 μmol/L, $p<0.001$) as well as manganese (2.50 vs. 0.02 μmol/L, $p<0.001$). The concentration of magnesium was not significantly different between patients with liver cirrhosis and controls (0.94 vs. 0.88 mmol/L, $p=0.132$). There were no differences in the concentrations of zinc, copper, manganese and magnesium between male and female patients with liver cirrhosis. Only manganese concentration was significantly different between Child-Pugh groups ($p=0.036$). Zinc concentration was significantly lower in patients with hepatic encephalopathy in comparison to cirrhotic patients without encephalopathy (0.54 vs. 0.96 μmol/L, $p=0.002$). The correction of trace elements concentrations might have a beneficial effect on complications and maybe progression of liver cirrhosis. It would be recommendable to provide analysis of trace elements in patients with liver cirrhosis as a routine.

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Chapter 1

Thalidomide and its Analogs: A Potential Immunomodulatory Alternative for Treating Liver Diseases and Cirrhosis

Eduardo Fernández-Martínez*
Laboratory of Medicinal Chemistry and Pharmacology
Cuerpo Académico de Biología de la Reproducción,
Área Académica de Medicina del Instituto de Ciencias de la Salud (I.C.Sa.)
Universidad Autónoma del Estado de Hidalgo (UAEH)

Abstract

Thalidomide is currently used for treating erythema nodosum leprosum, multiple myeloma, angiogenesis, rheumatoid arthritis, graft-versus-host disease, among others. Thalidomide effects are related to its capacity to inhibit the proinflammatory cytokine tumor necrosis factor-α (TNF-α) and, in consequence, causes immunomodulation on other cytokines. During the establishment of some diseases the balance between proinflammatory and antiinflammatory cytokines is disrupted, promoting a pathological state; thus, elevated levels of proinflammatory cytokines mediate several deleterious processes such as inflammation, necrosis, apoptosis and fibrosis. These events are present in acute and chronic degenerative liver diseases such as hepatitis, cholangitis, cirrhosis and hepatocellular carcinoma (HCC). Then, the immunomodulation on cytokines by drugs seems to be a pharmacological target to ameliorate liver damage and cirrhosis. In fact, there are not sufficient drugs for relief or cure of cirrhosis currently; some of these

* Address: Calle Dr. Eliseo Ramírez Ulloa no. 400,
Colonia Doctores, Pachuca, Hidalgo, México
C.P. 42090
Telephone: (52) +7717172000 ext. 4510 and 4512
E-mail: efernan@uaeh.edu.mx and tomedyfm@hotmail.com

few are expensive, unstable and palliative or may cause side effects. Novel thalidomide analogs have been synthesized with improved stability and potency as TNF-α inhibitory and immunomodulatory agents, besides low or none teratogenicity. Experimental assessment of thalidomide and its analogs in animal models of liver injury have afforded very hopeful outcomes. Thalidomide and two analogs have evidenced anticholestatic, antinecrotic and antifibrotic activities in bile duct ligation-induced biliary cirrhosis. Another analog protected D-galactosamine/endotoxin-treated mice from liver damage. Thalidomide ameliorated the alcoholic hepatic injury and prevented necrosis, cholestasis and fibrosis induced by CCl_4 in rats. Moreover, this drug salvaged from lethal hepatic necroinflammation and accelerated the recovery from established thioacetamide-provoked cirrhosis in rats. The antiinflammatory, antinecrotic and antifibrotic effects elicited by thalidomide and its analogs are mainly mediated by the inhibition on TNF-α through two different routes, as well as the down-regulation of nuclear factor-κB (NF-κB) signaling pathway and by diminishing adhesion molecules to prevent the progression of liver fibrosis and cirrhosis. Furthermore, thalidomide showed beneficial effects on HCC by decreasing angiogenesis and metastasis in murine models; therefore, diverse clinical phase I/II studies were carried out to evaluate its antitumoral or disease control outcomes. However, thalidomide as a single drug therapy yields very modest benefits, although in most cases this is well tolerated and offers disease stabilization. Different doses and the combination with other chemotherapeutic agents appear to enhance therapeutic effects; the assessment of the new thalidomide analogs in next clinical trials of HCC healing is strongly suggested. Thalidomide and its analogs may be a feasible option for the treatment of liver diseases and cirrhosis.

Introduction

History of Thalidomide

Thalidomide (Tha, α-N-phthalimidoglutarimide, Figure 1) was first synthesized in Germany in 1954 by the pharmaceutical company Chimie-Grünenthal GmbH. The former intended use for Tha was as a mild hypnotic-sedative agent similar to barbiturates but without their addictive or toxic effects (Keller et al., 1956; Somers, 1960). Grünenthal introduced Tha since 1956 in Germany wherein that was approved in 1957 as a safe sedative drug for sales over the counter. Soon thereafter, this drug was marketed in other countries including United Kingdom, the rest of Europe, New Zealand, Australia, Japan and Canada under the brand names such as "Contergan, Distaval, Talimol, Kevadon and Softenon"; however, Tha was never approved in United States because the Food and Drug Administration requested more information from Grünenthal concerning peripheral neuritis reports (Fullerton et al., 1961; Marriott et al., 1999; Teo, 2005). Meanwhile, Tha had been considered as a virtually non-toxic drug, due to its very low acute toxicity in rodent models (Somers, 1960; Williams, 1968); in addition, Tha became a very common sleep-inducing agent with very good antiemetic properties and, consequently, it began to be used by pregnant women for treating the nausea due to morning-sickness in the first trimester of gestation (Marriott et al., 1999; Teo, 2005). The reports of birth defects and deformed babies emerged at the end of 1956 to 1961, thus the strong suspicion regarding teratogenicity by Tha in man grew up; finally, in 1961 Lenz (1961; Lenz, 1992) published the first paper suggesting that Tha was responsible for limb deformities in newborn infants, this suggestion was rapidly confirmed by others as

well as experimentally in rabbits (McBride, 1961; Somers, 1962; Williams, 1968). Due to those terrible cases of amelia and phocomelia, ranging from 10000-12000 children around the world, Tha was withdrawn from the market in November 26, 1961 and during 1962. Nevertheless, by 1965 this agent had evidenced antiinflammatory properties, because Sheskin (1965) administered Tha as a sedative to leprosy patients suffering from erythema nodosum leprosum (ENL, a severe dermatological complication of Hansen´s disease formerly known as leprosy) and found amazing effects, given that clinical signs and symptoms of ENL were attenuated within 48 h. Such discovery established the basis to the future increased interest on Tha mechanism of action and the synthesis of novel safer analogs with non-teratogenic effects, as well as the further applications in the treatment of several inflammatory, degenerative and chronic diseases.

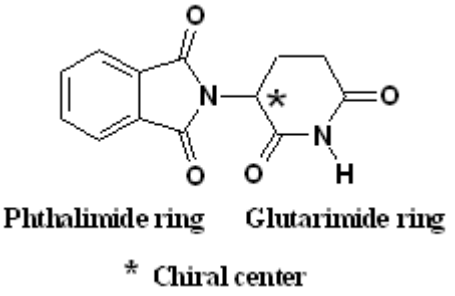

Figure 1 Thalidomide chemical structure.

Thalidomide Chemical Properties

The chemical name of Tha is 2-(2,6-dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione and it is composed by two imide moieties, the phthalimide ring and the glutarimide ring; its empiric formula is $C_{13}H_{10}N_2O_4$, with a molecular weight of 258.2 g/mol. Tha is a white, tasteless crystalline powder with a melting point of 269-271°C. This drug is sparingly soluble in water (6 mg/100mL), methanol, ethanol, acetone, and glacial acetic acid but readily soluble in acetone, dioxane, dimethyl formamide, pyridine, chloroform, and dimethyl sulfoxide. It is insoluble in ether and benzene (Somers, 1960). Its low solubility in water would suggest that its concentration in body fluids at any time would be small (Williams, 1968), so the efforts to get Tha dissolved have led to prepare solutions in alkaline pH that promotes the spontaneous hydrolysis of Tha; in consequence, such solutions contain Tha in addition to its hydrolysis products. The main route of hydrolysis of Tha at pH 6, 7.4 and 8 is cleavage of the phthalimido ring to α-(o-carboxybenzamido)glutarimide; this compound is reasonably stable at these pH values, however, as the pH is increased the bonds of glutarimide ring become susceptible to hydrolysis and, at pH 7.4 and 8 especially, considerable amounts of 2- and 4-phthalimidoglutaramic acids are formed (Schumacher et al., 1965a). It suggests that Tha biological effects may also be shared by its hydrolysis products or its metabolites, thus, this compound may act as a prodrug for one or more of those Tha derivatives (Fabro et al., 1965; Muller et al., 1996). In addition, Tha possesses a single chiral center in the glutarimide ring, an asymmetric carbon that originates the R and S enantiomers; thus, Tha is administered as a racemic mixture of (-)-(S)- and (+)-(R)-enantiomeric forms. It has been demonstrated for Tha

that there is a relationship between quirality and its biological effects (Blaschke et al., 1979); this point will be commented farther on.

Thalidomide and its Analogs: Characteristics and Mechanisms of Action

Nowadays, Tha has showed several mechanisms of action as an antiinflammatory and immunomodulatory drug because of its broad range of inhibitory and stimulatory effects on the immune system (Zwingenberger and Wnendt, 1995; Mujagić et al., 2002). However, so far the most plausible mechanism is the inhibition on the production of the important, pleiotropic, pronecrotic and proinflammatory cytokine tumor necrosis factor-α (TNF-α) (Sampaio et al., 1991), through enhancing the TNF-α mRNA degradation (Moreira et al., 1993). On this way, other authors have proposed that Tha has also immunomodulatory activity by two more routes: a) reducing the number of IgM plaque-forming cells and b) enhancing the secretion of the cytokine interleukin-2 (IL-2) in peripheral blood mononuclear cells (PBMC) (Shannon et al., 1997). Regarding other immunomodulatory effects, it has been observed that Tha has a costimulatory role in the upregulation of T helper 2 (Th2)-type immunity, because Tha increases the production of Th2-type (humoral) cytokines, for example interleukin-4 (IL-4) and interleukin-5 (IL-5); as well as inhibits the production of the Th1-type (cellular) cytokine interferon-γ (IFN-γ) in PBMC (McHugh et al., 1995; Marriott et al., 1999), while in T cells Tha induces a high production of IFN-γ and IL-2 (Corral et al., 1999).

There are other possible immunomodulatory and/or regulatory mechanisms of Tha on diverse endogen mediators of the immune and inflammatory response. It deserves special consideration the first report concerning the inhibition on the activation of the nuclear factor-κB (NF-κB) in HIV-infected primary macrophages (Moreira et al., 1997). NF-κB is a transcription nuclear factor that has received much attention since its discovery in 1986, because of its activation by many different stimuli and its diverse and prominent roles in maintaining the homeostasis, control of disease development, regulation of cell survival and activation of innate and adaptive immune responses, for instance, its activation provokes the production of proinflammatory cytokines including the most potent TNF-α (Sun and Karin, 2008). On this way, there are reports that support that Tha strongly suppresses at different levels the NF-κB activation induced by TNF-α and reactive oxygen species (ROS) such as H_2O_2, besides this inhibition is apparently not cell type specific, although these effects are not seen during the NF-κB activation by other inducers (Majumdar et al., 2002; Kim et al., 2004). This likely mechanism of action has prompted the design and synthesis of NF-κB inhibitors derived from Tha (Carcache de-Blanco et al., 2007).

Cyclooxigenase-2 (COX-2) is the inducible enzyme that catalyzes the synthesis of prostaglandins (PG) which are very well known potent endogen proinflammatory agents and that regulate the cytokines expression. COX-2 is induced by bacterial lipopolysaccharides (LPS) and it has been considered as a pharmacological target for the prevention and treatment of angiogenesis and cancer; indeed, Tha has evidenced promising effects as inhibitor of LPS-induced COX-2 (Fujita et al., 2001). Furthermore, novel Tha analogs have been recently synthesized and/or evaluated as inhibitors of COX-2 (Suizu et al., 2003; Fujimoto et al., 2006).

Other via of regulation by Tha is achieved through diminishing the nitric oxide (NO) production and its multiple biological actions by two possible ways, a) decreasing the TNF-α synthesis, since this cytokine as well as interleukin-1β (IL-1β) and IFN-γ are important mediators of NO production, because they regulate the expression of the inducible nitric oxide synthase (iNOS) (López-Talavera et al., 1996) and b) it has been also demonstrated that Tha possesses weak but significant inhibitory activity on iNOS, what encouraged the synthesis of Tha-related inhibitors of NOS (Shimazawa et al., 2004); moreover, some authors have designed their counterpart, some NO-donating Tha analogs as anticancer agents (Wang et al., 2009).

There is another feasible immunomodulatory mechanism of action of Tha, it has been showed that this drug binds to a pair of proteins identified as isoforms of the α_1-acid glycoprotein (α_1-AGP), suggesting a potential role for α_1-AGP as a mediator of the pharmacological effects of Tha; additionally, Tha analogs do not compete for that binding site (Turk et al., 1996; Niwayama et al., 1998). α_1-AGP is one of the major acute phase proteins in humans, rats, mice and other species; its concentration is elevated in response to systemic tissue injury, inflammation or infection, and these changes in serum protein concentrations have been correlated with increases in hepatic synthesis. The α_1-AGP expression is regulated by cytokines such as TNF-α, IL-6 and IL-1β; although the exact physiological role of α_1-AGP remains still to be completed, this protein is considered as a natural antiinflammatory and immunomodulatory endogen agent (Fournier et al., 2000; Hochepied et al., 2003).

Since Tha possesses valuable therapeutic properties but also teratogenic activity and other side effects, various groups of research in medicinal chemistry and pharmacology around world have synthesized and assessed diverse families of Tha analogs in order to augment the chemical stability, the potency as TNF-α inhibitors and their immunomodulatory efficacy, besides lowering the adverse effects (Corral et al., 1996; Marriott et al., 1998; Muller et al., 1998; Muller et al., 1999; Hashimoto, 2008; Zahran et al., 2008; Man et al., 2009). Some Tha analogs have been synthesized resembling its hydrolysis products (Muller et al., 1996) and others are similar to its hydroxylated metabolites that have been demonstrated to be potent immunomodulators (Yamamoto et al., 2009). Among the many families of novel and promising Tha analogs, there are two of them that are outstanding as potent TNF-α inhibitors; the first group is composed by molecules structurally and functionally very similar to Tha, known as immunomodulatory drugs because of their marked costimulatory properties on T cells promoting the secretion of IL-2 and IFN-γ, but these compounds also inhibit the production of IL-1β, IL-6 and IL-12 as well as greatly increase the synthesis of IL-10, the main antiinflammatory Th2-type cytokine in stimulated PBMC (Corral et al., 1999; Schafer et al., 2003). The most important members of this immunomodulatory group are pomalidomide (CC-4047, Actimid™) and lenalidomide (CC-5013, Revlimid™) (Teo, 2005); both drugs belong to a novel generation of amino-substituted Tha analogs in the phthalimide ring (Muller et al., 1999) (Figure 2).

On the other hand, the second group of effective TNF-α inhibitors was synthesized resembling the structure of Tha hydrolysis products (Corral et al., 1996; Muller et al., 1996), they also increase IL-10 (Marriott et al., 1998; Corral et al., 1999) and possess biological activity as potent phosphodiesterase-4 (PDE-4) inhibitors while Tha is not, therefore, it cannot be excluded that one or more of the Tha metabolites or degradation products may inhibit that enzyme (Muller et al., 1998). PDE-4 is the primary enzyme that catalyzes the

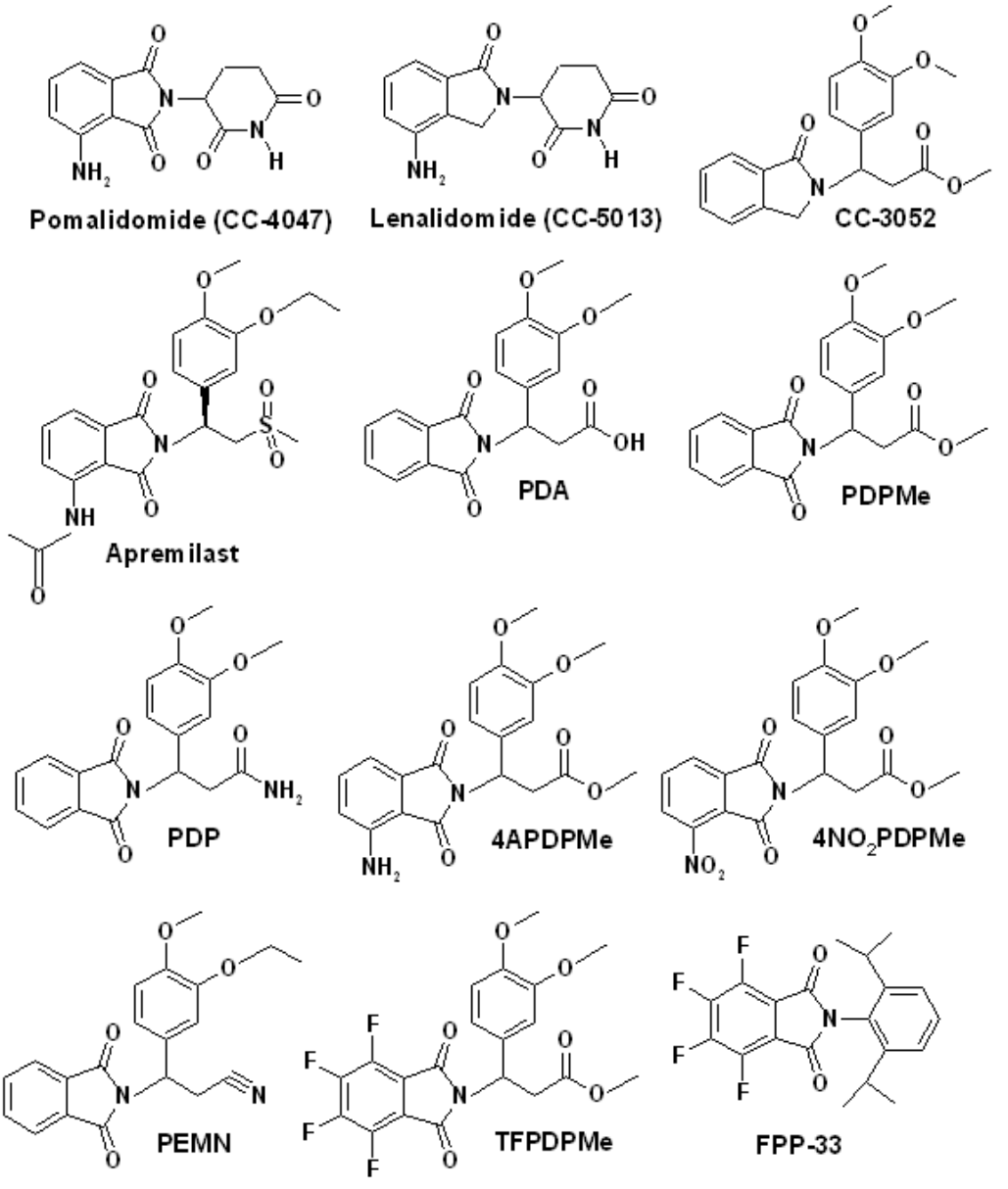

Figure 2 Some structures of thalidomide analogs.

hydrolysis of adenosine 3´,5´-cyclic monophosphate (cAMP), as a result, the inhibitors of PDE-4 are cAMP-elevating agents (Bender and Beavo, 2006). It is well documented that augmented levels of cAMP inhibit the TNF-α production (Cheng et al., 1997; Kast, 2000). Thus, inhibition on PDE-4 has been shown to be an effective via for inhibition of TNF-α production in activated monocytes and PBMC by this group of compounds, especially for perfluorinated Tha analogs (Muller et al., 1998; Niwayama et al., 1998). In addition, stable analogs of cAMP, adenylyl cyclase activators or PDE inhibitors are capable of reducing the activation of NF-κB and in consequence lowering TNF-α as well as other proinflammatory cytokines (Gantner et al., 1997). Some compounds that belong to this group are: PDA, 3-

phthalimido-3-(3,4-dimethoxyphenyl)-propanoic acid; PDP, 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanamide; PDPMe, methyl 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanoate; $4NO_2$PDPMe, methyl 3-(4-nitrophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate; 4APDPMe, methyl 3-(4-aminophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate; TFPDPMe, methyl 3-tetrafluorophthalimido-3-(3,4-dimethoxyphenyl)-propanoate; PEMN, 3-phthalimido-3-(3-ethoxy, 4-methoxyphenyl)-propanitrile (Muller et al., 1996; Muller et al., 1998). Currently, apremilast (CC-10004) is perhaps the most promising drug of this family (Man et al., 2009) (Figure 2).

Concerning the mechanisms of action of Tha and its biological effects, there are reports about the pharmacological differences between the two Tha enantiomers, while (+)-(R)-Tha exhibited significant positive influences on all sedative effects, the (-)-(S)-Tha had a significant effect in the opposite direction, because it has shown the dose-dependent teratogenic activity (Blaschke et al., 1979), besides a superior effect in preventing splenomegaly in chicken embryos (Field et al., 1966) and in immunomodulation (Muller et al., 1999). Other studies have reported that this drug possesses bidirectional immunomodulatory properties, inhibiting or enhancing the TNF-α production depending on the enantiomer administered, R or S (Miyachi et al., 1996), as well as these differential effects have been observed in diverse cellular cultures or by using diverse cytokine inducers or immune stimulatory agents; thus, Tha enhances 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced TNF-α production by human leukemia HL-60 cells, while it inhibits TPA-induced TNF-α production by another leukemia cell line called THP-1, although Tha inhibits TNF-α production by both cell types when they are stimulated with okadaic acid (OA) (Miyachi et al., 1997a; Miyachi et al., 1997b). Furthermore, some research groups are looking for TNF-α bidirectional modulators based on Tha derivatives, for example the compound FPP-33 (Figure 2) (Hashimoto, 2008; Fernández-Braña et al., 2009). Therefore, experimental differences in effectiveness between thalidomide and its analogs might be observed due to variations in potency, stability, metabolism, pharmacokinetics, bioavailability, enantiodependence, cellular type or to a bidirectional cytokine regulation; besides the dose, via of administration, time courses, animal species and schedule of treatment could be other striking factors capable of leading to a variation in the desired pharmacological immunomodulation. Although further studies are required to clarify and optimize immunomodulatory effects, the hopeful outcomes of these compounds on cytokines, at systemic and hepatic level, suggest a wide perspective to use them as immunomodulatory agents in liver diseases; this will be analyzed later on. Tables 1 and 2 summarize some immunomodulatory effects on very important cytokines as well as some possible mechanisms of action of Tha and its most remarkable analogs.

Thalidomide and its Analogs: Pharmacokinetics and Metabolism

The therapeutic dose of Tha as a racemic mixture is normally 100-400 mg/day for multiple myeloma patients and higher doses of 600-1600 mg/day in graft-versus-host disease treatments (Eriksson et al., 1995; Mujagić et al., 2002; Kamikawa et al., 2006). The absolute bioavalability of Tha after oral intake is limited as the drug is slowly absorbed from the gastrointestinal tract, the total absortion of Tha increases proportionally with the increase in

Table 1. Immunomodulatory properties on cytokines of some thalidomide analogs

Analogs	TNF-α IC_{50} (μM)	IL-1β IC_{50} (μM)	IL-6 IC_{50} (μM)	IFN-γ in T cells	IL-10	Times more potent than Tha inhibiting TNF-α
Thalidomide (Tha)	194	↓ Yes	↓ Yes	↑ Yes	↑ Yes	1.00
$4NO_2$PDPMe	64	NA	NA	NA	NA	3.03
PDA	60	↓ Yes	NA	NA	↑ Yes	3.23
PDP	12-13	↓ Yes	NA	↓ Yes	↑ Yes	14.92
PDPMe	2.90	↓ Yes	↓ Yes	NA	Yes	66.89
CC-3052	1.22	↓ ---	↓ ---	NA	↑ Yes	159.01
4APDPMe	0.45	↓ Yes	↓ Yes	NA	↑ Yes	431.11
TFPDPMe	0.26	0.40	0.30	NA	NA	746.15
PEMN	0.12	↓ Yes	↓ Yes	NA	↑ Yes	1616.66
Lenalidomide	0.10	↓ Yes	↓ Yes	↑ Yes	↑ Yes	1940.00
Apremilast	0.077	NA	NA	NA	NA	2519.48
Pomalidomide	0.013	↓ Yes	↓ Yes	↑ Yes	↑ Yes	14923.07

--- No effect or slightly; ↑, Increases its production; ↓, Inhibits its production; NA, not available; IC_{50}, inhibitory concentration-50 (micromolar, μM) on TNF-α production as well as their effects on other cytokines produced by LPS-stimulated peripheral blood mononuclear cells (PBMC), except for IFN-γ in T cells. Tha ↓ IFN-γ in PBMC.

Table 2. Possible molecular mechanisms of action of thalidomide and some analogs

Analogs	PDE-4 IC_{50} (μM)	Binding to α1-AGP	TNF-α mRNA Degradation
Thalidomide	>500	Yes	↑ Yes
$4NO_2$PDPMe	NA	NA	NA
PDA	NA	NA	No
PDP	9.40	NA	No
PDPMe	2.50	NA	No
CC-3052	3.00	NA	No
4APDPMe	NA	NA	NA
TFPDPMe	4.70	No	No
PEMN	0.13	NA	NA
Apremilast	0.074	NA	NA
Lenalidomide	>100	NA	NA
Pomalidomide	>100	NA	NA

IC_{50}, inhibitory concentration-50 (micromolar, μM) on phosphodiesterase-4 (PDE-4) enzyme activity; ↑, increases its degradation; NA, not available; α1-acid glycoprotein (α1-AGP).

dosage from 200 to 1200 mg given once or twice daily. However, peak plasma concentrations increase in a less than proportional manner and the time to peak plasma concentration is delayed, indicating that Tha poor aqueous solubility affects the rate of dissolution and absorption after oral intake. In healthy men, peak plasma levels (maximal concentration, C_{max}) of 0.8-1.4 µg/mL were obtained in a mean of 4.4 h following a single 200 mg oral dose (Mujagić et al., 2002; Richardson et al., 2002a). In mice, fed with a diet containing 0.03 % w/w of Tha, this drug reached a mean plasma concentration of 0.8 µg/mL (Shannon et al., 1981) which is an equivalent concentration of 0.9 µg/mL quantified in the blood of a man following a single oral dose of 100 mg of Tha (Faigle et al., 1962; Shannon et al., 1997); furthermore, concentrations as high as 5.0 µg/mL of Tha in the blood have been reported when patients were given 200 mg four times a day (Vogelsang, et al., 1992). A study in eight healthy male volunteers, whom received once an oral dose of 200 mg of Tha, reported a volume of distribution of 120.7 L, plasma C_{max} of 1.15 µg/mL, time to maximal plasma concentration (t_{max}) at 4.39 h, a mean absortion half-life of 1.7 h and a mean elimination half-life at 8.7 h in a monocompartament model with renal excretion of 0.6 % of initial dose; that elimination half-life is about three times longer than that observed in animals, besides that the total body clearance rate is relatively slow 10.41 L/h (Chen et al., 1989). Other authors have reported pharmacokinetic parameters for Tha in multiple myeloma (MM) patients in order to offer more accuracy in data in that disease, for example, after a single dose of 200 mg of Tha the C_{max} was 1.39 µg/mL, t_{max} at 4.8 h, area under curve (AUC) 81.0 µmol·h/L and the elimination half-life at 7.6 h; all those parameters were also compared with data from mice and rabbits administered with Tha (Chung et al., 2004). Differences among races should be taken into account when Tha is administered for the treatment of any illnesses because the pharmacokinetic parameters may be affected as well as lower or higher effective doses would be required (Kamikawa et al., 2006).

In spite of the efforts to separate the R and S Tha enantiomers in order to get also both pharmacological activities apart without interference on each other, it has been observed that Tha enantiomers racemize in vitro (Knoche and Blaschke, 1994) as well as after the administration of one pure enantiomer it is racemized in vivo; the pharmacokinetic parameters have been reported, the mean rate of chiral inversion of (+)-(R)-Tha and (-)-(S)-Tha in blood at 37 °C were 0.30 and 0.31/h, respectively. In addition, rate constants of degradation were 0.17 and 0.18/h as well as mean rates constants for in vivo inversion were 0.17/h (R to S) and 0.12/h (S to R), predominating (+)-(R)-Tha at equilibrium; also, mean elimination rate constants were 0.079/h (R) and 0.24/h (S). Furthermore, a considerable faster rate of elimination of the (-)-(S)-Tha was seen and the mean apparent terminal half-life of the Tha enantiomers was 4.7 h (Eriksson et al., 1995). The interconversion of enantiomers under quasi-physiological conditions has been observed inclusive with Tha analogs, as the case of pomalidomide. The S-isomer of pomalidomide, which is more effective as immunomodulatory agent than the R-isomer, undergoes a rapid racemization in 2 h, suggesting that there is no advantage in developing the single isomer (Teo et al., 2003). Pharmacokinetic data for lenalidomide is still in development although in healthy volunteers the oral absorption is rapid, reaching the C_{max} between 0.625 and 1.5 h post-dose. Food reduces the C_{max} by 36 %, its pharmacokinetics is linear and the AUC of 0.8 to 1.2 µmol·h/L increases proportionately with dose. In patients suffering from MM the C_{max} occurs between 0.5 and 4 h and renal half-life of elimination is around 3.1 to 4.2 h (Richardson et al., 2002b).

Regarding the family of Tha analogs inhibitors of PDE-4, the only related data to pharmacokinetics are the half-lives reported in human plasma at 37 °C that reflects the drug stability (Corral et al., 1996), for example, as the case of PDP its half-life is around 8 h, for PDPMe approximately 3 h and an analog which resembles to the 4APDPMe that has a half-life of about 4 h. The most stable Tha analog is CC-3052, which exhibits increased stability in human plasma with a half-life of around 17.5 h versus 1.5 h for Tha (Marriott et al., 1998). Apremilast pharmacokinetic parameters were reported in female rats orally administered with 10 mg/kg, such as C_{max} of 1100 ng/mL, AUC of 1400 ng·h/mL, half-life of 5 h with 64 % absorbed (Man et al., 2009). Differences in half-lives may reflect the solubility and stability of each compound in organism and therefore their bioavailability; then, this is a very important factor because some analogs could be metabolized or eliminated from organism at different rates without reaching a significant immunomodulatory effect, a dose adjustment may be required accordingly.

When Tha is administered orally to animals, only a small amount of the unchanged drug is excreted in the urine and the major portion of the compound is broken down and excreted as at least 20 transformation products (Schumacher et al., 1965b); in dogs, the little unchanged Tha is excreted in feces preferentially. The biotransformation of Tha can occur by non-enzymatic hydrolysis, just by spontaneous rupture catalyzed by alkaline pH, as it has been mentioned above. Also, Tha is metabolically labile because of its biotransformation by hepatic P450 enzyme-mediated hydroxylation, specifically CYP2C (Ando et al., 2002); two major metabolites of Tha are 5-hydroxyTha and 5´-hydroxyTha to form then a multitude of metabolites that have been isolated (Lepper et al., 2006). The hydrolysis products have been detected in diverse species as well as the proportion of hydroxylated metabolites is higher in mice than in rats and rabbits while these are almost undetectable in healthy volunteers and MM patients (Chung et al., 2004). Hence, the possibility should be considered that the teratogenic side effect or/and other multiple pharmacological activities might be due to the interspecies differences in its pharmacokinetics and metabolites, including their enantiomers, rather than Tha itself (Chung et al., 2004; Yamamoto et al., 2009). Lenalidomide is eliminated unchanged through kidneys (two thirds of dose) in healthy volunteers and it has been thought it that undergoes a higher hepatic metabolism than Tha.

Current Uses for Thalidomide and Some Analogs

Thanks to the discovery by Sheskin (1965) that Tha possessed striking effects on the patients suffering from the cutaneous manifestations of ENL, in July 16, 1998, thalidomide reemerged, since the Food and Drug Administration of United States approved its use for the treatment of such disease (Annas and Elias, 1999). A short time later after 1965, Tha was successfully tried in the graft-versus-host reaction in rats and man (Field et al., 1966; Vogelsang et al., 1992); inclusive, there are current reports on this Tha application (Ratanatharathorn et al., 2001). Based on its immunomodulatory properties that evoke an antiinflammatory effect, Tha has been tested in clinical studies for treating MM wherein it has shown high efficacy (Venon et al., 2009); in addition, its analog lenalidomide gained the FDA approval in June 2006 for treating relapsed and refractory MM (Chen et al., 2009; Magarotto and Palumbo, 2009). Tha inhibitory effects on angiogenesis (D'Amato, et al., 1994) opened its potential use for the treatment of many diverse diseases depending on that process, such as

cancer solid tumors (Richardson et al., 2002a) as well as for the use of Tha analogs in the management of those ailments (Marriott et al., 1999; Teo, 2005). Rheumatoid arthritis is a chronic and inflammatory autoimmune-related disease; Tha and its analogs have also been tried in this illness with hopeful results (Gutierrez-Rodriguez et al., 1989; Oliver et al., 1999). Tha and lenalidomide showed to be a successful tool in the treatment of Behçet's disease (Direskeneli et al., 2008; Green et al., 2008). Furthermore, thalidomide has beneficial properties to controlling the aphthous ulcers and cachexia associated with HIV/AIDS (Jacobson et al., 1997; Marriott et al., 1999). Crohn's disease is an autoimmune inflammatory bowel disease in which Tha has been reported to be an effective treatment for patients with refractory episodes; additionally, there are current trials to evaluate Tha analogs in such illness (Mansfield et al., 2007; Gordon et al., 2009).

As Tha and its analogs are clinically or experimentally being tried in many chronic, degenerative and inflammatory diseases, there is a potential risk of producing teratogenic outcomes in possible pregnant patients being administered with these drugs, hence, it has been created the system for thalidomide education and prescribing safety (STEPS) that is a strict as well as comprehensive program to control and monitor access to Tha or its analogs (Zeldis et al., 1999). Although Tha and some analogs display teratogenic effects it is important to keep in mind that their main therapeutic applications are for the treatment of severe, chronic and degenerative diseases wherein patients must not get pregnant (Annas and Elias, 1999).

Cytokines and Liver Damage

Immunomodulation on Cytokines: Restoring the Proinflammatory and Antiinflammatory Disequilibrium

Cytokines are proteins or peptides, some of them glycosylated, produced by cells, mainly immune cells, in response to a variety of inducing stimuli, with growth differentiation and activation functions that regulate the nature of the immune responses or that influence the behavior of other different cell types. Cytokines are involved in nearly every facet of immunity and inflammation, from induction of the innate immune response to the generation of cytotoxic T cells and the development of antibodies by the humoral immune system, as well as they coordinate the functions of the immune system with the rest of body. The combination of cytokines that are produced in response to an immune insult determines which arm of the immune system will be activated. Consequently, cytokines are most distinguished for their activities associated with inflammation, immune reactivity, tissue injury or repair and organ dysfunction. At present, at least 70 candidate cytokines are known or genetically predicted, they are grouped as interferons (IFNs), interleukins (ILs), colony-stimulating factors (CSFs), transforming growth factors (TGFs) and tumor necrosis factors (TNFs) (Clemens, 1991; Simpson et al., 1997; Rose-John, 2002; Kidd, 2003; Steinke and Borish, 2006). The distinctive pattern of effect of each cytokine depends on concentration-dependent binding to specific receptors on the surface of the target cells and subsequent activation of cellular machinery at the cell membrane level, or in some cases, at the level of the nucleus

and genetic machinery (Kidd, 2003); in this regard, two excellent reviews are recommended (Grötzinger, 2002; Ishihara and Hirano, 2002).

One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) cell types activity. The Th1/Th2 hypothesis arose from 1986 research suggesting mouse T-helper cells expressed differing cytokine patterns and other functions. This hypothesis was adapted to human immunity, with Th1 and Th2 cells directing different immune response pathways. Th1 cells drive the type-1 pathway of cellular immunity to fight viruses and other intracellular pathogens or eliminate cancerous cells. Th2 cells drive the type-2 pathway of humoral immunity, allergic responses and up-regulate antibody production to fight extracellular organisms such as elimination of parasites; overactivation of either pattern can cause disease, and either pathway can down-regulate the other. However, such hypothesis cannot explain several immune actions because of human cytokine activities rarely fall into exclusive pro-Th1 or -Th2 patterns. Th1 cells produce several characteristic cytokines, most notably IFN-γ, IL-2 and IL-12 whereas Th2 cells produce a set of cytokines, most notably IL-4 and IL-5. IL-10, formerly assumed to be the major means by which Th2 cells down-regulate Th1 cells, is also produced in comparable amounts by other cell types (Farrar et al., 2002; Kidd, 2003).

As commented above, cytokines are categorized into Th1 and Th2-type cytokines, and it seems that Th1 cytokines behave as proinflammatory mediators involved in the pathogenesis of several diseases, included liver injury; for example, IL-12 and IFN-γ are known to be representative proinflammatory Th1 cytokines and play a crucial role in the host defense against bacterial infection in liver (Seki et al., 2000; Masubuchi et al., 2009), as well as the most important proinflammatory cytokines TNF-α, IL-6 which also possesses antiinflammatory activities, and IL-1β (Rizzardini et al., 1998; Cartmell et al., 2000). Additionally, numerous cytokines have predominantly antiinflammatory effects, including IL-1Ra, transforming growth factor-β (TGF-β) although this is a profibrogenic cytokine in liver as it will be discussed later, IL-22, and the most important antiinflammatory cytokine IL-10 (Louis et al., 2003; Steinke and Borish, 2006).

When an etiological agent is present in the organism this causes a disruption on the normal antiinflammatory/proinflammatory cytokines equilibrium, often up-regulating excessively the proinflammatory cytokines and as a consequence down-regulating the antiinflammatory ones; if this event is strong enough in an acute manner or chronically persistent, this may set the basis for a disease. Thus, the immunomodulation is intended to reestablish the balance of cytokines into the normal homeostatic levels, without depleting any of them or exacerbating others. Th1/Th2 regulation is exceedingly complex, but its importance is unquestionable, particularly in the study of diverse diseases and autoimmune disorders. This is an active area of research for the design of immunomodulatory therapies proposed either to dampen overreactive responses or to strengthen weak ones. Magic bullets and master switches may be rare commodities in this area; nevertheless, defining all the mechanisms controlling these processes and the use of immunomodulatory compounds such as Tha and its derivatives will help make rational therapies to manipulate Th1/Th2 balance during diseases (Farrar et al., 2002).

Cytokines Role in Deleterious Processes of Liver Damage

Cytokines have been implicated in the pathogenesis and progression of chronic liver disease (CLD). Indeed, the cellular source and biological target of cytokines are not restricted to cells of the immune system, as in the liver endothelial cells, stellate cells (Ito cells, fat-storing cells or activated myofibroblasts), hepatic resident macrophages called Kupffer cells (may be the most important source in liver) and hepatocytes are capable of producing and responding to a number of different cytokines. Additionally, the liver is an important organ in the metabolism of cytokines. All cells normally resident in the liver have the capacity to produce cytokines, which by stimulating surrounding cells (paracrine effect) or themselves (autocrine effect) leads to a further cytokine production and an amplification of an inflammatory response. While some cytokines are released by resting cells in liver, the concentrations and variety of cytokines released are considerably increased following stimulation by a variety of inducers, such as bacterial endotoxin or lipopolysaccharides (LPS), viruses, chemical agents, cancer, liver ischaemia-reperfusion and alcohol consumption (Simpson et al., 1997). Hepatic uptake of circulating cytokines is inhibited by alcohol and this may contribute to the elevated levels of TNF-α and IL-6 observed in such patients, in fact, cytotoxic cytokines likely induce liver cell death by both necrosis and apoptosis in alcoholic liver disease. Anticytokine therapy has been highly successful in attenuating cell injury/death in a variety of toxin-induced models of liver injury, including alcohol-related liver injury (McClain et al., 1999).

There are many hepatic disorders or diseases wherein the effect of cytokines has been proved as a key part of those deleterious processes, as well as during liver injury and inflammation; furthermore, cytokines also are implicated in the normal hepatic function and metabolism as well as in liver regeneration (Simpson et al., 1997; Galun and Axelrod, 2002; Gao, 2005). Particularly, the proinflammatory and pronecrotic TNF-α functions as two edged sword in the liver, this cytokine is required for normal hepatocyte proliferation during liver regeneration. It functions both as a comitogen and to induce the transcription factor NF-κB which has antiapoptotic effects. On the other hand, TNF-α is the mediator of cholestasis and hepatotoxicity in many animal models, including those involving the toxins concanavalin-A and LPS. TNF-α has also been implicated as an important pathogenic mediator in patients with alcoholic liver disease and viral hepatitis (Green et al., 1996; Bradham et al., 1998).

In experimental animal models, such as the bile duct ligation (BDL) in rats that induces a stable and frank secondary biliary cirrhosis by chronic cholestasis, the plasma and liver levels of proinflammatory/antiinflammatory cytokines and NO are modified when compared to normal rats; those changes are related to biochemical markers of cholestasis, necrosis and fibrosis (Fernández-Martínez et al., 2006). Whereas in clinical studies, the serum levels of IL-1β, IL-6, TNF-α, IFN-γ, and C-reactive protein (CRP) have been found elevated in patients with CLD. Cirrhotic patients with CLD show higher serum levels in IL-1β, IL-6, TNF-α, and CRP than noncirrhotic cases. Elevated concentrations of cytokines represent a characteristic feature of CLD regardless of underlying disease (Tilg et al., 1992). Regarding the cytokine gene expression in cirrhotic and noncirrhotic human liver, data suggest that TGF-β has a predominant role in liver fibrosis. Whereas, IL-1β, 6, 8, TNF-α, and IFN-γ, appear to participate in the pathogenesis of the mild to severe inflammatory phenomena seen in alcoholic and post-hepatitis C liver cirrhosis, respectively. Also, data indicate that neither

IFN-γ nor IL-10, at least at the levels observed in post-hepatitis C liver cirrhosis, are able to counteract the fibrotic/inflammatory processes (Llorente et al., 1996).

Summarizing there is increasing evidence that several cytokines mediate the deleterious hepatic inflammation, apoptosis, and necrosis of liver cells, as well as the characteristic chronic processes cholestasis and fibrosis, previous to the end stage cirrhosis (Tilg, 2001). Throughout CDL the relationship among chronic hepatocellular damage, liver inflammation and cirrhosis has not been clearly defined, but cytokines could be the common link between these complex pathological outcomes (Llorente et al., 1996).

Thalidomide Effects on Liver Damage and Cirrhosis

Thalidomide Effects on Liver Damage

Alcoholic liver disease is maybe one of the most important hepatopathies around the world. In this regard, the effect of Tha has been studied in an animal model of alcohol-induced liver damage; the sensitization of Kupffer cells to LPS and the overproduction of TNF-α are critical for progression of alcoholic liver injury. The treatment with ethanol for 8 weeks caused marked steatosis, necrosis, and inflammation in the liver. These pathologic parameters were diminished markedly by treatment with Tha. In a 4-week ethanol group, the LPS-induced liver damage was aggravated and Kupffer cells were sensitized to LPS. Coadministration of thalidomide with ethanol prevented the sensitization of those cells completely. Furthermore, thalidomide abolished the LPS-induced increase in CD14 (this is a functional LPS receptor on macrophages/monocytes and neutrophils) expression and intracellular calcium concentration $[Ca^{2+}]i$ elevation in the macrophages. Moreover, thalidomide reduced the LPS-induced TNF-α production by Kupffer cells by decreasing TNF-α mRNA. Thus, Tha prevented alcoholic liver injury through suppression of TNF-α production and abolishment of Kupffer cells sensitization (Enomoto et al., 2002; Enomoto et al., 2004)

Activation of Kupffer cells by LPS plays a pivotal role in the onset of pathophysiological events that occur during endotoxemia and septic shock, as well as $[Ca^{2+}]i$ is involved in LPS-stimulated cytokine production, as the case of TNF-α which is mostly produced by Kupffer cells. TNF-α plays a key role in the initiation and progression of multiple organ failure syndrome induced by septic shock as well as in the cholestasis provoked by sepsis (Van Amersfoort et al., 2003; Moseley, 2004). Enomoto and coworkers (2003) determined whether Tha could prevent LPS-induced liver injury, they found that LPS caused focal necrosis with neutrophil infiltration in the liver. Moreover, LPS dramatically increased ALT/AST. These pathologic parameters and increases of serum transaminases were diminished markedly by Tha. In isolated Kupffer cells, LPS-induced increases in [Ca2+]i and TNF-α production were suppressed by treatment with Tha. To further explore the mechanism by which Tha directly abrogated Kupffer cell sensitivity to LPS, they determined the effect of Tha in vitro on LPS-induced [Ca2+]i response and TNF-α production. With the addition of Tha to the culture media before LPS, these parameters were suppressed. They concluded that Tha prevents LPS-induced liver injury via mechanisms dependent on the suppression of TNF-α production from

Kupffer cells. The immunomodulatory effects of Tha have been evidenced in other two models of sepsis, the first of Escherichia coli sepsis in vivo in rat as well as in in vitro by using human monocytes (Giamarellos-Bourboulis et al., 2003), and the second in sepsis by multidrug-resistant Pseudomonas aeruginosa (Giamarellos-Bourboulis et al., 2005); in those studies Tha inhibited the microbial-induced NO, TNFα and IL-1β but not IL-6, as well as increased the survival rate in septic rats.

Concerning the evaluation of Tha analogs in animal models of liver injury as hepatoprotective drugs, there are three current reports. Thiele and coworkers (2002) synthesized and assessed the immunomodulatory and hepatoprotective properties of a Tha analog named TFBA, which was found to be an TNF-α, IL-6 and IL-10 inhibitor in isolated and stimulated monocytes; this drug is not either a PDE-4 inhibitor or costimulator of T cells. When TFBA was administered to mice with hepatic injury by galactosamine/LPS, it diminished the ALT activity, TNF-α production but not IL-6; however this drug increased the hepatoprotective IL-10. Furthermore, a serial of Tha analogs have been synthesized and evaluated as immunomodulatory agents in liver and plasma in an acute model of LPS-induced septic challenge in rat. Animal groups were twice administered with Tha or its analogs in an equimolar dose. Two hours after last dose, rats were injected with saline or LPS. The cytokines TNF-α, IL-6, -1β and -10 were quantified and studied in plasma and liver. After two hours of LPS-induction, different patterns of measured cytokines were observed with Tha analogs administration evidencing their immunomodulatory effects in both tissues. Interestingly, some analogs decreased significantly plasma and hepatic levels of LPS-induced proinflammatory TNF-α and others increased plasma concentration of antiinflammatory IL-10. Tha analogs also showed slight effects on the remaining proinflammatory cytokines in both tissues. Differences among immunomodulatory effects of analogs might be related to potency, mechanism of action, and half lives (Fernández-Martínez et al., 2004). Finally, hepatic glycogen metabolism is altered by NO during endotoxic shock and the previously tested serial of Tha analogs immunomodulate the endotoxin-induced cytokines which regulate the NO release. Therefore, the short-term effects of those Tha analogs were assessed on the hepatic glycogen store and on the plasma and hepatic NO in an acute model of endotoxic challenge in rat. Endotoxin caused inverse dose-dependent effects increasing plasma NO and lowering hepatic glycogen. Tha analogs showed short-term regulatory effects on glycogen, some of them increased it. Plasma NO was almost unaffected by analogs but hepatic NO was strikingly modulated. Analogs slightly up-regulated the liver IFN-γ (a known NO-coinducer) and two of them increased it significantly. Due to their interesting effects the Tha analogs may be used as a pharmacological tool due to their short-term regulatory effects on glycogen and NO during endotoxic shock, since drugs that increase glycogen may improve liver injury in early sepsis (Fernández-Martínez et al., 2008).

Thalidomide Effects on Fibrosis, Cirrhosis and its Complications

Cholestasis is defined as a disorder of cholepoiesis and bile secretion as well as mechanical or functional stoppage of the bile flow in intrahepatic or extrahepatic bile ducts, with bile components passing into the blood. Persistent cholestasis with concomitant inflammatory and connective tissue reactions as well as all forms of chronic cholangitis may lead to irreversible cholestasis and, after long-term, to biliary fibrosis (excessive

accumulation of collagen) with preserved liver structure or to the last phase known as biliary cirrhosis (Kuntz and Kuntz, 2006). Secondary biliary cirrhosis induced in rat by BDL is a widespread experimental model (Kountouras et al., 1984). Tha and two analogs, PDP and PDA, have been assessed as hepatoprotective agents during BDL (Fernández-Martínez et al., 2001; Fernández-Martínez et al., 2009). Tha showed a poor improvement on biochemical markers of liver damage when compared to those afforded by PDP. That analog protected from liver injury since it diminished alkaline phosphatase (AP, cholestasis marker) and alanine aminotransferase (ALT, indicator of necrosis) activities, as well as bilirubins (indicator of cholestasis) and prevented collagen accumulation significantly; while thalidomide showed only modest beneficial effects on bilirubins and ALT. PDP diminished the increase in plasma TNF-α levels induced by BDL, while thalidomide not only failed to inhibit this cytokine, but slightly increased it. PDA is a water soluble analog that also improved cholestasis, necrosis and fibrosis outcomes in the BDL model. Histopathology showed remarkable liver damage amelioration due to Tha and even better for both analogs. The effectiveness of these Tha analogs has been related to its potency as TNF-α and PDE-4 inhibitors, therefore, inhibiting proinflammatory cytokines results in decreased necrosis, cholestasis and fibrosis in biliary secondary cirrhosis (Tilg, 2001).

Carbon tetrachloride (CCl_4)-induced liver damage is a very used animal model, wherein cytokines are involved too (Recknagel et al., 1989; DeCicco et al., 1998); chronic administration of this hepatotoxicant produces cirrhosis similar to that induced by alcoholism. In that model Tha has showed promising effects in several papers. Muriel and coworkers (2003) reported by first time the effect of Tha during the chronic intoxication with CCl_4 that induced 33.3% mortality, while Tha cotreatment reduced it to 13.3%. The serum activities of ALT, γ-glutamyl transpeptidase (GGTP, marker of cholestasis) and AP increased 3, 2 and 4-fold by CCl_4 treatment; Tha completely prevented elevation of these enzymes. Hepatic lipid peroxidation increased about 20-fold and glycogen was abolished in CCl_4-cirrhotic rats; Tha completely diminished the former and partially the later. Histology showed that CCl_4-treated rats receiving Tha had minor histological alterations and thinner bands of collagen. The antifibrotic effect estimated by collagen was partial but significant. Thus, Tha ameliorated the cirrhosis induced by CCl_4. Similar results were obtained by other authors using this model, in addition, they have found that Tha might exert an effect on the inhibition of oxidative stress via down-regulation of NF-κB signaling pathway to prevent the progression of liver cirrhosis; it may be due to the oxidative stress parameters such as superoxide dismutase, glutathione peroxidase and malondialdehyde as well as the expression of NF-κBp65, TGF-β (the most potent profibrogenic cytokine) and the tissue inhibitor of metalloproteinase-1 (TIMP-1, this protein inhibits the collagenase activity) were significantly diminished in CCl_4-cirrhotic rats treated with Tha (Lv et al., 2006a). In addition, some authors have proposed that Tha is able to prevent or to reverse, in an established CCl_4-induced cirrhosis model, the liver damage and fibrosis by down-regulation of NF-κB activation because of the inhibition on the degradation of the inhibitor of NF-κB (IκB) as well as NF-κB-induced adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, these adhesion molecules are very important in the process of recruitment and migration of inflammatory cells provoked by endothelial activation in cirrhosis; furthermore, as a consequence, levels of hyaluronic acid, laminin, procollagen-III and collagen-IV are diminished by the antifibrotic effects of Tha (Lv et al., 2006b; Paul et al., 2006; Lv et al., 2007; Xiao et al., 2007).

Tha has shown to be a very good hepatoprotective as well as antifibrotic agent in two more models of cirrhosis. Yeh and coworkers (2004) demonstrated the hepatoprotective and antifibrotic effects of Tha in the model of thioacetamide-induced cirrhosis, given that this drug improved the survival rate, reduced the expression of TNF-α, TGF-β, TIMP-1 and TIMP-2 in rats; moreover, they verified in vitro that the Kupffer cells isolated from Tha-treated rats had suppressed the production of TNF-α and TGF-β. Furthermore, similar antifibrotic effects reported for Tha have been studied in the model of dimethylnitrosamine-induced cirrhosis. In such experiment Tha also diminished in vivo the fibrosis scores of cirrhotic livers from intoxicated rats, the content of collagen as well as the NF-κB positive cells were reduced, and that the mRNA expressions of TGF-β, collagen 1α2, TNF-α and iNOS genes were attenuated by Tha. The same authors tried Tha on rat hepatic stellate cells (HSC-T6) in vitro, they found that Tha inhibited in a concentration-dependent manner the NF-κB transcriptional activity induced by TNF-α, incluiding IκB kinase-α (IKKα) and IκB phosphorylation, as well as lowered the TGF-β-induced collagen deposition in these stellate cells (Chong et al., 2006). For further reference regarding the NF-κB activation and its regulation in liver as well as the relationship TGF-β/liver fibrosis four excellent reviews are recommended (Fallowfield et al., 2006; Gieling et al., 2008; Iimuro and Brenner, 2008; Sun and Karin, 2008).

In addition to the hopeful results obtained by Tha as discussed for experimental models of fibrosis and cirrhosis, it rises interesting to comment briefly about the experimental and clinical effects of Tha on the portal hypertension, that is a clinical syndrome very common in cirrhotic patients, whom later may develop gastroesophageal variceal bleeding (Sanyal et al., 2008). The first report concerning this issue was in an animal model of portal hypertension, wherein rats were administered with Tha; this drug inhibited the TNF-α synthesis, reduced the NO production, blunted the development of hyperdynamic circulation and decreased the portal pressure (López-Talavera et al., 1996). Other two experimental approaches have been recently done by using cirrhotic BDL-rats; fist, the chronic administration of Tha improved the portal-systemic collateral vascular responsiveness to arginine-vasopressin in cholestatic rats, which was partially related to the inhibition of the cytokine vascular endothelial growth factor (VEGF) (Chang et al., 2009), and second, Tha decreased portal venous pressure as well as the intrahepatic resistance by reducing the hepatic thromboxane-A_2, a potent vasoconstrictor (Yang et al., 2009). An open pilot study in patients with stable alcoholic-cirrhosis and esophageal varices were administered with Tha or with pentoxifylline (immunomodulatory drug and PDE inhibitor); Tha, but not pentoxiphylline, reduced the hepatic venous pressure as well as TNF-α levels, that suggested future controlled clinical trials (Austin et al., 2004). Finally, a patient study case of intractable bleeding from hypertensive gastropathy secondary to extrahepatic portal vein obstruction was managed successfully by Tha (Karajeh et al., 2006).

Thalidomide in Experimental and Clinical Hepatocellular Carcinoma (HCC)

One of the first approaches to use Tha in liver diseases was perhaps its application in primary biliary cirrhosis (PBC), an idiopathic autoimmune disease with many clinical and pathological similarities to other illnesses wherein Tha had been successful; thus, a double-blind placebo controlled pilot study was performed, nonetheless, there were no improvements

in liver function tests or in liver histology. A number of patients treated with Tha reported an improvement in pruritus. That study suggested that Tha was unlikely to be affective in PBC (McCormick et al., 1994). Later, after the renaissance of Tha for the treatment of MM, the next obvious step was using this drug in several clinical trials for the treatment of HCC, which is the fifth most common cancer in the world and the third cause of cancer-related mortality. Cirrhosis is the main risk factor underlying HCC, and there is a clear association between chronic infections with hepatitis B and C virus, excessive alcohol consumption, cirrhosis and HCC; however, HCC also occurs in a noncirrhotic liver and the need of a systemic therapy is urgent (Witjes et al., 2009). Experimentally, Tha alone has significantly evidenced to inhibit angiogenesis and metastasis of HCC in the nude mice model, besides its inhibitory effects on TNF-α (Zhang et al., 2005a). Also, Tha in combination with paclitaxel (Zhang et al., 2005b) or with doxorubicin (Yang et al., 2005) afforded synergistic and significant necrosis and growth inhibitory effects on tumors, reduced angiogenesis and metastasis. These experimental results encouraged the Tha assessment in clinical trials.

Tha effects in clinical evaluations for treating HCC have yielded varied outcomes. Some authors have documented very promising effects, since individual case studies (Quek et al., 2006) or several patients in phase II clinical studies have responded to Tha therapy alone or in combination with other drugs (Wang et al., 2004; Demeria et al., 2007) and with radiotherapy (Hsu et al., 2006). Although Tha as a single treatment might not have very good results, because of the modest effects obtained in most cases (3-6 %), for example reducing the tumors size and/or achieving the stabilization of the disease for a longer period (Lin et al., 2005; Chuah et al., 2007); consequently, authors strongly recommend the use of Tha in combination with other chemotherapeutic agents, including the new immunomodulatory Tha analogs, to treat early small but not large tumors (Walder, 2005; Pinter et al., 2008). Generally Tha side effects are reported as well tolerated for patients, although few publications claim that this drug is not well tolerated when it is administered with other adjuvant agents (Cappa et al., 2005; Schwartz et al., 2005). As a unique case, it has been reported the successful treatment with oral Tha of a male patient (52 years old) who was suffering from hepatic epithelioid hamangioendothelioma (HEH) and lung metastasis, that cancer type is a very rare vascular tumor of the liver with an unpredictable malignant potential; Tha inhibited the growth and progression of the tumor (Mascarenhas et al., 2004).

Adverse Effects by Thalidomide: Hepatotoxicity

As commented above, Tha is a relatively safe drug compared with other known teratogens due to its very low acute toxicity, LD_{50} of >5000 mg/kg in mice and rabbits (Somers, 1960; Williams, 1968). The low toxicity of Tha has been observed in man, as 20 cases of accidental or intentional overdosage had been reported and all recovered eventfully (Somers, 1960), it may be possible that the absence of toxicity is due to the limited absorption. Regardless that, the common side effects during treatment with Tha are sedation, constipation, fatigue, skin rash and peripheral neuropathy (Singhal et al., 1999); less frequently bradycardia, hypotension and hypothyroidism have been described during prolonged treatments, but often well tolerated by patients, as it has been commented for Tha administration in MM and HCC clinical trials.

However, recent case reports concerning extremely rare but existing Tha hepatotoxicity in patients have been published. Post marketing surveillance of Tha since reintroduction in 1998 has identified one case where the cause of death was thought to be directly due to treatment with Tha (Clark et al., 2001). One case of Tha-induced fulminant hepatic failure which proved to be fatal in a 64-year-old woman has also been described (Hamadani et al., 2007). Almost the rest of cases have also been elder patients (57-79 years old) and just one younger woman (36) undergoing refractory MM with some complications as hypertension, diabetes, renal failure or leukemia and one with stable chronic hepatitis C; none of them had previously manifested hepatic alterations as well as their liver function tests in plasma were normal before beginning Tha treatment (Trojan et al., 2003; Dabak and Kuriakose, 2009; Levesque and Bradette, 2009). In addition, there are documented cases of hepatotoxicity but due to lenalidomide treatment in MM patients (Hussein, 2005; Hussain, 2007). Two patients with advanced HCC whom were under Tha therapy developed tumor lysis syndrome (TLS), similar to other two patients that received Tha for the treatment of MM (Spencer et al., 2003; Lee et al., 2006). Most cases returned to normal or quasinormal plasma values of liver damage biochemical markers (ALT, AST, AP and bilirubins), after Tha dosage cessation. Therefore, it becomes increasingly important to identify patient groups that may be particularly susceptible to specific adverse drug effects and to identify conditions under which specific adverse events may be more likely to occur. Oncology patients may represent a patient population with increased susceptibility to Tha-associated adverse effects (Clark et al., 2001).

It is necessary to comment that in no one of the diverse experiments in animal models of liver damage herein reviewed, and performed by several authors, Tha or its analogs administered in acute or chronic manner have showed by themselves either any sign of hepatotoxicity or a negative modification on the liver injury indicators during their evaluation, as well as they have not affected the normal plasma and liver cytokine profiles in control groups; what is more, even when some models are really drastic or severe (CCl_4, BDL, LPS, dimethylnitrosamine and thioacetamide) Tha or its analogs have resulted hepatoprotective agents, that has also been assessed by histopathology. There is only one report about exacerbation of acetaminophen hepatotoxicity by Tha, nevertheless authors did not find any significant influence on normal plasma levels of liver damage markers by the only administration of Tha (Kröger et al.; 1995).

Conclusion

In spite of the current medical advances and the development of new expensive therapies for the treatment of liver diseases and their last stage cirrhosis, nowadays there are not enough nor successfully effective and secure systemic drugs to guarantee a much better quality of life or the cure to the patients suffering from those illnesses (Muriel and Rivera-Espinoza, 2008). Nevertheless, immunomodulation seems to be a complex but very promising pharmacological approach to reestablish the cytokine imbalance during the liver damage and cirrhosis, this in order to manipulate or possibly to control the deleterious and beneficial processes that those mediators of inflammation regulate and, as a consequence, to maintain their levels into the normal liver and systemic homeostasis. Therefore, on the basis of all the reports herein

reviewed, including pro and con analyzed, the use of Tha and its analogs is suggested as potential hepatoprotective immunomodulatory drugs, although it is very clear that further and deeper basic as well as clinical research is necessary to detail the ratio risk/benefit.

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Chapter 2

Role of the Energy State of Liver Cell in Cirrhosis Development and Treatment

Victoria Chagoya de Sánchez,[1] Francisco Hernández-Luis,[2] Mauricio Díaz-Muñoz [3]and Rolando Hernández-Muñoz[1].

[1] Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular Universidad Nacional Autónoma de México, México 04510 D.F. México

[2] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, México 04510 D.F. México

[3] Departamento de Neurobiología Celular y Molecular, Campus UNAM-Juriquilla, Instituto de Neurobiología, 27230 Querétaro, Qro. México

Abstract

Cirrhosis is one of the most common causes of mortality worldwide because hepatic dysfunction constitutes a potentially lethal condition. Its etiology is variable being the most important alcoholic liver disease, viral chronic hepatitis and there's no treatment for prevention or regression of this pathology. At the same time, there is an increased risk of sepsis, bacterial peritonitis, variceal bleeding, portal hypertension and development of hepatocelular carcinoma. In this chapter, we briefly describe the normal structure and function of the liver including the importance of energy participation, in the normal and cirrhotic liver. Among the liver dysfunctions of this pathology exists a decrease in energy parameters in the blood of alcoholic and nonalcoholic patients with cirrhosis. In experimental cirrhosis induced with CCl_4, a strong correlation between energy parameters of the liver and the red blood cells indicated that blood parameters could be a reflection of liver damage. Our experience regarding the studies of acute and chronic experimental hepatotoxicity using the nucleoside adenosine as an hepatoprotector suggest that the nucleoside effect increasing energy availability by itself, played and important role preventing the mitochondrial dysfunction induced by the hepatotoxics like ethanol, CCl_4 in acute an chronic models. Regardless of the mechanism involved in the effects of adenosine on CCl_4-induced cirrhosis in rats, the maintenance of the energy parameters of

the liver cell during a chronic insult could be relevant to preserve liver function and the delay the fibrogenic effect of the toxin. More interesting is the beneficial effect of adenosine and adenosine derivative compound IFC305 on the preestablished CCl_4 cirrhosis associated with an improved liver function. In summary, adenosine and its derivative, through reversed cirrhosis, liver dysfunction and energy parameters of the liver cells could be considered as a tool to study the interrelationship between mitochondrial function and hepatotoxicity. Besides, it could be considered as a potential therapeutic agent for the treatment of chronic hepatic diseases.

Introduction

In most mammals the liver is the largest organ in the body and is constituted by 4 major lobes. It is situated in the right upper quadrant of the abdominal cavity and plays a central role in metabolic homeostasis, mainly as a primary regulatory site for food biochemical transformations and energy metabolism. The liver processes nutrients from the diet to obtain energy, and then distributes the energy substrates such as glucose, ketone body, purines and lipoproteins to extra hepatic tissues to help to maintain the energy status of the whole body. In this chapter, we briefly describe the normal composition and liver function, including the importance of energy participation, in the normal and cirrhotic liver.

Hepatic Circulation

The liver has a unique anatomical relationship with other organs mainly by a dual blood supply which makes possible its very important role in the intermediary metabolism. The total blood flow in normal adult is between 1,500 and 1,900 ml/min [1], and varies according to sex, age, exercise and feeding conditions. There are 2 main sources of liver blood: one third is delivered by the hepatic artery and the rest by the portal vein flowing within sinusoids [2]. In cirrhosis the arterial supply is increased while the portal-systemic shunting decreases the venous blood. The portal vein receives blood from the small intestine, colon and pancreas; within the liver its radials accompany the hepatic artery branches in a parallel intrahepatic distribution. Portal venous content of oxygen is high and contribute over 60% of the total oxygen supply to the liver under normal conditions. The physiological blood flow is mediated by the vascular resistance and the arterial pressure, and may be mediated by several factors, including adenosine [3]. Reduction in portal venous flow leads to an increase in hepatic arterial circulation which is also mediated by adenosine [4]. Under normal conditions, the liver contains a very large amount of blood in relation with its weight and may function as blood reservoir. The bile duct is formed by epithelial tissue specialized to resist the corrosive effect of bile; it accompanies the hepatic artery and portal vein constituting the portal triad of the liver [5].

Liver Composition

The structural unit of the liver is called acinus [6]. In the liver there are different types of cells: hepatocytes, which constitute the hepatic parenchyma and represents 70-80% of the total cells, whereas the other 20-30% is formed by endothelial cells, cells of the bile duct, oval cells, Kupffer cells (KC), Pit cells and hepatic stellate cells (HSC).

Hepatocytes occupy 80% of the liver volume, constitute the parenchymal cells with a high metabolic activity, and contain a complete set of organelles: mitochondrias, peroxisomes, lysosomes, Golgi complex, and a well organized cytoskeleton. Hepatocytes are epithelial cells that form cords, with high polarity thanks to the tight junctions localized between adjacent cells. The basolateral membrane faces the sinusoid, whereas desmosomes and gap junction are located within the lateral domain [5]. The space between cords of hepatocytes is the sinusoidal space while the space between hepatocytes and endothelium is de space of Disse. The complex of the hepatocytes, hepatic artery, portal vein, bile ducts, lymphatic vessels and nerves form the triad or portal space.

Endothelial cells constitute the wall of the hepatic sinusoid and are separated from the parenchymal cell by the space of Disse. They possess pores or fenestras that permit the free exchange of fluids [7]; in cirrhosis the fenestras are obliterated. These cells show a large endocytic activity, and secretion of several mediators such as interleukin-1, interleukin-6, interferon and nitric oxide which are parachrine modulators.

Kupffer's cells are the fixed macrophages of the liver, its origin is uncertain. One school suggests that they are derived from mononuclear stem cells from the bone marrow whereas others suggest that they are derived from circulating monocytes [8]. They are localized in the lumen of periportal sinusoids. The main function of these cells is the phagocytosis and the production of superoxide anions, hydrogen peroxide, hydrolytic enzymes, and eicosanoids which facilitate antigen destruction and inmunomodulatory functions [9].

Pit cells are intrahepatic leucocytes, large granular cells anchored to the endothelium. These cells possess natural killer cell activity [10, 11] and play a role in citotoxic activity toward tumor and virus-infected cells [9, 12].

The hepatic stellate cells (HSC) also known as lipocyte, fat-storing cell, perisinusoidal cell, Ito cell, and vitamin A-storing cell, are quiescent in normal conditions. They have an important role in the synthesis and degradation of the extracellular matrix proteins being key mediators in hepatic tissue repair and in the fibrotic onset involved in cirrhosis [13, 14].

Biliary epithelial cells participate in the bile formation, which is transported down the bile canaliculum, where they formed the bile ductules or canals of Hering. These cells have the potential to become oval cells or liver stem cells. The appearance of oval cells may be an important sign of liver recovery in some pathological conditions [5].

The hepatic tissue free of cells represents 20% of the liver volume, and constitute the extracellular matrix (ECM) localized in the Disse space. It contains structural proteins, being the most abundant the collagen type I, with collagen type III, V, VI, VII also present, glycoproteins such as fibronectin, tenacin and laminin, entactine and perlecan that constitute the basal membrane. Their function is essential to maintain the hepatic architecture and the organization of the entire organ. The matrix components include ligands that activate signaling pathways within the cells regulating cellular proliferation, survival and

differentiation [15]. Hepatocytes contribute about 80 to 90% on the synthesis of liver collagen which is degraded by the metalloproteinases [16].

Liver Metabolism

The liver has an intense participation in the intermediary metabolism which mainly occurs within the hepatocytes. In carbohydrate metabolism, there is glucose uptake, glycogenesis or glycogen synthesis, glycogenolysis or glycogen degradation, gluconeogenesis or glucose formation from other sources than carbohydrates. All of these metabolic pathways influence the blood glucose levels or glycemia. In protein metabolism, deamination and interconvertion of the aminoacids are very active. Ammonium is eliminated through the urea cycle in periportal hepatocytes whereas is incorporated in glutamine in pericentral hepatocytes. It has a high activity of protein synthesis, endogenous and exogenous such as blood serum proteins mainly albumin, coagulation factors, acute phase proteins, apolipoproteins, among others. In lipid metabolism, the liver is the principal organ for cholesterol synthesis, phospholipids, triacyglycerides, ketone bodies, lipoproteins, bile acids, and show a very high activity of peroxisomal and mitochondrial β-oxidation of fatty acid. The liver also generates purines and pyrimidines for their own use and for distribution to other tissues in the form of adenosine, inosine and hypoxanthine [17]. In the hepatic tissue other specific functions take place such as the storage of glycogen, vitamins A, D and B12, iron, and blood; in addition bile is secreted and the damaged erythrocytes are eliminated. The liver is the only tissue that has a detoxification function for harmful endogenous compounds such as ammonia and for exogenous toxic substances. The hepatic metabolism is under biochemical, endocrine and neural regulation.

Within this complex system, the structural and functional coordination of each component of the ECM and each cell population has a precise role to execute in order to reach the homeostasis that maintains the structure and function of the normal hepatic tissue. Several metabolites, cytokines, growth factors, either endogenous or exogenous, and specially the energy supply, contribute to preserve the cell-cell and cell-ECM communication, and to regulate the gene expression responsible for normal hepatic physiology.

Energy Supply in the Liver

In mammalian cells energy is mainly generated as ATP. It is produced by the oxidative phosphorylation and glycolysis and is used by diverse consuming pathways such as synthesis of proteins, glycogen, fatty acids, urea, purine and pyrimidines. The process of mitochondrial oxidative phosphorylation utilizes the reducing equivalents (H^+) from the nutrients, carbohydrate and lipids, once in the mitochondrial matrix the electrons flow down the electron transport chain in the inner mitochondrial membrane, and the H^+–ATP synthase convert ADP and Pi to ATP. The ATP inside the mitochondria is then exchanged for cytosolic ADP by the inner-membrane translocase. The flux through the mitochondrial ATP-synthase is regulated by the intramitochondrial concentration of its substrates ADP and Pi. The ADP level in the cell results from the breakdown of ATP consequent to the workload.

This process has to be connected with mitochondrial metabolic pathways such as the tricarboxylic acid cycle, the oxidation of fatty acids, and the mitochondrial and cytosolic dehydrogenase systems dependent to NAD^+/NADH playing an important role in the transport of reducing power from mitochondria to cytoplasm and vice-versa [18, 19]. The regulation of the whole process is complex but in normal conditions must fulfill the energy demands of the tissue under physiological conditions. Mitochondrial dysfunction will result in an energetic unbalance that would be the cause of degenerative diseases.

Control of Energy Metabolism

The ATP molecule plays a central role in energy control, and then, the knowledge of the factors that control its production and its consumption is basic to evaluate its role in the regulation of the intermediary metabolism. The sole molecule of ATP is not an adequate indicator of the energy state, considering that ATP is related in reversible manner with ADP and AMP. Hence, the adenine nucleotide system contains 3 components ATP, ADP and AMP. Atkinson proposed the adenylate energy charge hypothesis [20]. The energy charge value oscillates between 0–1 according to relative concentrations of ATP, ADP and AMP. When all adenine nucleotides are converted into ATP, the system is fully charged, and fully discharged when only AMP is present (Energy Charge = [ATP] + ½ [ADP) / [ATP) + [ADP) + [AMP]. Atkinson's hypothesis was tested *in vitro* systems showing that the flux of ATP-regenerating reactions decrease when energy charge increase and the reactions utilizing energy increase [21, 22]. However the significance of the metabolic control of adenylate system has been questioned in the intact cell [23]. We showed that adenosine administration *in vivo* is capable to increase the energy charge in the liver (from 0.86 to 0.93) [24]. This finding has been confirmed *in vitro* in perfused rat liver [25], liver slices [26] and isolated hepatocytes [27]. Nevertheless, the mechanism by which adenosine increases the hepatic energy charge has not been clarified. Previous work with adenosine as liver metabolic regulator showed that the nucleoside administration to normal rats increase hepatic glycogen biosynthesis [28] and diminished fatty acid oxidation in the liver [29]. Considering that the former change correspond to an ATP-utilizing system and the latter to an ATP-regenerating system lead us to think that control metabolism of energy charge is also observed *in vivo*. Other parameter related with the energy metabolism and mitochondrial function is the cell redox state which has an important regulatory role in metabolic fluxes [30].

Cell Redox State in the Liver

It is frequently to assume that the phosphate anhydride bonds of ATP are "high-energy" bonds which are capable of storing energy and driving reactions in otherwise unfavourable directions. However, it is rather the extent to which the observed mass-action ratio is displaced from the equilibrium which defines the capacity of the reactants to work, instead any attribute of a single component. In this regard, any hypothetical cell could utilize any reaction to transducer energy from the mitochondrion, as it occurs with the glucose-6-phosphatase reaction, where that reaction is maintained ten orders of magnitude away from

the equilibrium. In this concern, theoretically the glucose-6-phosphatase could eventually form ATP, which it not occurred during the metabolism [31].

From here, that besides the phosphorylation potential and the mitochondrial capacity to perform phosphorylative oxidation would depend on an additional metabolic constant represented by the oxidation-reduction (Redox) potentials [32]. Both the mitochondrial respiratory chain and the photosynthetic electron-transfer chains operate as a sequence of reactions in which electrons are transferred from one component to another [33]. Here, while many of these components simply gain one o more electrons in going from the oxidized to the reduced form, in others the gain of electrons induces an increase in the p*k* in one or more ionisable groups on the molecule, with the result that reduction is accompanied by the gain of one or more protons.

All the oxidation-reduction reactions can quite properly be described in thermodynamic terms by their Gibbs energy changes; since these reactions involve the transfer of electrons electrochemical parameters can be employed. Although the thermodynamic principles are the same as for the Gibbs energy change, the origin of oxido-reduction potentials in electrochemistry sometimes obscure this relationship [34]. The additional facility afforded by an electrochemical treatment of a redox reaction is the ability to dissect the overall electron transfer into two half-reactions, involving respectively the donation and acceptance of electrons, such as it occurs in the following reaction $NADH \leftrightarrow NAD^+ + 2e^-$ [35]. In this case, a reduced-oxidized pair such as $NADH/NAD^+$ is termed a *redox couple* [31]. The ΔG which is available for a redox reaction is proportional to the difference in the actual redox potential ΔEh between the donor and acceptor redox couples. In the case of the mitochondria, the *E*h (at pH 7.0) for the $NADH/NAD^+$ couple is about -280 mV and the *E*h (at pH 7.0) for the O_2/H_2O couple is +780 mV [36]. From here, that the redox potential difference ΔEh_7 of 1.16 V is the measure of the thermodynamic disequilibrium between the couples, which provide the electrochemical driving force for ATP synthesis [31].

Alterations in cell redox state have been implicated in injured hepatocytes, especially regarding alcohol effects on the liver. Oxidative stress is a well-documented cause of alcoholic liver damage [37]. Ethanol is metabolized via alcohol dehydrogenase (ADH), the microsomal ethanol-oxidizing system (MEOS), and catalase in the peroxisomes, although the alcohol dehydrogenase pathway is responsible for a majority of ethanol metabolism. In this pathway, nicotinamide adenine dinucleotide (NAD^+) is reduced by a transfer of hydrogen to NADH, with concomitant production of acetaldehyde. The $NADP^+$ can also be reduced to NADPH, and this increase in reducing equivalents in the cytosol (NADH and NADPH) changes the redox potential of the cell. Hydrogen equivalents from ethanol, but not NADH, are transferred from the cytosol to the mitochondria via a shuttle mechanism such as the malate cycle, the fatty acid elongation cycle, and/or the α-glycerophosphate cycle. This replaces the citric acid cycle as the source of hydrogen, and the mitochondria become more reduced [38, 39].

As to cirrhosis, a possible role of disturbed cell redox state (more oxidized or reduced) is much less known. However, since the cell redox state is an important regulatory factor of several metabolic fluxes [31], it has been suggested that also collagen metabolism could be highly influenced by changes in the redox state. The increase in hepatic reduced power, in the form of NAD(P)H favours conversion of glutamate to proline, which is an immediate precursor of collagen [40]**,** and a decreased mitochondrial NAD+/NADH ratio also promotes a diminished degradation of this amino acid as demonstrated in the flight muscle

mitochondria [41]**.** Moreover, reduced redox in mitochondria also impairs proline transport into the organelle, then influencing the rate of its oxidation through reactions involving collateral enzymatic steps from the Krebs cycle [42], which as a whole would elicit an increase in liver proline, as a fundamental collagen component which consequently can lead to liver fibrosis.

In models of experimentally-induced rat liver cirrhosis, mitochondrial function and structure in cirrhotic livers from rats show a variety of changes as compared to control livers. Mitochondrial ATP production is reduced in rats with CCl_4- or thioacetamide-induced liver cirrhosis and in rats with secondary biliary cirrhosis. Similarly, in humans with liver cirrhosis, mitochondrial cytochrome a + a3 content is elevated and has been used to assess the risk for hepatectomy. In rats with secondary biliary cirrhosis, compensatory strategies include increased mitochondrial volume per hepatocyte and possibly increased extramitochondrial ATP production (increased glycolysis). Thus, a variety of adaptive mechanisms are used to maintain mitochondrial function in cirrhotic livers [43]. The rate of ATP production is significantly decreased in mitochondria from cirrhotic rats, using several substrates. In contrast, the static head (state 4) phosphate potential is not altered in control mitochondria, which has led to conclude that liver cirrhosis promotes a loss of hepatocytes which is paralleled by reduced oxygen uptake and reduced mitochondrial enzyme activities [44]. These reports are in agreement with the statement that in perfused cirrhotic livers (secondary biliary cirrhosis), a reduced cytoplasmic and mitochondrial redox states occur accompanied by a diminished electron-transport chain activity [45].

Intracellular Calcium Dynamics in the Liver Relationship with Energy Necessities

Mitochondrial physiology, and the concomitant ATP production, is highly dependent of the fluxes of calcium from and into the organelle. It is well reported that several dehydrogenases that form part of Krebs cycle are modulated by intramitochondrial calcium: pyruvate dehydrogenase, NAD^+-isocitrate dehydrogenase and oxoglutarate dehydrogenase. The calcium sensitivity of these enzymes is the foundation of the mitochondrial response to calcium in situations of high energy demand. By other hand, calcium may become to be an altering factor in the bioenergetic of the cell when it is increased to high levels and for prolonged time in the intracellular milieu. In these cases, the mitochondria can not modulate the entry and exit of calcium, and start an intense production of free radicals that eventually lead to the activation of the apoptotic program by releasing the cytochrome C [46].

In physiological conditions calcium mobilization within hepatocytes and other liver type cells can be transient or oscillatory, and can occur as localized or global events [47]. In the liver as well as other tissues and organs, calcium plays a dual role in cellular signaling: 1) it acts as a cation, modulating the electrical properties of the plasma membrane, and 2) it coordinates metabolic and transcriptional responses during transduction events.

Several important metabolic functions of the mammalian liver have been shown to be located in zones with respect to the complex microcirculation of the organ. In this context, it is recognized the existence of at least 2 types of hepatocytes: periportal and perivenous. Periportal hepatocytes are better adapted to oxidative reactions, and biochemical pathways

such as urea synthesis and gluconeogenesis take place within there. Perivenous hepatocytes are better suited for anaerobic environment with an enhanced glycolitic activity. Besides, in these hepatocytes the metabolic transformation of xenobiotics and the N_2-handling by glutamine formation are highly expressed [48]. Hepatic calcium channels and ATPases also depict unequal distribution between the periportal and perivenous zones (unpublished data). So far, no report has been published regarding how metabolic zonation is altered in the cirrhotic liver.

Intracellular calcium is a well known factor that regulates responses associated to signaling transduction as well as biochemical networks, due to its ubiquitous role as a metabolic coordinator. Fluctuations of cytoplasmic calcium codify a message that is interpreted in a spatiotemporal manner by metabolic and transcriptional factors, thus regulating a variety of cellular processes such as cell proliferation and apoptosis, differentiation, muscle contraction, neurosecretion, gene expression, among others [47]. This fine-tuned handling of intracellular calcium is the result of a coordinated activation of ion channels, metabolic pumps, and exchangers in organelles such as the endoplasmic reticulum, mitochondria, and nucleus. Some of the most influential elements involved in intracellular calcium dynamics are the calcium-release channels inositol 1, 4, 5-trisphosphate receptor (IP_3R) and ryanodine receptor, as well as the sarco-endoplasmic reticulum calcium ATPase (SERCA). All of these proteins are highly expressed in the liver [47]. Some factors present in the liver that are recognized to mobilize intracellular calcium are: IP_3, cyclic adenosine 5´-diphosphoribose (cADPR) which activates R y R, NO, reactive species of oxygen (ROS) such as H_2O_2 and O_2^-, nicotinic acid adenine dinucleotide phosphate (NAADP), diacylglycerol, arachidonic acid, sphingosine and sphingosine-1-phosphate, and calcium itself [47].

It is well accepted that Ca^{2+} plays a fundamental role in cellular regulation. In hepatic tissue this second messenger is involved in glucose metabolism (glucogenolytic and gluconeogenetic activities), protein folding, mitochondrial function, gene transcription, apoptosis, cell proliferation, bile secretion and more [49]. The mechanism by which this cation modulates all these processes is based in the existence of temporal and spatial sensors of calcium transients and oscillations that seems to constitute a type of metabolic modulatory code [50]. Intracellular Ca^{2+} dynamics takes place by the coordinated action of numerous proteins responsible for calcium mobilization outward and inward of the cytosolic space. The liver expresses the two principal intracellular calcium release channels: IP_3R type 1 and 2, and the type 1, reported as a truncked but functional channel-protein [51]. The hepatic proteins that extrude cytosolic calcium are represented by the SERCA splicing isoform 2b and the plasmatic membrane calcium ATPase (PMCA) including the isoforms 1, 2 and 4 [52]. Liver tissue exhibit a distinctive heterogeneity in its anatomical functionality that depend of it blood and hence nutrient and oxygen supply. Hepatocytes close to portal vein are named periportal hepatocytes and comprise a cell population in which predominate glucogenolysis, gluconeogenesis, bile formation and lipid catabolic activity. While in the case of hepatocytes near to central vein, they are called perivenous hepatocytes shows glycolytic and detoxification activity as their main biochemical tag. It was reported that hepatic IP_3R shows a heterogeneous distribution along the hepatic acinus and bile duct epithelia [53], but there are not reports about other calcium-handling proteins zonational distribution.

Liver calcium signaling responds to energetic status and hence to feeding condition. Hepatocytes of fasting rats show significant increase in intracellular calcium levels [54]. Rawson *et al*., also demonstrated that rats treated with 2,5AM (2,5-anhidro-D-mannitol)

showed an important reduction of ATP levels and also a marked elevation of intracellular calcium [55].

Even though calcium mobilization has been shown in several cellular types in the liver, an especially relevant mention regarding cirrhosis onset is the activation of stellate cells. The transformation of stellate cells into myofibroblast involves the participation of a number of intracellular signaling pathways, including the entrance of calcium into the stellate cell [56].

Physiopathology of Cirrhosis

At present, it is still recognized that the precise etiology of cirrhosis is not known. Although some of the settings in which cirrhosis develops are obvious, the mechanisms by which these situations or agents are translated to the clinic-pathologic picture of cirrhosis are unclear. Circumstantially, it appears clear that cirrhosis is associated with excessive alcohol consumption, viral hepatitis, drug-induced liver injury, prolonged extrahepatic biliary obstruction, late stages of some parasitic diseases, as well as some genetically transmitted metabolic disorders such as hemochromatosis and Wilson's disease.

Studies with laboratory animals, although productive of great volume of data, have not still provided a reliable animal model with which to study this problem. However, the demonstration by Rubin and Lieber [57] of the production of alcoholic cirrhosis in baboons is a landmark in liver disease, since these investigators showed for the first time that cirrhosis, much like human alcoholic cirrhosis, can be reproducibly caused by the administration of alcohol in laboratory animals.

In any case, the most prominent feature in cirrhosis is that of liver fibrosis. Hepatic fibrosis is a dynamic process whereby the liver responds to conditions of persistent damage, which leads to deposition of fibrillar extracellular matrix, altered hepatocyte regeneration, deranged microvascular architecture and terminally cirrhosis. For more than a century and a half, the description of a liver as "cirrhotic" was sufficient to connote both a pathological and clinical status, and to assign the prognosis of a patient with liver disease. However, as our interventions to treat advanced liver disease have progressed (e.g., antiviral therapies), the inadequacy of a simple one-stage description for advanced fibrotic liver disease has become increasingly evident. Here, the understanding the range of potential outcomes based on the severity of cirrhosis is essential in order to predict outcomes and individualize therapy, which needs a reformulation of the concept of cirrhosis from a static to a dynamic process [58]. Cirrhosis is defined histologically as a diffuse process in which the normal anatomical lobules are replaced by architecturally abnormal nodules separated by fibrous tissue [59]. Progressive histological stages have been defined in the process leading to the development of cirrhosis, and with some grade of accurately measuring, scales as that named METAVIR [60], and other scoring systems [61, 62] attempt to semi-quantitatively define progressive fibrosis based on the pattern and relative amounts of scar within a liver biopsy specimen. Moreover, since normal liver has only a small amount of fibrous tissue in relation to its size, continued liver injury produces a progressive accumulation of extracellular matrix, or scar, leading to patterns of fibrosis deposition. In this context, once fibrosis reaches the final stages, the diagnosis of cirrhosis is established and the process is considered "end-stage" from a pathological perspective [63].

Liver fibrosis has been considered to be an irreversible process involving the progressive replacement of the hepatic parenchyma with collagen rich extracellular matrix. Acute injury of the liver is associated with a classic wound-healing process in which inflammation triggers scar-formation that is subsequently resolved to enable regeneration of the damaged hepatic parenchyma. However, in the chronic injured liver, repeated and overlapping phases of inflammation and wound-healing overwhelm the normal regenerative process and instead promote the net deposition of fibrillar collagen. From here, that cirrhosis is characterized by a profound architectural distortion of the liver lobule characterized by thick bands of fibrotic tissue that bridge hepatic vessels and which surround nodules of regenerating hepatocytes, promoting two forms, micronodular and macronodular cirrhosis [64]. In both cases the consequence is functional perturbations of the liver evidenced by jaundice, anorexia and fatigue.

In normal non-fibrotic liver the extracellular matrix is of a low density basement membrane-type with fibrils of collagens I, III, IV and VI mainly concentrated in the portal triad, capsule and around large vessels, with only scattered collagen fibrils present in subendothelial spaces. During liver fibrosis, not only is there increased ECM production of approximately six times greater than normal, but there are also profound changes in its composition [65]. The normal low density basement membrane type matrix is progressively replaced by an interstitial scar-type matrix with high fibrillar collagen content, evoking a deleterious effect on the biochemical function of hepatocytes and ultimately results in hepatic necrosis and subsequent loss of hepatic function [66].

In the context of cirrhosis, it is now considered that the principal cell responsible for promoting the deposition of cross-linked fibrillar collagen in the injured liver is the hepatic myofibroblast, since they are scarce in the uninjured liver but their numbers dramatically increase at sites of infection, inflammation and injury. are a wound-healing cell generated locally from the transdifferentiation of resident perisinusoidal hepatic stellate cells and periportal fibroblasts [67], but they also seem to be generated via the recruitment and differentiation of bone-marrow-derived mesenchymal stem cells [68, 69]. Normally hepatic stellate cells reside in the space of Disse where they function as a major store of retinol and in its form, produce low levels of extracellular matrix proteins. Upon liver injury, hepatic stellate cells lose their vitamin A, proliferate and subsequently transform to a myofibroblast like phenotype in which they acquire contractile, proinflammatory and fibrogenic properties, which are important as aids to wound closure while their secretion of collagen-rich extracellular matrix promotes the formation of a temporary scar. Furthermore, hepatic myofribroblasts are the principal source of matrix metalloproteinase-2 which in conjunction with other enzymes, degrade normal subendothelial matrix, facilitating replacement with high density interstitial matrix [70].

In normal liver, matrix homeostasis is highly developed with controlled degradation of excess extracellular matrix by a family of matrix metalloproteinases (MMPs) including MMP-1 and MMP-8 [71], while activated hepatic myofibroblasts produce vast quantities of Type I and Type III collagen and in addition secrete the tissue inhibitors of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2) which operate to block the collagenolytic activity of a wide range of MMPs [72]. As a consequence, normal matrix homeostasis is severely disrupted in favor of net deposition of fibrillar collagen and the presence of regenerative nodules. Cross-linking of the collagen fibrils is involved in the stabilization of the extracellular matrix, which is mediated by transglutaminase mediated cross-linking of

lysines and in addition by development of pyridinoline cross-links [73]. It is this disruption of the liver architecture that primarily leads to the impairment of liver function found in cirrhotic individuals.

The component of auto-immunity and genetically acquired metabolic disorders add other components to the generation and physiopathology of cirrhosis. It has been established that congenital hepatic fibrosis, and indeed Caroli's disease closely resemble each other pathophysiologically, in that both occur as a result of ductal plate malformation. The persistence of these immature duct elements stimulates the formation of portal fibrous tissue, and it is this periportal fibrosis that contributes to the clinical picture of recurrent cholangitis or portal hypertension and associated symptoms. On the other hand, long standing portal hypertension is known to result in secondary portal vein thrombosis, and eventually portal vein cavernous transformation, which is firmly believed that this event is actually a component of the disorder, present at the onset rather than developing at a later stage [74].

Here, the hepatic stellate cell (HSC) is also at the center of these hepatic fibrotic processes associated with liver disease, and has also been shown to play a role in the progression of the disease in congenital hepatic fibrosis. It is widely accepted that transforming growth factor (TGF)-β is a potent growth inhibitory and pro-fibrotic cytokine which plays a pivotal role in the physiological process of wound healing as well as in the pathogenesis of organ fibrosis [75]. TGF-β expression has been shown to be increased in a wide range of fibrotic diseases. Initiation of hepatic stellate cell activation is primarily induced by TGF-β1 derived from Kupffer cells. TGF-β1 mediates its pro-fibrotic actions by stimulating fibroblasts and related cell types, including the hepatic stellate cell in the liver, to secrete a wide range of extracellular matrix proteins. In pathological conditions this leads to accumulation of fibrotic matrix or in a more physiological context to the efficient healing of wounds [76, 77]. Latent TGF-β is also activated by MMP-9, another product of Kupffer cells. TGF-β has other important actions, namely its immunomodulatory properties and its anti-proliferative effects on epithelial cells, including hepatocytes. Degradation of the basement membrane and extracellular matrix constituents, and the remodeling of this matrix are important processes of embryonic development. Basal laminar components such as laminin and type α-I collagen along with the coordinated expression of proteolytic enzymes are thought to be essential for the normal development of intrahepatic bile ducts [78, 79]. Most of the proteolytic enzymes involved in these processes belong to the matrix MMPs and the serine proteinases, in particular the plasminogen activator (PA)/plasmin system [80]. Both tissue PA (tPA) and urokinase type PA have been shown to contribute to the plasminogen-dependent lysis of basement membrane laminin in human carcinoma cell lines. Furthermore, plasmin contributes to the activation of MMP-9 and MMP-13 which also play an important role in the degradation of basement membrane components including type α (-I collagen. It has been postulated that biliary overexpression of plasminogen and tPA leads to the generation of excessive amounts of plasmin, and subsequent plasmin dependent lysis of the extracellular matrix molecules [81].

Overexpression of the osteopontin gene has also been implicated in the pathophysiology of biliary atresia, as well as congenital cholestatic syndromes such as CHF and Caroli's disease. Osteopontin is a stimulant of fibroinflammation, and its overexpression has been shown to be regulated by the presence of excessive amounts of regulatory factors such as NF-κB and TGF-β1 [82]. Moreover, it has been demonstrated decreases in the levels of the microRNA miR15a in the livers of patients with different liver fibrotic processes. Here, this

event results in an increase in the expression of a cell-cycle regulator known as cell division cycle 25A gene product (Cdc25A), which is directly responsible for cellular proliferation and cystogenesis in vitro. In fact, NF-κB (nuclear factor κB) is a heterodimeric transcription factor that is constitutively expressed in all cell types and has a central role as a transcriptional regulator in response to cellular stress. The NF-κB signaling has a role in the maintenance of liver homoeostasis as well as in the pathogenesis of a wide variety of conditions affecting the liver, including viral hepatitis, steatohepatitis, cirrhosis and hepatocellular carcinoma, probably through its canonical pathway, including the IKK (IκB as inhibitor of κB kinase) complex and the RelA subunit [83].

Finally, among a constellation of complications involved in the physiopathology of cirrhosis, two of them deserve brief comments. Liver cirrhosis is associated with number of hematological complications and coagulation disturbances. Severe coagulopathy of liver disease is more frequently seen in acute liver failure, but still remains important complication of liver cirrhosis and chronic liver failure and decreased production of blood coagulation factors by the liver plays a key role in altered haemostasis in liver diseases. Altered fragile balance of blood coagulation proteins and infection are associated with both worsening coagulopathy and bleeding risk. Additional haemostatic abnormalities in patients with severe liver diseases are thrombocytopenia, chronic disseminated intravascular coagulation, accelerated fibrinolysis, hypofibrinogenemia and dysfibrinogenemia [84].

Hepatic encephalopathy is one of the most important clinical manifestations in decompensated liver cirrhosis. Accepted concepts regarding the pathophysiology of hepatic encephalopathy are that the endogenous neurotoxic substances, including ammonia: 1) escape from catabolism by the liver due both to the impaired function of the cirrhotic liver and also to the presence of portal systemic shunting; 2) circulate at elevated concentrations in the systemic blood flow; 3) reach the brain through the blood-brain barrier; and 4) impair cerebral function leading to disturbances of consciousness. The majority of these toxic substances is produced in the intestine by the bacterial flora, and is absorbed into the portal venous flow. The epidemiology of liver cirrhosis depends particularly on its etiology, and shows a marked geographic difference worldwide between Western, and Asian countries. Hepatic encephalopathy developed at an annual rate of 8% in cirrhotics in Far Eastern studies. In Eastern and Far East countries, therapeutic options are similar to those in the western hemisphere, but pronounced application of dietary restriction, antimicrobial agents, disaccharides, shunt obliteration and branched chain amino acids is noted. Thus, hepatic encephalopathy remains a serious complication of liver cirrhosis [85].

Energy Metabolism in Hepatotoxicity

The causes of acute toxicity are multiple: the contact with organic solvents such as ethanol, halogenated hydrocarbons like carbon tetrachloride, inhibitors of protein synthesis, some medicaments such as corticosteroids, valproic acid, acetaminophen or alterations of physiological process including biliar flux or immunological problems. The mechanisms are also diverse: the oxidative stress produced by an excess of reactive oxidative species (ROS), or the diminution of antioxidant defense such as glutathione, mitochondrial and endoplasmic reticulum damage that interfere with energy metabolism, protein synthesis and an increase in

cytosolic calcium. Hepatic steatosis is an early sign of hepatotoxicity associated with a decrease of liver ATP [86-88], and its prevention by ATP administration [86] has been questioned by other authors [87, 88]. In our experience, using 3 experimental models of acute hepatic toxicity, by ethanol [89], cicloheximide [90] and carbon tetrachloride [91] administration, the fatty liver formation was accompanied by a decrease in energy status [89], with a diminution of energy charge 0.80 in control vs. 0.76 in ethanol treated groups. Adenosine administration increased the energy charge to 0.85, and reduced the ethanol-induced fatty liver [24]. Similar effects were observed in the diminution of the fatty liver induced by cicloheximide and carbon tetrachloride upon adenosine administration. Further evidence of the participation of mitochondrial energy metabolism in the damage induced by ethanol and its protection by adenosine treatment is a pronounced increase of ethanol oxidation *in vivo* [92] and in hepatocytes obtained from adenosine-treated rats [92], showing that adenosine increased the electron flux in the mitochondrial respiratory chain by the utilization of reducing equivalents (NADH) generated during ethanol catabolism in the cytosol and favoring acetaldehyde removal by enhancing the NAD^+/NADH ratio in the mitochondria [92]. Moreover, the protection exerted by adenosine during carbon tetrachloride acute hepatotoxicity is based in an anti-lipoperoxidative action associated to uric acid generated during its catabolism, and by an increase in mitochondrial glutathione peroxidase [93]. These results show the importance of mitochondrial dysfunction in acute hepatotoxicity and the beneficial effects of improving the energy metabolism with adenosine treatment.

Most of chronic hepatotoxicity consequences in cirrhosis, which is a degenerative liver disease characterized by a lost of equilibrium between formation and degradation of proteins of the MEC, mainly collagen, which is accumulated by a diminution of its degradation, result in an alteration of hepatic architecture and consequently lost of hepatic function. Its etiology is diverse: chronic exposition to drugs and toxic substances, chronic viral infections mainly hepatitis B and hepatitis C, biliar disease, cardiovascular congestive disease, autoimmune disease, metabolic alterations, and cryptogenic cirrhosis in which the cause cannot be established. Cirrhosis is one of the most common causes of death in many countries, affecting more than 5% of the population worldwide. Incidence of cirrhosis is growing as a result of the widespread occurrence of chronic hepatitis as well as the evident lack of an established therapy [94]. The patients frequently died of digestive hemorrhage, hepatic insufficiency, or cancer. Investigating the protective effect of adenosine administration in an experimental model of liver cirrhosis induced by CCl_4 administration 3 times a week during 4 and 8 weeks [95], we found that control cirrhotic group showed an active hepatic fibrogenesis since the levels of liver collagen were 2 – 3 times higher after the CCl_4 treatment. Measuring the energy parameters of hepatic adenine nucleotide, the hepatotoxic treatment induced almost 20% decrease of ATP and the energy charge decrease (from 0.85 to 0.81). In contrast, the group treated with adenosine at 4 and 8 weeks recuperated the ATP levels and the energy charge almost to normal values. But the most interesting findings were a decrease of 50% of the collagen accumulated, explained mainly by an enhanced collagenase activity. The effect of adenosine was also associated with a striking improvement of liver function, decreasing necrosis and increasing albumine synthesis. We assume that the maintenance of the energy parameters of the liver cell during a chronic insult could be relevant to preserve liver function and to delay the fibrogenic effect of the toxin. In order to understand the metabolic significance of the mitochondrial disturbances found in the cirrhotic animals, the transport of cytoplasmic reducing equivalents to the mitochondrial electron chain by the malato-aspartate

shuttle was measured [92] showing an important decrease in the animals treated with CCl_4 (50%) which was prevented by adenosine treatment. Some other parameters of mitochondrial function were evaluated in this model such as mitochondrial oxidation of substrates in sites I and II, ATP synthesis, membrane electrical potential ($\Delta\Psi$), ΔpH, ion transport, and membrane composition. All of them were damaged by the CCl_4 treatment and were partially or completely improved by adenosine treatment. The possibility that the nucleoside stabilizes the membrane damaged by the hepatotoxin has also been suggested [91, 92, 95]; however the mechanism involved in the protective action of the nucleoside on the mitochondrial alterations induced by the hepatotoxin remains to be fully elucidated.

Collagen Metabolism and Cell Redox State

Correlationship of mitochondrial injury associated to CCl_4–induced liver fibrosis/cirrhosis was studied in the same model of CCl_4 evaluating the mitochondrial function *in vivo* through the assessment of mitochondrial and cytoplasm redox states in relation with collagen synthesis and degradation [30]. Collagen is a proline-rich protein, and proline is synthetized from glutamate in a redox-control reaction [96, 97]. The rate of collagen synthesis was gradually enhanced by CCl_4 treatment and this effect was accompanied by a progressive loss of collagenolytic activity in the cirrhotic animals. The simultaneous administration of adenosine partially blocked the stimulated collagen synthesis induced by the hepatotoxin and maintained high levels of hepatic collagenase activity. The correlationship of those parameters with the cytoplasmic NAD/NADH ratio, result in a highly significant inverse correlation with collagen synthesis ($r=0.81$, $p< 0.005$), whereas the collagenase activity had less influence ($r=0.51$, $p<0.1$). On the other hand, mitochondrial NAD/NADH ratio showed a striking inverse correlation with the rate of collagen synthesis ($r=0.84$, $p<0.005$), and a highly significant linear relationship with collagenase activity ($r=0.93$, $p<0.001$). Adenosine administration prevented mitochondrial damage and the shift of the cell redox state avoiding the enhancement of free proline and the rate of collagen synthesis. The changes in redox state mainly reflect the events occurring in the parenchymal cells (hepatocytes) where CCl_4 exerts its toxic action. It is possible that adenosine preventing the mitochondrial damage induced by the hepatotoxin could maintain normal communication with mesenchymal cells in order to maintain an increased collagen turnover suggesting that preserving the mitochondrial function possibly arrest the sequence of events leading to cirrhosis [98-100].

Oxidative Stress and Proliferative Potential in Experimental Cirrhosis

Oxidative stress has been postulated as a major molecular mechanism involved in CCl_4 hepatotoxicity [93-95]. As it was previously commented in this chapter, in acute CCl_4 poisoned rats, adenosine was able to decrease necrosis, fatty liver, and oxidative stress mainly by a diminution of the ROS and by an increase in antioxidant defenses such as glutathione

peroxidase. The link between lipoperoxidation and chronic liver fibrosis has been proposed by several authors [30, 101-104], stressing the hypothesis that hepatic lipid peroxidation plays an important role in the etiology of hepatic fibrogenesis [103]. In rats rendered cirrhotic by CCl_4 continuous treatment, oxidative stress increases mainly in the microsomal and cytosolic fractions and decrease the antioxidant defense such as glutathione at 4 weeks of treatment. At 8 weeks of treatment, micronodular cirrhosis developed whereas oxidative stress was greatly attenuated, persisting only in the microsomes. Simultaneous administration of adenosine, a reliable hepatoprotector that prevents the onset of liver fibrosis, was able to block the oxidative stress generated by the hepatotoxin but elicited a selective subcellular distribution of lipoperoxidation mainly in the nuclear fraction [104]. This observation is similar to the one obtained in hepatectomized rats in which an increase in nuclear lipoperoxidation was also observed [105,106]. In the cirrhotic rats treated with CCl_4 , adenosine induced a highly significant increase in nuclear lipidperoxidation at 4 and 8 weeks, suggesting that adenosine-treated cirrhotic rats showed a liver "physiological" lipoperoxidation that improves its proliferative potential [104], whereas the same process in cirrhotic animals is highly disturbed [102]. This possibility was tested by the 5 to 8 fold increase in the activity of thymidine kinase which is rate limiting ezyme of DNA synthesis. The adenosine-mediated preservation of cellular energy availability linked to an efficient mitochondrial function, and maintenance of the liver redox state as the main driving forces of the metabolism pathways, could be factors participating in the adenosine-induced prevention of the onset of oxidative stress, and the recuperation of regenerative potential diminished in cirrhotic rats [104].

Energy Metabolism in Patients with Liver Damage

The evidences of the important role that energetic metabolism plays in development and reversion of cirrhosis in an experimental model induced with CCl_4 compelled us to investigate the alterations of the energy parameters in patients with liver damage. Considering the essential participation of the liver in the maintenance of the normal levels of nucleosides for the synthesis of purine nucleotides in extrahepatic tissues such as the blood cells, which are unable to synthesize purines [107, 108], the liver is able to maintain a constant adenine nucleotide pool in the red blood cell through a constant purine supply [109,110]. Moreover, the circadian variations of purine nucleotides in the blood and liver of the rat further supports the liver-blood relationship [109] that was confirmed studying *in vivo* correlation between liver and blood energy status in experimental cirrhosis induced with CCl_4 and treated with adenosine [110]. The changes in adenine nucleotides and energy parameters in the liver and blood ATP/ADP ratios showed a strong linear correlation ($R=0.93$, $p<0.001$). After 4 weeks of treatment the correlation ATP/ADP was $r=0.94$, $p<0.001$ and at 8 weeks of treatment ($r=0.90$, $p<0.001$) diminished by the hepatotoxic treatment in liver and blood. This relationship could also be a useful tool for diagnose and to follow the progress of some degenerative liver diseases [110]. On these bases, blood adenine nucleotides were determined in patients with alcoholic hepatitis (AH n=8)), alcoholic liver cirrhosis (ALC, n=9), non alcoholic liver cirrhosis (NALC, n=7), and amoebic liver abscess (ALA, n=4) [111]. A decrease of 28% to 39% in blood ATP levels was observed among the patients with hepatitis and cirrhosis groups respectively ($p<0.05$) whereas no significant changes in blood ATP

levels were detected in the ALA group. Although, total blood adenine nucleotides were significantly diminished in AH, ALC, NALC groups, the AH patients retained their energy relationships (ATP/ADP, energy charge, total nucleotides) within normal range. On the other hand the cirrhotic groups independently of their etiology failed to maintain an adequate ATP/ADP ratio, energy charge in the blood suggesting decreased energy availability in their blood cells. Based on these results, it is possible to assume that the changes in blood adenine nucleotides and in the energy parameters of the blood of patients with frank cirrhosis, independently of the etiology are related with the liver damage. Moreover, the liver damage decreases the energy availability in extrahepatic tissues which depend on the liver supply of purines for adenine nucleotide synthesis.

Reversion of Preestablished CCl_4-Induced Micronodular Cirrhosis

The beneficial effects of adenosine described in this chapter were studied in a prevention model that involved the simultaneous induction of cirrhosis with the administration of the nucleoside. But a more common situation was the one of the patients who developed severe cirrhosis and the diagnostic was established until the deficiency of the hepatic functions was evident. Therefore in the experimental model, adenosine effect was tested in well-established cirrhosis after 10 weeks of CCl_4 administration. After completing the hepatotoxic administration, part of the animals were treated with adenosine for 5 or 10 weeks while other group was treated with saline [112]. The cirrhotic state was characterized by increased liver fibronectine and collagen type I and III, enhance expression of alfa-1 collagen mRNA, portal hypertension and liver dysfunction. Several weeks after suspension of the toxic, the collagen showed an enhanced type I/III ratio, associated with a deficient collagenolytic activity in cirrhotic livers. Liver expression of some metallo-proteinases (MMPs) and of tissue inhibitors of MMPs (TIMPs) also indicated a reduction of collagen breakdown in cirrhotic livers. In the same way, parameters indicatives of oxidative stress, mainly protein oxidation, were persistently augmented. All these events were coincident with diminished regenerative capacity of the cirrhotic liver. In contrast, cirrhotic animals treated with adenosine showed blocked fibrogenesis, increased collagen degradation associated to reduction of liver TIMPs levels, and normalizing collagen-type ratios. In addition, the nucleoside promoted and effective hepatocyte's proliferation in the cirrhotic liver and accelerated normalization of parameters indicative of liver function and oxidative stress showing that adenosine could be considered as a potential therapeutic agent in the treatment of chronic hepatic disease as fibrosis/cirrhosis.

Adenosine and its Derivates

There are important limitations in the transference of the findings in experimental models to a clinical development. Then it was very important to understand the effect of the nucleoside adenosine in the cirrhogenic process. Adenosine is a purine nucleoside present

within and outside cells that acts as chemical messenger with autocrine, paracrine and endocrine actions. It is mainly formed by the novo purine synthesis, phosphohydrolysis of adenine nucleotides or by hydrolysis of S-adenosyl homocysteine. Extracellular adenosine can exert its function through activation of adenosine receptors (A1, A2a, A2b, A3) or can be transported into the cells by the nucleoside transporter. Within the cell, adenosine is phosphorylated by adenosine kinase, is deaminated to inosine by adenosine deaminase or is converted to S-adenosyl-homocysteine by S-adenosyl-homocysteine hydrolase [113]. Its metabolism is very active resulting in a short half live of the nucleoside. The physiological actions of adenosine are multiple, in the central nervous tissue [114] the cardiovascular system [115], modulating the immune response [116] and as a metabolic modulator [113].

Among the last actions is the contribution of the maintenance of energetic homeostasis in the liver, blood and brain [114]. Several derivatives of adenosine were tested, in order to find a possible mechanism of action. The aspartate of adenosine (IFC305) showed interesting results that are presented in Figure 1. **A.** Protective effect of adenosine and IFC305 versus a lethal dose of CCl_4 (2.5 ml/kg of body weight of 1:4 dilution of CCl_4 in vegetable oil, administered intraperitoneally). The hepatotoxin induced 90% death of the tested animals 1 to 2 days after its intragastric administration. Concomitantly administration of adenosine (200 mg/kg if b.w.) with the CCl_4 provided a 69% of survival even 3 days after intoxication. Using a fourth of the dose of IFC305 its protective effect was close to 80% showing a better protection against a lethal dose of the hepatotoxic than the parental compound. No further mortality was observed in rats treated with the nucleoside o IFC305 after 3 days of CCl_4 poisoning. **B.** Time course of liver clearance of adenosine or IFC305. Intact rats were intraperitoneally injected with 50 mg/kg containing 5 µCi of [^{14}C]-adenosine or aspartate salt of [^{14}C]-adenosine with a specific activity of 23.5 µCi/mmol. Hepatic levels of labeled compounds were determined in perchloric acid extracts by HPLC. The results show that the administration of the same concentration with similar specific activity of radio labeled adenosine alone or as aspartate salt, resulted in different permanence in the liver. Adenosine reached a maximal peak of absorption at 20 min while IFC305 at 30 min. Adenosine level rapidly declined to practically undetectable levels between 60-120 min, while IFC305 was still increased between 20-40 min, presenting a significant liver concentration even 120 min after its administration showing that the liver clearance of the IFC305 is much slower than that of adenosine, this effect of the adenosine derivative could be explained by a 20% diminution of the activity of adenosine deaminase enzyme encharged to degradate adenosine. **C.** Effect of adenosine in comparison with IFC305 in collagen content and collagenase activity in cirrhotic rats. The CCl_4–induced cirrhosis has been described in detail, as well as the analytical methods for collagen content and collagenase activity [95]. The 5 fold increase in liver collagen at T0 (suspension of the CCl_4) was maintained 4 to 8 weeks and liver collagen breakdown was gradually decreased.

In animals receiving adenosine (200 mg/kg three times a week) nearly 50% diminution in collagen content; when IFC305 (50 mg/kg three times a week) was administered instead of adenosine, the beneficial effects was slightly more potent. Both compounds increase liver collagenase activity in the same magnitude. Demonstrating that IFC305 is capable to reverse preestablished micronodular cirrhosis in rats stimulating the collagenolytic activity. **D.** Effect of adenosine in comparison with IFC305 on the liver proliferative capacity in cirrhotic rats. The experimental model and the analytical methods were previously described [112]. Cirrhotic rats showed an increase in DNA synthesis (assessed by thymidine kinase activity, in

controls 0.22± 0.03 nmoles TMP formed/h/ mg of cytosolic protein)) but this effect disappeared 8 weeks later. Administration of adenosine to cirrhotic rats for 8 weeks leads to an important increase of DNA synthesis. On the other hand administration of IFC305 (lower dose for 8 weeks also elicited DNA synthesis but in a lower magnitude. **E.** Effect of adenosine and IFC305 in liver function in cirrhotic rats. Liver function was drastically disturbed, some parameters indicative is the cell necrosis that lead to an increase in transaminases (ALT, AST), after 8 weeks after cessation of CCl_4 and accompanied by a decrease in serum albumine and an increase in bilirrubin. Treatment with both adenosine and IFC305 largely ameliorate liver function as reflected for the normalization of the measured parameters. Control values: ALT activity= 28 ± 3 IU/L, AST =165 ± 21 IU/L; albumin=4.1 0.4 mg/dL, and bilirrubin (Total) 0.9 ± 0.1 mg/dL. We can conclude from these studies that the beneficial actions of adenosine on experimental cirrhosis can be potentiated by using an aspartic-salt derivative which has a longer half life in the liver and its beneficial effects on CCl_4-induced damage [117].In this work we demonstrated that the treatment with IFC305 at a 50 mg/kg dose on CCl_4-induced liver cirrhosis in rats reversed hepatic fibrosis and modified global gene expression profile in cirrhotic livers using 5K DNA microarray analysis. Hierarchical clustering of 413 differential genes (Selected by Z-score value), 125 were not substantially affected by IFC305, and 289 were more affected by the treatment some up-regulated and other down-regulate but after the IFC305 treatment the expression tended to be normalized. Some specific genes were analyzed by quantitative RT-PCR, the fibrogenic genes, such as transforming growth factor β (*Tgfb*), collagen type 1 (*Col1a*), fibronectin 1 (*Fn1*) increased its expression in cirrhotic groups and IFC305 treatment diminish its expression supporting the antifibrogenic action of the compound. Moreover, the reduced transcript level of *Pparg* **i**n cirrhotic liver is being re-established by the compound indicating a probable prevention of HSCs activation and/or a reversion of activated HSCs to a quiescent state**.** It also modified the urea cycle rate limiting enzymes argininosuccinate synthase (ass1) and carbamoyl-phosphate synthetase 1 (*Cps1*), which suggest that IFC305 could have an additional possible effect of aspartate favoring the urea cycle in the liver [117]. Knowing the pivotal role that hepatic stellate cell (HSC) plays in liver fibrogenesis leading to cirrhosis, mainly through their activation from a quiescent adipogenic state to a proliferative myofibrogenic conditions. The effect of IFC305 was tested in primary cultured rat HSC result in a suppression of its activation maintaining a quiescent cell morphology including lipid droplets content also induce a decrease of fibrogenic genes expression and up-regulate three antifibrogenic genes (*MMP-13, Smad 7, and PPARγ*). Furthermore, IFC305 was able to repress the platelet-derived growth factor (PDGF)-induced proliferation of HSC. This inhibition was independent of adenosine receptors and more related with adenosine metabolism and pyrimidine starvation. [118].

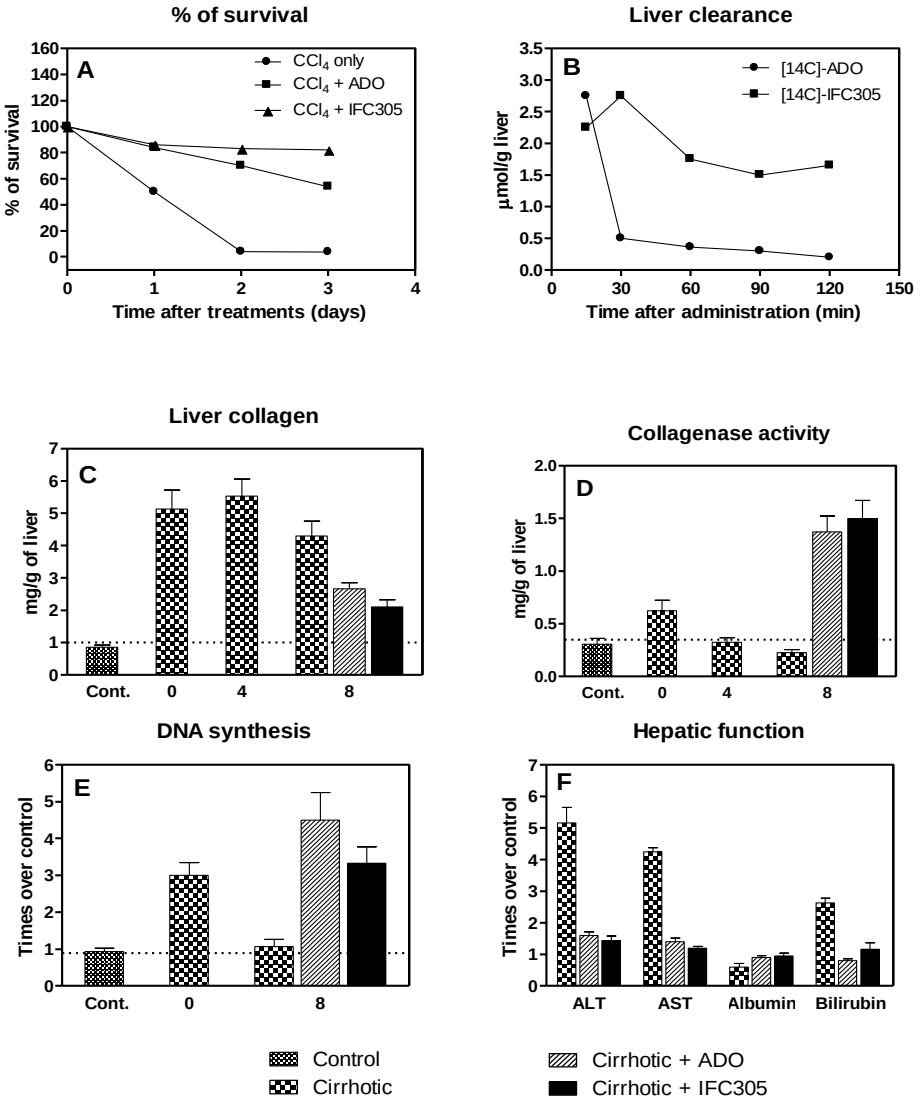

Figure 1. Comparative effects of adenosine and IFC305: A) Effects on CCl_4- induced mortality in rats. Intact Wistar rats were treated with 2.5 ml/kg of CCl_4 of body weight by intragastric administration (CCl_4- only). Another group or rats were concomitantly administrated with adenosine (200mg/ kg of b.w) or IFC305 (50 mg/ kg of b.w) and CCl_4 (CCl_4 + ADO or CCl_4 + IFC305 respectively). B) Time course of liver clearance. Intact rats were intraperitoneally injected with 50 mg/ kg containing □.□Ci of [^{14}C]-adenosine or aspartate salt of [^{14}C]-adenosine with a specific activity of 23.5 µCi /mmol. Liver levels of labeled compounds were determined in perchloric acid extracts by HPLC. C) and D) Effects of chronic administration of adenosine or IFC305 on liver collagen content and collagenase activity in cirrhotic rats. Wistar rats were rendered cirrhotic by chronic treatment with CCl_4. Animals were intraperitoneally injected (0.4 g/kg) three times a week during 8 weeks. After suspension of CCl_4 (0), a group was intraperitoneally treated with Adenosine (200 mg/ kg of b.w) (CCl_4 + ADO) or IFC305 (50mg/ kg of b.w) (CCl_4 + IFC305) three times weekly, during 4 or 8 weeks. Collagen content or collagenase activity in the liver was measured as described elsewhere (61). E) Effects on the liver proliferative capacity of cirrhotic rats. DNA synthesis was assessed by activity of thymidine kinase of liver rats treated as in C-D. F) Hepatic function. Parameters indicative of hepatic function of rats treated as in C and D were assessed by diagnostic kits from SIGMA Chem. Co (St. Louis, MO).

Antifibrotic Therapy

Nevertheless, the scientific advances providing novel insights into the molecular mechanisms participating in hepatic fibrosis have not resulted in an effective antifibrotic therapy yet. Recent reviews on these subjects [119] comment about several potential therapeutic targets but unfortunately clinical development has been disappointed. The information described in this chapter provides us with scientific evidence in vivo and in vitro, to support the potential use of adenosine derivative IFC305 preventing fibrosis and cirrhosis development. Although the experimental model of CCl_4-induced cirrhosis has been considered an adequate model of human cirrhosis [120] we are aware of the differences in time of development of cirrhosis and also the liver size of the experimental animal versus the human liver. The preclinical studies: toxicity, genotoxicity, teratogenicity and carcinogenicity results were satisfactory leading us to test the compound in small group of patients with cirrhosis from hepatitis C, resulting in an improvement of hepatic functions, and life quality with not adverse consequences at the dose and via of administration used. Other benefit of this compound is its physiological degradation without compromised the detoxification machinery of the liver that is affected in cirrhosis. These findings lead us to initiate the clinical trial which is in progress.

What do we expect from this treatment?

1) Prevention of cirrhosis in patients with viral hepatitis that potentially can develop the disease.
2) When the cirrhosis is established, prevention of its progression.
3) Slow but constant recuperation of the hepatic functions.
4) Diminution of clinical implications of cirrhosis such as bleeding, encephalopathy, portal hypertension and ascitis.
5) Slow but progressive restructure of the architecture of the hepatic tissue.

Conclusion

The high metabolic activity of the liver for its own and other tissues functions, require high energy demand, which is satisfied by their numerous mitochondria contained, through the oxidative phosphorylation process, as the major generator of cell ATP. The information provided in this chapter based in our own and other researchers' works shows evidence of the important role of bioenergetics in a degenerative disease such as cirrhosis. The diverse etiology of this pathology like alcoholism, chronic viral infections mainly of type B and C, metabolic diseases generate an energetic unbalance reflected in a decrease in energy parameters like ATP and energy charge favoring catabolic process, induction of fatty liver and necrosis. More over decrease the ATP-dependent functions like protein synthesis, albumin, coagulation factors, signalization pathways among others; increase oxidative stress, favoring deregulation of proteins of the extracellular matrix, present chromosomal instability altering gene expression, modify the calcium dynamic, favors the differentiation process of the hepatic stellate cells to a myofibrogenic phenotype, and lost of regenerating capacity. All these conditions modify the metabolic fluxes and cycles like the urea cycle. The molecular

mechanisms participating in the reversion total or partially of these events mainly the energy status of the cell by treatment with adenosine or its derivative IFC305 are in study in our laboratory but at the moment it is a promise to obtain a successful antifibrotic therapy.

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Chapter 3

End Stage Chronic Liver Disease – Yesterday, Today and Tomorrow

N. C. Nayak and Deepali Jain
Department of Pathology
Sir Ganga Ram Hospital, New Delhi

Abstract

Cirrhosis and end stage chronic liver disease (CLD), common in adults globally, are largely caused by environmental agents and factors and are prone to develop serious complications including hepatocellular carcinoma (HCC). Till the mid 1960s dietary protein deficiency was considered the cause of cirrhosis in chronic alcoholics and in the malnourished population of developing countries. Soon it was established that alcohol is directly hepatotoxic and that protein-calorie malnutrition does not cause cirrhosis. The major break through on cirrhosis came with the discovery of hepatitis B virus (HBV), and by mid 1970s it was clear that chronic HBV infection caused most CLD and HCC in developing countries and a smaller proportion in developed western countries. From 1990 on, a large segment of CLD not attributable to alcohol abuse or HBV infection was recognized as due to HCV infection. Wide use of blood transfusion and increasing intravenous drug abuse in the post-world war II period led to rapid increase in HCV related CLD and HCC. By early 21st century HBV related diseases decreased significantly in countries that adopted universal control measures for this virus infection including vaccination. In developed countries there was some reduction in HCV related CLD also but in other parts of the world it rose progressively while HBV related CLD showed small or no decline. In recent years alcohol consumption and alcohol related CLD have rapidly increased in developing countries. Intrahepatic biliary tract diseases and autoimmune liver disease have all through constituted a small proportion of CLD. On the other hand, a sizeable number of end stage CLD currently evolve from nonalcoholic fatty liver disease (NAFLD), most of which clinically present as cryptogenic. Also, cases of noncirrhotic portal fibrosis (NCPF), a generally benign liver disease known by several other names, have presented as end stage cryptogenic cirrhosis needing liver transplantation.

Etiologic types of end stage CLD are changing over time. HBV and HCV related CLD are preventable and even curable if detected early. HBV related CLD, once highly prevalent is steadily declining and is expected to become minimal by mid twenty first century. HCV related ones are likely to come down in time though more slowly, while alcoholic CLD is probably going to persist or even increase during the next few decades. End stage CLD is rare in children, caused mainly by genetic or developmental anomalies. Unique forms of non-Wilsonian copper overload CLD are rare and ill understood.

Introduction

Prolonged ill health due to diseased liver, often terminating in fatal complications has been known in different ancient medicines for several centuries, but the first documentation in modern medical writing of this chronic disease and the appearance of liver at autopsy in such patients was made during the late 18th and early 19th century by English (John Brown-1642 to 1700; Matthew Baillie-1761 to 1823), French (Theophile Laennec-1781 to 1826) and Italian (Geovanni Morgagni-1682 to 1771) physicians [1]. Laennec is credited to have christened the disease 'Cirrhose' derived from the Greek word 'Kirrhos' meaning tawny (tan or yellow-brown) because of the changed colour of the liver he saw at autopsy and recorded it in his famous treatise of 1819 on invention of the stethoscope [2]. Following this, for some time the disease was referred to as Laennec syndrome or Morgagni-Laennec syndrome but very soon the name **Cirrhosis,** English equivalent of Laennec's term was universally accepted and used. The livers seen at autopsy in cases dying of cirrhosis were not only of a yellow-brown rather than the normal dark brown hue but were also shrunken and diffusely nodular. In due course however, several cases of cirrhosis were seen to have nodular livers without yellowish discoloration and the diagnosis of the disease cirrhosis came to be applied only to those with livers that were diffusely nodular as well as diffusely fibrotic but not necessarily yellow-brown [3]. Even though this definition of the disease on the basis of morphologic appearance is widely divergent from the original meaning of the term Cirrhosis, the name has continued to stay simply because an alternate justifiable one has not been forthcoming.

As for other diseases, health professionals have all along persisted in seeking the cause/s of cirrhosis so that measures for curing, arresting early and even preventing this potentially fatal chronic liver disease are identified and instituted. Interestingly, both Laennec and before him Matthew Baillie had associated the disease with excessive alcohol consumption. At that time and later on alcoholics with cirrhosis were consistently in poor general health with significant under nutrition, findings which led to the concept that cirrhosis of alcohol abusers is caused by nutritional deficiencies. When European countries and Britain colonized different parts of southern and eastern Asia, Africa and South America, cirrhosis was reported by their physicians in malnourished non-alcohol addicted populations of those areas. This combined with some experimental studies in which cirrhosis could be induced in rats and mice raised on protein deficient diets helped substantiate the nutritional etiology of all liver cirrhosis in humans. This view was prevalent for several decades from late 19th till a little after the mid 20th century [4-6].The phenomenal advances in science and technology during the last 4 decades of the last century however brought in unprecedented new information on several human diseases including cirrhosis and cirrhosis-like chronic liver diseases. Protein calorie malnutrition was shown to cause fatty liver but not cirrhosis, alcohol was confirmed to

be directly hepatotoxic and cirrhogenic, while several other common causes of cirrhosis were identified. While earlier alcohol abuse was the only known causal association of cirrhosis and in the vast majority of patients the cause was not apparent, by the end of the last century the cryptogenic (of hidden or unknown cause) segment constituted only about 15% of cirrhosis cases.

Over time, improved health care and education, socio-economic development and availability of effective drugs and vaccines in both developed and developing countries helped reduce the incidence and prevalence of several etiologic types of cirrhosis. On the other hand, certain health care practices, and life style and ecological changes resulted in emergence of new etiologic types of cirrhosis as well as increase of some older ones. Thus, prevalence patterns of different etiologic types of cirrhosis continued to exhibit temporal changes varying from one geographic area to another. Till the mid 1960s not only the etiology of cirrhosis but also the criteria of its morphologic identification were unclear and controversial. The discovery of hepatitis B virus (HBV) was soon followed by reliable reports that established this agent as globally the most important and common cause of chronic liver disease and cirrhosis. At this opportune time a World Health Organization expert group, for the first time suggested definitive morphologic criteria for identifying cirrhosis and a classification of cirrhosis on etiologic basis [3]. As these concepts became globally accepted and applied, more well defined data on prevalence of cirrhosis in different geographic regions and their etiologic bases became available. Also, certain protracted liver diseases presenting with some or several clinical features of cirrhosis and having variable grades of hepatic fibrosis could now be differentiated from cirrhosis on adequate morphologic assessment. It was clear that in these latter diseases the natural history is far more benign than in cirrhosis having almost no fatal complications. On morphologic criteria, cirrhosis results from repeated or continuing parenchymal injury leading to destruction of the function-related structural architecture throughout the liver and is thus of irreversible nature. Given time it will end up being totally unresponsive to any treatment and strong susceptibility to fatal complications including hepatocellular carcinoma. Cirrhosis, irrespective of its cause, has therefore been considered the only such end stage chronic liver disease for which the possible answer is organ replacement by transplantation. However, as liver transplantation became widely used around the world it was seen that some explant livers from patients transplanted for end stage cirrhosis did not fulfill the morphologic criteria for cirrhosis and belonged to some or other category of non-cirrhotic chronic liver disease.

This chapter will deal with the currently accepted causes of all end stage chronic liver disease (CLD),mostly cirrhosis but also noncrrhotic diseases diagnosed as cirrhosis, the evolution of our knowledge on identifying these causes, and the temporal changes in incidence and prevalence of the various etiologic types of end stage CLD.

Etiologic Types of Cirrhosis and Other End Stage CLD

Cirrhosis by definition is a morphologic alteration of the liver characterized by complete destruction of its normal architecture resulting from formation of nodular areas of hepatic parenchyma surrounded and separated by fibrous septa, both these involving the entire organ.

Thus, livers having widely distributed fibrous septa but without parenchymal nodules through out, livers with diffuse nodularity of the parenchyma but no accompanying fibrosis in all parts, and livers with parenchymal nodules separated by fibrous tissue limited to one or a few regions but not involving the entire organ, do not qualify as cirrhosis. True cirrhosis is an irreversible change since the altered hepatic architecture, unlike in non-cirrhotic livers does not reverse after complete and long cessation of the injuring agent/s' action. Therefore, in established cirrhosis the resultant altered functions may remain compensated for variable lengths of time but in due course they decompensate and end stage complications occur which become fatal. The worst of these complications is development of hepatocellular carcinoma (HCC), one of the most fatal cancers in humans. The beginning of all cirrhosis, as expected, is from injury to liver cells by an agent that either continually operates or acts at frequent intervals before the previous damaged and dead liver cells are replaced by regeneration. Over extended periods of time the slow but continuing parenchymal loss, incomplete regeneration, and repair by fibrosis lead to the morphologic change of cirrhosis. Intrahepatic vascular alterations in the fibrous septa and compression of efferent venous channels by parenchymal nodules cause portal hypertension and both intra- and extra-hepatic portal-systemic venous shunts, both of which are responsible for most of the complications of cirrhosis. High susceptibility to development of HCC is ascribed to accumulation of mutations during repeated injury of hepatocytes and in several cases to additional carcinogenic effect of some agents causing cirrhosis.

Being a long drawn process to develop, cirrhosis is essentially a disease of adults and older individuals. A much smaller incidence is seen in early and mid childhood while the condition is rare in adolescence and early adulthood between age 14 and 25 yrs. Cirrhosis seen in the pediatric age are almost exclusively those related to metabolic or developmental abnormalities. On the other hand the vast majority of cirrhosis and other end stage CLD occurring in adults are related to environmental agents and factors. These etiologic types of cirrhosis are therefore potentially preventable. Identifying the etiology of end stage CLD in patients and the prevalence of the various etiologic types in the community are important not only for proper management of individual patients but also for planning and instituting adequate health care facility for these diseases in the region. The etiologic associations of end stage CLD are listed below (Table 1) separately for the common and the rare ones in adults and for the relatively important ones in children.

In the subsequent sections only the more important and common causes of cirrhosis will be dealt with in detail regarding the evolution of knowledge on the particular etiologic association, ways of identifying the etiology in a case of cirrhosis / end stage CLD and the changing scenario of its prevalence with time. Those in which no etiology can be ascertained using all diagnostic modalities are referred to as Cryptogenic (unrevealed etiology).

Table1. Etiologic Association of Cirrhosis/End Stage CLD

Common	Rare
ADULTS	
Alcohol (Ethanol)	Metabolic Hemochromatosis Alpha-1-Antitrypsin deficiency
Hepatitis B virus	Noncirrhotic Portal Fibrosis (NCPF)*
Hepatitis C virus	Nutritional
Nonalcoholic Fatty Liver Disease (NAFLD)	Mixed Hepatitis B/C virus and Alcohol Hepatitis B/C virus and NAFLD
Intrahepatic Biliary Tract Disease Primary Biliary Cirrhosis (PBC) Primary Sclerosing Cholangitis (PSC) Drugs, Chemicals, Biological toxins Auto Immune Liver Disease (AILD)	Cryptogenic Parasitic, Cardiac **
CHILDREN	
Biliary Atresia	Glycogen storage diseases
Wilson's disease	Galactossemia NonWilsonian diseases with high hepatic copper***

*Known by several other names, this is a noncirrhotic chronic liver disease that has recently been recognized to be a cause of end stage disease clinically indistinguishable from cirrhosis.
**Almost all of these have marked fibrosis but are not true cirrhosis
*** Indian childhood cirrhosis and similar diseases.

1. Alcohol Related Cirrhosis/End Stage CLD

1.1. Evolution of Knowledge

Alcohol in one or other form of fermented beverage is being used by people of various societies and civilizations around the globe from as long back as the Neolithic period dating to around 10,000 BC [7]. Association of alcohol abuse with cirrhosis, however, has been recorded much more recently, starting in the late 18th century. Since these alcoholics with liver cirrhosis were consistently ill nourished and emaciated it was believed that the liver disease in them was caused not by drinking too much alcohol but by continued lack of proper nutritional intake. During this period British and other European physicians practicing modern medicine in the respective countries' colonies in Africa, Asia and the Americas repeatedly observed cirrhosis in the malnourished populations, several of whom were not alcohol abusers. The view that almost all liver cirrhosis, including those in chronic alcoholics is causally related to nutritional deficiency got further support from the following: 1) Small

laboratory animals raised on diets poor or deficient in proteins like methionine and cystine developed cirrhosis; 2) Laboratory animals given water with alcohol to drink but normal diet failed to show cirrhosis; 3) In under developed regions of the world with repeated crop failures and continuing shortage of food, protein-calorie deficiency diseases (Kwashiorkor or Marasmus) was endemic among young children and cirrhosis frequent in adults [5]. The concept of nutritional etiology for cirrhosis thus got a strong hold which continued till the mid 1960s and a confusing, multi-axial nomenclature was used to describe three pathologic types of cirrhosis – Nutritional, Posthepatitic and Postnecrotic, the cirrhosis in alcoholics belonging to the first category [4]. This tide turned when results of more detailed epidemiological and clinical studies on humans and proper experimental studies on rodents as well as nonhuman primates were evaluated. It was shown in humans as well as in laboratory animals that alcohol is directly injurious to the liver cell irrespective of the nutritional status of the host [8]. Hepatic damage starts with steatosis, leading to steatohepatitis, progressive fibrosis and finally, if unchecked, to cirrhosis. Up to the stage of established cirrhosis the changes reverse if alcohol is stopped. In contrast protein-calorie malnutrition in animals and humans induces only steatosis which can be severe and extensive, but does not lead to significant fibrosis and never to cirrhosis. In fact, preformed collagen in the liver gets resolved in protein deficiency states. Also, children with severe protein-calorie malnutrition when followed never develop cirrhosis [5, 9, 10].

Considerably robust data now exist to indicate that alcohol is a major cause of a range of liver disease world wide. The important determinants for development of these diseases are dose, duration and type of alcohol consumed, drinking patterns and some genetic predisposition [11]. Fatty liver develops in 90% of individuals drinking about 60g/day of alcohol [12] or even less [8] despite adequate diet and good nutrition. Continued alcohol consumption of >40g/day increases the risk of progression to cirrhosis to 34% and fibrosis or cirrhosis to 37% [13]. Morphologically, the cirrhosis caused by alcohol is characteristically of the ‘Micronodular’ type [3] but can occasionally be of the ‘mixed Micro-Macronodular’ type that is generally seen in cirrhosis caused by hepatitis B and C virus [3]. The amount of alcohol ingested has been shown to be the most important risk factor for development of alcoholic liver disease. The chances of developing cirrhosis increase with ingestion of 60-80 g/day of alcohol for ≥ 10 years in men and > 20g/day in women, while the odds of developing cirrhosis with a daily alcohol intake of >30g/day is 13.7 compared with non-drinkers [14, 15]. However, despite consuming alcohol at high levels, only a fraction of 6-41% of these individuals develop cirrhosis [15, 16]. This may well be due to a genetic predisposition to both alcoholism and alcoholic liver disease [17], though the specific genetic abnormalities for these have not as yet been identified [11].

The type of alcoholic drink used appears to be important for onset and progression of alcoholic liver disease, beer and spirits being much more damaging to the liver than wine [18]. Another risk factor that has been shown to be related to occurrence of cirrhosis in alcoholics is the pattern of drinking. Consuming alcohol outside of meal times increases the risk of developing alcoholic liver diseases 2.7 fold compared with drinking alcohol only at meal times [19]. Further evidence of an indirect but strong support of the etiologic role of alcohol abuse in liver cirrhosis is from reports showing that for an increase in per capita alcohol consumption in a population group there is a significantly higher increase in cirrhosis both in men and women [20].

1.2. Identification of Alcohol as Etiology

Unlike for chronic hepatitis virus infections which are also important causes of cirrhosis there are no serological tests that help in unequivocally identifying alcohol as the etiology of this disease. It has been suggested that a serum AST/ALT ratio of more than 2 is strongly indicative of advanced alcoholic liver disease, particularly cirrhosis [21, 22]. This however is only suggestive but not diagnostic of cirrhosis caused by alcohol abuse. Some emphasis has been given in the past to histological features in liver biopsy that are considered characteristic of alcohol induced injury [23-25] but more recently it has been shown that these same features are also present in nonalcoholic steatohepatitis (NASH)/nonalcoholic fatty liver disease (NAFLD) [26-28]. Also, in advanced stages of alcoholic cirrhosis including the end stage disease, the important histological hall marks of injury to the liver by alcohol namely, fatty change and Mallory hyaline are inconspicuous and focal or even absent (Nayak NC, unpublished data). Pathological study of liver biopsy, explant and autopsy liver from patients with end stage CLD therefore may fail to provide definitive evidence for incriminating alcohol in the etiology. Thus, the most dependable evidence of labeling a case of cirrhosis/end stage CLD as etiologically related to alcohol is to obtain a reliable history of alcohol abuse over a long period from the patient and persons close to him/her [29] in the absence of other identifiable causes. It is however, common for alcoholics to deny alcohol abuse and under report intake of alcoholic drinks [30] and the physician must carefully collect this information with adequate support from other laboratory data [31].

1.3. Prevalence and Temporal Changes

As indicated earlier, even though occurrence of cirrhosis and its fatal outcome in chronic alcoholics had been recognized since late 18th century, the cause of liver damage in these patients was considered to be nutritional deficiency and not alcohol itself till a decade after the mid 20th century [4].When it became established that alcohol directly damages the liver causing increasing steatosis, progressive fibrosis and finally cirrhosis it came to be realized that this agent is a major cause of cirrhosis worldwide [11]. Although its prevalence among all etiologic types of cirrhosis has not been determined, global mortality data on liver cirrhosis indicate that in general, countries with high per capita consumption of alcohol have much higher prevalence of cirrhosis than those with low alcohol consumption. These data in the absence of more objective information on frequencies of precisely identified alcohol related cirrhosis would indicate that alcohol abuse causes liver cirrhosis. Thus, alcohol related cirrhosis and end stage CLD are frequent in several countries of Europe and the Americas where as many as 5-20 % of the general adult population have been estimated to consume large amounts of alcohol [11, 15, 32-34]. In contrast, even in regions of the world where social drinking of small amounts of alcohol at meal times has been in practice since long but heavy drinkers are few, relatively less alcohol related cirrhosis was encountered [35, 36]. In India and some other countries of South Asia where alcohol drinking used to be uncommon, liver diseases caused by alcohol was very rarely encountered till the last 2 or 3 decades of the 20th century (Nayak NC, Unpublished data).

Table2. Temporal changes in estimated prevalence of major etiologic types of cirrhosis/end stage CLD in different global regions from mid 20th to mid 21st century

Global Region	Etiological Type	Approximate frequency (%) at different time periods					
		Up to 1950#	1950-1970*	1970-1990**	1990-2010***	2010-2030	2030-2050
DEVELOPED COUNTRIES							
	Nutritional	80	-	-	-	-	-
	Alcohol related	-	70	60	50	60	60
	HBV related	-	-	30	10	3	1
	HCV related	-	-	-	30	20	4
	NAFLD related	-	-	-	5	15	35
	Cryptogenic	20	30	10	5	2	-
DEVELOPING COUNTRIES							
	Nutritional	90	-	-	-	-	-
	Alcohol related	-	5	10	15	28	35
	HBV related	-	-	70	40	20	5
	HCV related	-	-	-	30	30	23
	NAFLD related	-	-	-	10	20	35
	Cryptogenic	10	95	20	5	3	2

Cirrhosis in alcoholics and in malnourished populations considered to be due to nutritional deficiency
***** Alcohol and not malnutrition established as directly causing cirrhosis; ** HBV established as causing cirrhosis
******* HCV and NAFLD established as causing cirrhosis. NCPF shown to cause end stage CLD.

In most developed countries of the western world where drinking alcohol has been common since old civilizations, a gradual decline in consumption of spirits and of alcohol related deaths occurred from 1970 through 1990 [15, 34]. In more recent years however, these rates have not only stabilized but have steadily increased in these countries. On the other hand, in developing countries, particularly in those in which the economy has been growing fast like the two highly populous ones, China and India, that earlier had low incidence of alcoholic liver disease including cirrhosis, there has been a steep rise in consumption and abuse of alcoholic drinks, and correspondingly in liver cirrhosis [35, 36] (Nayak NC, Unpublished data). In a recent study designed to categorize the etiologic types of end stage CLD through morphologic evaluation of whole native explant livers from adults in our living donor liver transplant program, 86 of 372 (23.1%) were labeled as alcohol related or alcoholic cirrhosis (Nayak NC, Unpublished data). We and others have predicted that an increasing prevalence of alcohol related cirrhosis/end stage CLD is expected globally over the next several decades [33]. This increase is partly direct, due to progressive rise in alcohol abuse and partly indirect because of control and therapeutic success on hepatitis B and C virus infections during the same period of time. Unlike other common causes of cirrhosis known today, drinking alcohol has long been woven into the socio-cultural fabric of most human societies and is increasingly being globalized. It is unlikely that in the future times this practice will decline. Thus, alcohol related cirrhosis is here to stay, holds a major share in the realm of chronic liver disease and in due course is likely to become the most important cause of cirrhosis and end stage CLD globally (Table 2).

2. Hepatitis B Virus Related Cirrhosis/End Stage CLD

2.1. Evolution of Knowledge

Liver cirrhosis unassociated with alcoholism and alcohol abuse had been known since the early 1940s but that these livers resulted from episodes of inflammation and necrosis was soon afterwards recognized by pathologists who introduced the terms ‘Posthepatitic’ and ‘Postnecrotic’ cirrhosis respectively [4]. Where as two types of acute viral hepatitis, ‘Infectious hepatitis’(Type A) and ‘Serum/Syringe hepatitis’(Type B), were known by then, only some years after the world war II it became apparent that a proportion of the war veterans who had suffered from this acute hepatitis later developed chronic liver disease. Simultaneously, pathologists working in West and East Africa reported that in these regions post-viral hepatitis cirrhosis was common and interestingly these livers frequently harbored primary hepatocellular carcinoma (HCC). The precise nature of this virus inducing non-alcoholic type of posthepatitic/postnecrotic cirrhosis with strong predisposition to develop HCC, however had to await the discovery of ‘Australia antigen’ during mid 1960s that was very soon identified as the surface component of the agent of type B hepatitis and named Hepatitis B virus (HBV) [37].Within a few years of this epoch making discovery the entire structure of this virus, its molecular biology, immune reactions to acute and chronic infection and the nature of protective antibody were all worked out. By the later part of 1970s specific test systems to detect HBV infection markers in the serum and tissues of patients and general population became available for extensive clinical and epidemiological studies on chronic liver disease and HCC, and a safe, effective vaccine against diseases caused by this virus was developed. Evidence that chronic HBV infection induces chronic hepatitis followed by

cirrhosis and finally leading to HCC were as follows: 1) Widely applied serologic tests on population groups of different regions showed a direct relationship between rates of seropositivity for markers of chronic HBV infection and prevalence of nonalcoholic cirrhosis of the posthepatitic/postnecrotic types and of HCC [38]; 2) Patients of liver cirrhosis with and without HCC who were not alcoholic, tested positive for HBV infection markers.; In areas with high prevalence of posthepatitic cirrhosis and HCC as well as HBV carrier rates, individuals most prone to develop cirrhosis and HCC were those getting infected from theirs mothers at birth, in early infancy or in early childhood [37-39]; 3) HBV virus components could be demonstrated in cirrhotic liver cells and rarely in HCC cells by very specific immunostains and in tissue cultures [40-42]; 4) Chimpanzees, a subhuman primate susceptible to HBV infection develop chronic hepatitis, cirrhosis and HCC when chronically infected by the virus [43]; 5)Natural infection of certain animals and birds by species specific hepatitis virus, like Woodchuck hepatitis virus (WHV), Ground squirrel hepatitis virus (GSHV) and Duck hepatitis virus (DHV) induced CLD and HCC in the respective animal/bird [37, 44] and 6) Implementation of control measures including vaccination recommended against HBV infection [37, 45, 46] in some countries over last several years has resulted in considerable decline in both cirrhosis and HCC [47-51].

2.2. Identification of HBV as Etiology

The diagnosis of HBV related chronic liver disease is mainly based on detection of markers of this virus infection and of virus components in blood and of the virus components in liver tissue of patients along with absence of other known causal agents/factors. Past infection is assessed by presence of IgG class antibody where as ongoing infection is confirmed by demonstration of the surface antigen of virus-HBsAg, HBV DNA and HBeAg. The latter two are particularly indicative of active replication of virus and the titer of HBV DNA indicative of the degree of viral replication. In the liver, intracellular localization of HBV in hepatocytes shows an extremely random distribution [41]. Therefore, non detection of viral components in liver biopsy material which represents only a very small fraction (about 1/50,000 part) of the organ does not exclude intrahepatic presence of HBV. On the other hand the virus and virus components present in blood circulation represent the total pool of intrahepatic material secreted out from the liver into blood. History of blood or blood product transfusion, intravenous drug abuse, multiple injections etc. are important but not essential, because hepatitis C virus (HCV) and some other infectious agents causing liver injury are also transmitted through these routes. Patient's mother being HBV carrier with HBe Ag positivity will be evidence in favor of neonatal infection that strongly predisposes to development of cirrhosis.

Morphologic features of the liver are also not specific for HBV etiology. Grossly the cirrhosis is of the mixed macro-micro nodular type and microscopically, chronic inflammation is prominent and fatty change is very rare. These changes, as indicated earlier are also seen in the advanced cirrhotic stage of alcohol induced liver injury, as well as in HCV and NAFLD related cirrhosis [52].

2.3. Prevalence and Temporal Changes

Prevalence of HBV related cirrhosis/end stage CLD started to be documented only after mid 1970s when reliable serological tests for identifying HBV infection became available and widely used. It was soon established that this virus infection is globally present, though

variably endemic in different geographic regions. Several countries of East and South East Asia and of Africa are highly endemic while most developed Western countries have low endemicity and other countries of Asia, southern Europe and South America are of intermediate endemicity. Prevalence of HBV related cirrhosis parallel these infection rates. Transmission of HBV occurs through the hematogenous route on entry of infective material by transfusion of blood/ blood products, needle sticks, and abrasion of skin and mucous membranes. Non-immune adults may develop acute icteric or non-icteric hepatitis from which the great majority recover spontaneously, mounting an immune response. Only in 2-5% of infected adults the hepatitis becomes chronic and may progress to cirrhosis. On the other hand if infection occurs very early in life during birth to early childhood at which time the body's immune system is not fully mature and competent, an immune tolerance leads frequently to chronicity of hepatitis. These individuals become candidates for development of HBV related cirrhosis and later of HCC. In its natural history the time scales for this sequence of HBV infection through chronic hepatitis to cirrhosis and finally HCC has been averagely estimated at 10, 10 and 8 years respectively [53, 54]. In the post-world war II period, from early 1950s through mid 1990s blood and fluid transfusions, injectible therapies and procedures, and intravenous drug abuse were extensively practiced, which helped in significant increase in HBV infection and liver diseases resulting from it during this and subsequent periods. An assessment report indicated that at the end of the 20th century, world wide there were 2 billion people infected by HBV among whom 350 million were HBV carriers, and 0.5 to 1.2 million died per year from HBV related chronic hepatitis, cirrhosis and HCC, the count for the last named being 350,000 per year [55].

As the biologic response to HBV infection in neonates, infants and young children is distinctly different from that in adults, the magnitudes of infection load, carrier rate and chronic liver diseases caused by this virus in any population group and geographic region would depend largely on the extent of mother to infant virus transmission [53, 54]. Thus, most East Asian populations and populations of sub-Saharan Africa have high prevalence of HBV related cirrhosis and HCC as well as high HBeAg positive HBV carrier rates in women. Countries and population groups with moderate and low prevalence of Cirrhosis and HCC have corresponding HBV carrier rates in women. Improvement in general health care and sanitation practices, and implementation of control measures for virus transmission have resulted in gradual reduction of variable degrees in prevalence of HBV related cirrhosis and HCC in most areas by the end of the first decade of the present century [49, 51, 55, 56] (Nayak NC, Unpublished data). Control measures including universal vaccination of infants will effect primary prevention of HBV infection and resultant liver diseases and in due course will reduce the burden of HBV related cirrhosis and HCC. Clinically established chronic hepatitis prior to development of cirrhosis can also be reversed by therapeutic elimination of HBV. Several generations of effective antiviral drug and combination therapies are now available to reduce the HBV infection and disease load. This together with the control measures, particularly wider implementation of universal vaccination programs is expected to significantly reduce the global burden of HBV related cirrhosis and HCC three to four decades from now (Table 2).

3. Hepatitis C Virus Related Cirrhosis/End Stage CLD

3.1. Evolution of Knowledge

Success in accurate serological diagnosis of hepatitis A and B and mapping of the world wide prevalence of acute and chronic diseases caused by these two viruses revealed that several cases of clinically acute and chronic hepatitis did not fall into either of these two categories. They were referred to as NonA-NonB hepatitis for about one and half decades starting mid 1970s, mainly with two distinctly different types of clinical and epidemiological characteristics. One was enterically transmitted manifesting as acute sporadic and epidemic hepatitis [57, 58] resembling HAV disease and the other parenterally transmitted leading to chronic hepatitis and cirrhosis resembling HBV induced liver disease. Towards the end of the 1980s both the viral agents for these diseases were identified and named hepatitis E virus (HEV) and hepatitis C virus (HCV) respectively. HCV, a RNA retrovirus not only induces prolonged inflammation of the liver but like HBV induces chronic hepatitis, cirrhosis and HCC. There are however, several differences between HCV and HBV in their biological interaction with the host. HCV gains entry into the human body exclusive by the hematogenous route, largely through blood transfusion, to some extent through intravenous drug abuse but only very rarely through maternal-perinatal transmission (unlike HBV). Thus HCV infection that frequently leads to chronic liver disease starts mostly at much older age than HBV infection. The initial acute viral injury by HCV is generally so mild that it is almost always asymptomatic and unapparent, while close to 85 per cent of infected individuals develop chronic inflammation, which if not detected and treated in time will proceed to cirrhosis and a fair proportion of these to HCC in due course [59, 60].

With high quality immunological and molecular techniques available before the end of the last century, very soon after HCV was identified it was cloned, host immunity responses were deciphered and diagnostic tests for presence and severity of ongoing and past infection were established. Epidemiologic and clinical studies carried out in different regions of the world helped in working out the HCV infection and carrier rates, the magnitudes of HCV related chronic liver diseases including HCC and the status of CLD caused by this virus in combination with other etiologic agents – HBV, alcohol, metabolic syndrome. Availability of reliable tests to indentify HCV infected blood helped in controlling and eliminating transmission of virus to individuals needing blood and blood product transfusion. Attempts at making an effective and safe vaccine are underway and one or more are likely to be available in foreseeable future [61].

3.2. Identification of HCV as Etiology

As for HBV related cirrhosis/end stage CLD, confirmation of the role of HCV in CLD is essentially based on definitive demonstration of seropositivity for the virus infection markers on sensitive and specific immunological tests in the absence of evidence for other etiological agents/factors. These include anti-HCV antibody and HCV RNA, the latter being more specific as it represents active virus replication. Also, as in case of HBV DNA, the quantitative assay of HCV RNA units would indicate the degree of virus replication. History of blood transfusion or of intravenous drug abuse will serve as supplementary evidence since this is the most important route of transmission for HCV. Morphologic features of HCV related CLD are often characteristic and seem to serve as fair guide to suggest this etiology.

Histologically, lymphoid cell aggregates distributed randomly in the fibrous septa in cirrhosis and chronic hepatitis are seen in the liver in close to 85 per cent of cases. In a recent double blind review of a large series of explant livers from our patients of end stage CLD who underwent living donor liver transplantation, this morphologic feature was encountered in 85.6% of HCV seropositive cases as against in 13.2% of HCV seronegative ones (Nayak NC, Unpublished data). The other microscopic feature that is a fairly common accompaniment of lymphoid cell aggregates is focal macrovesicular fatty change of hepatocytes [62]. The cirrhosis is of the mixed macro-micro nodular type. These gross and microscopic features are suggestive but not diagnostic of HCV etiology. Detection of HCV and its components by specific immunohistochemical techniques has had inconsistent results and is therefore not used as an aid in pointing to HCV etiology of CLD.

3.3. Prevalence and Temporal Changes

Mapping of regional and global prevalence of HCV infection, carrier state and of HCV related chronic liver disease including cirrhosis and HCC have been carried out only starting in the last decade of the 21st century when specific serodiagnostic tests came into wide use. Since the early 1990s figures for these seem to have increased almost in logarithmic scales in some of the developed countries [36, 59, 63]. This is understandable from the steep post-world war II rise in health care practices of unrestricted use of blood transfusions and injectible medicine as well as intravenous drug abuse and increased international travel [64]. The lag period from first infection by HCV to development of fully established cirrhosis is about 25 years and to occurrence of HCC on the cirrhotic liver is an additional 10 to 12 years. The summated effects of the virus transmission factors and of the time span of pathologic changes induced by the virus from first infection as mentioned above will determine the incidence, point prevalence and temporal changes of HCV related cirrhosis/ end stage CLD and HCC. The degree and extent of control and preventive measures against HCV infection and HCV induced diseases will help in the spread and occurrence of these chronic diseases. Current statistics reveal that globally, a pool of 170 million persons, comprising approximating 3% of world population constitute the HCV infected carrier pool, 27% and 25% of cirrhosis and HCC respectively are etiologically related to HCV, while a little less than half of 446,000 deaths from cirrhosis and nearly a third of 483,000 deaths from HCC in the world are attributable HCV infection [59, 64, 65].

In most developed countries introduction of control measures for HCV infections for almost 15 years now has resulted in a significant drop in new infection by this virus but because of the long latent period of approximately 25 years from time of infection to occurrence of cirrhosis, individuals infected by HCV prior to 1990 are and will be for some more years, presenting with cirrhosis. This has resulted in the somewhat paradoxical situation of a fall in HCV infected cases but a significant rise in HCV related cirrhosis/end stage CLD [36, 60, 63]. On the other hand, in the same regions all types of HBV related diseases including CLD have declined considerably as preventive measures for HBV infection have been instituted since 1970s, two decades earlier than those for HCV infection. These temporal changes in relative proportions of HBV and HCV related cirrhosis/end stage CLD have been different in developing countries because control measures for these viral infections have not been adequately implemented. Thus, while HBV infection and related cirrhosis/end stage CLD have shown slight reduction, HCV related diseases have registered a moderately upward trend. In India, while during late 1970s HBV related cirrhosis constituted a little more than

70% of all posthepatitic/postnecrotic cirrhosis [40], three decades later this figure had dropped to about 35% of hepatitis virus associated end stage CLD, the remaining being related to HCV (Nayak NC, Unpublished data).

With existing control measures for both HBV and HCV infection continued in developed countries and increasingly more widely applied in developing countries and an effective HCV vaccine soon becoming available, CLD related to these two viruses are likely to gradually decline through the coming decades and possibly get nearly eliminated from the presently developed countries by A.D 2050 (Table 2).

4. NAFLD Related Cirrhosis/End Stage CLD

4.1. Evolution of Knowledge

In the early years of 1980s a chronic liver disease manifesting with morphologic changes of alcoholic hepatitis was described in confirmed nonalcoholics and named as Nonalcoholic steatohepatitis (NASH). It soon became apparent that the disease was far more common than presumed, was associated with type 2 diabetes, insulin resistance, metabolic syndrome with obesity/ increased body mass index (BMI) and hypothyroidism [66-69]. Pathologic changes in the liver as seen in aspiration or core biopsy material had a spectrum ranging from only steatosis through steatohepatitis to additional mild or moderate fibrosis [26-28, 52]. The preferred name for the disease became Nonalcoholic fatty liver disease (NAFLD), NASH being only one of the more important stages in its progression. Epidemiologic and clinical studies across the world during the past decade and half have revealed that NAFLD has emerged as a highly prevalent chronic hepatopathy in both developed and developing countries, being of epidemic proportions in the former and gaining rapid strides in the latter. This high incidence is due largely to life style changes coming with economic development, along with increased prevalence of obesity and diabetes. In the early reports on NAFLD it was considered a nonprogressive disease with steatosis and inflammation as the essential pathologic change in liver. But as more studies, some with long follow up, were under taken it became clear that simple steatosis without any significant inflammation was not uncommon in this disease [28, 67] and that both these morphologic alteration can be followed by progressive fibrosis and ultimately cirrhosis [70]. It was also seen that individuals without any of the usual risk factors known for NAFLD can harbor this disease and have pathologic changes in the liver identical to those in patients with risk factors. With liver transplantation becoming widely practiced for end stage CLD, several cases diagnosed as cryptogenic cirrhosis were finally, after examination of explant livers, labeled as NAFLD related cirrhosis [52, 68]. It has been estimated that 20 to 25 per cent cases of NAFLD progress to cirrhosis.

4.2. Identification of NAFLD as Etiology

NAFLD related cirrhosis/end stage CLD is suspected when patients labeled as cryptogenic cirrhosis after all common and other known causes have been carefully excluded by adequate history, clinical examination and laboratory investigations, have combinations of risk factors for NAFLD. In those cases where these factors are absent, as known now for a good proportion of NAFLD, additional evidences are needed. One such evidence is hepatic steatosis which if significant can be observed on ultrasound imaging. Morphologic feature in

liver biopsy has been generally accepted as gold standard for diagnosis of NAFLD. This consists of fatty change, inflammation, Mallory hyaline and fibrosis, a picture identical to the one in alcoholic liver disease. History of alcohol abuse is to be conclusively excluded before these changes are taken as indicative of NAFLD. But lately it has been shown that in the cirrhotic stage of NAFLD the hepatic changes seen in early and established phase of the disease, particularly steatosis are very inconspicuous or even absent [52, 71].Thus, where as liver biopsy is helpful in diagnosis of early and established phase of NAFLD, this is generally not true for the cirrhotic stage of disease. Also, it has been shown that in NAFLD one or even two biopsy specimens may fail to show the diagnostic features of the disease because of sampling error [72]. A small set of histologic features in liver that may point to a case of cryptogenic cirrhosis as NAFLD related has been suggested by us recently [52].

4. 3. Prevalence and Temporal Changes

The disease known today as NAFLD or NASH was not recognized and not recorded before 1980. But 20 years later its prevalence is accepted to be high world wide and in many developed western countries it is presently considered to be the commonest type of chronic liver disease. The disease however was, for some time, considered to be benign and indolent without any major serious outcome. More recently it has been appreciated that an as yet uncertain proportion of it progress to cirrhosis that is difficult to distinguish from other common etiologic types of cirrhosis on clinical grounds alone. In our own experience from cases of end stage CLD undergoing liver transplantation, NAFLD cirrhosis accounts for a little less than one fifth of all end stage CLD (62 of 372) and more than 10 % of these (7 of 62) have superimposed HCC (Nayak NC, Unpublished data). With the increasing incidence of NAFLD as well as of associated conditions like obesity, diabetes, insulin resistance etc. in both developed and developing countries and a good proportion of it terminating in cirrhosis, it can be assumed that in the coming decades, probably even beyond mid 21st century NAFLD related cirrhosis/end stage CLD will constitute a major component of liver cirrhosis across the world.

5. Multiple Etiology Related Cirrhosis/End Stage CLD

Since all major factors/agents causing cirrhosis/end stage CLD are closely associated with the human environment, it is only natural that some of these will operate in variable combinations. This is particularly true of etiologic agents/factors that are available or present over wide geographic areas. It is not very unusual to see cases of cirrhosis/end stage CLD that have had chronic exposure to different combinations of hepatitis viruses, alcohol abuse and risk factors for NAFLD. In the event of such combined assault the cirrhosis generally evolves faster and has a worse prognosis than when caused by a single etiologic agent/factor. Such multiple etiology related cirrhosis, which luckily are much less often encountered than single etiology related ones, include cases of HCV infection combined with alcohol abuse or NAFLD [11, 73, 74]. The reasons for the rarity of combined causes in cirrhosis are unknown.

6. Cryptogenic (of Unknown Etiology) Cirrhosis/End Stage CLD

At any given time the etiology of cirrhosis/end stage CLD can be identified only from the knowledge and awareness of different etiology and ways to recognize them that exist and are available at that time. Cases where a diagnosis of cirrhosis or end stage of CLD is made but in which a known cause or causes can not be identified are assigned to the category of 'cryptogenic', meaning that at that point in time the cause remains undetermined. Thus, with advancing knowledge on the various causes of chronic liver diseases and the possibility of the latter's progression to cirrhosis this segment of 'cryptogenic cirrhosis' has gradually shrunken over time (Table 2). In developed countries the proportion of this group has reduced from about 20% in mid 1990s to about 5% in current years [71]. Now that a proportion of the common condition of NAFLD is known to progress to cirrhosis, several of these cases earlier categorized as 'cryptogenic' are now identified as NAFLD related. Availability of whole explant native livers from cases transplanted for end stage CLD, has helped in assigning definitive categories of etiology to cases given a pre-transplant diagnosis of cryptogenic cirrhosis after detailed morphological examination and adequate clinicopathologic correlation [52, 71]. In such a series at our centre the final category of cryptogenic cirrhosis has reduced to a mere 2.7% only (Nayak NC, Unpublished data).

7. Noncirrhotic Portal Fibrosis (NCPF) Related End Stage CLD

An indolent form of chronic liver disease that used to be endemic in India and Japan but infrequent in developed western countries clinically mimics cirrhosis closely and presents with features of sustained portal hypertension. The first detailed account of this condition separating it from cirrhosis and its pathologic features characterized by absence of cirrhosis, variable but significant portal fibrosis and obliterative changes in different orders of intrahepatic portal vein branches were reported from India around early 1970s [75, 76]. These were very soon confirmed by Japanese investigators and the names Noncirrhotic portal fibrosis (NCPF) and Idiopathic portal hypertension (IPH) were given to the disease in India and Japan respectively, though a similar or same condition encountered elsewhere became known by other names as well [77]. Unlike cirrhosis, NCPF has been known to have generally a benign course with no fatal outcome if variceal bleeding is prevented by endoscopic or surgical procedures [78, 79]. Incidence of NCPF seems to be fast declining in endemic areas like India and Japan [79]. Progression of this disease to end stage CLD with development of complications that could only be relieved by organ replacement was not known until liver transplantation came into wide use. In recent years, some reports have described end stage CLD cases, mostly having a pre-transplant diagnosis of cryptogenic cirrhosis, in which native explant livers did not show cirrhosis and were categorized as NCPF type noncirrhotic liver disease [77, 80, 81]. From our liver transplant centre we have described a series of such cases [77] that had the characteristic morphologic features of NCPF initially reported by us [75].

These studies on transplanted patients have established that NCPF cases, if untreated, may progress to present as end stage chronic liver disease indistinguishable from any other etiologic type of true cirrhosis and often misdiagnosed as cryptogenic cirrhosis. In our recent

report a few criteria that may help in arriving at a pre-transplant diagnosis of NCPF in such cases have been suggested [77].

8. Nutrition and Cirrhosis/End Stage CLD

As has been emphasized earlier the term Nutritional cirrhosis is almost extinct now and rightly so. Nutritional deficiency, particularly protein-energy malnutrition does not lead to cirrhosis. On the other hand, over nutrition and grossly imbalanced nutrition (true malnutrition) will result in obesity and metabolic syndrome which as mentioned earlier will induce NAFLD and cirrhosis/end stage CLD. In the past gastro-intestinal by-pass surgery for morbid obesity was often followed by fatty liver disease identical to alcoholic liver injury. This was very likely post operative exacerbation of pre-existent NAFLD in the obese subjects. Protein-calorie deficiency and deficiency of other specific nutrients, however do have prognostic and therapeutic implications in patients with alcohol related cirrhosis and possibly other types of cirrhosis as well [11].

Cirrhosis in Children

This present chapter has been designed to deal with cirrhosis in adults because firstly, in clinical practice almost all cirrhosis occur and manifest in adulthood and secondly, the unfolding of all knowledge on etiology of cirrhosis that has had great impact on modern health care has been on the disease in adults. Cirrhosis in children is almost always related to some inherited metabolic abnormality (e.g. Wilson's disease and Storage diseases) or to acquired or inherited developmental defects manifesting from early infancy (e.g. Biliary atresia). It would however be a serious omission if at least a brief mention is not made of an unique, enigmatic and biologically perplexing type of cirrhosis affecting young children of a restricted geographic region and of a somewhat similar disease rarely encountered in children elsewhere. Initially described from India under the name of Indian childhood cirrhosis, this usually rapidly fatal disease commonly encountered in young children of the Indian subcontinent manifests with a morphologically characteristic type of liver injury and cirrhosis having marked hepatic copper overload [82]. The disease is distinctly different from and unrelated to Wilson's disease. In the first ever etiology based classification of liver cirrhosis recommended by a WHO expert group a special berth was assigned to Indian childhood cirrhosis because of its unique nature and striking morphologic features, even when its etiology was unknown [3]. Though the etiology and pathogenesis of the disease has remained unsolved some workers have suggested that the copper overload seen in the active stage of disease is secondary to liver injury of unknown nature in a child born with a transient genetic defect in copper homeostasis. Other investigators however believe that in these children the copper overload which is of dietary origin initiates and propagates a toxic liver injury. Rare instances of non-Wilsonian copper overload disease seen in children from other parts of the world have also been claimed by some workers there to be due to excessive copper intake resulting in hepatotoxicity. The controversy on the origin and role of copper overload in Indian childhood cirrhosis seem to have been settled recently in support of the nontoxic and

non dietary role of copper on the basis of data from a very large, well controlled multicentre study sponsored by the Indian council of medical research [83]. A similar conclusion has also been reached in a controlled epidemiological study on drinking water containing high levels of copper and incidence of liver disease in children in eastern USA [84]. For some as yet unexplained reason the prevalence of Indian childhood cirrhosis, once of such common occurrence in India seem to have sharply declined in recent years.

Conclusion

Cirrhosis, a globally common irreversible chronic liver disease (CLD) and few other noncirrhotic CLD do progress to end stages that terminate in fatal complications. Being caused mostly by environmental agents and factors that act over several years these diseases manifest mostly in adulthood. Cirrhosis associated with alcohol abuse, a commonly encountered condition was considered to be due to malnutrition till the mid 1960s when it was convincingly shown to result from direct toxic injury by alcohol. Following the discovery of Hepatitis B and C viruses in mid 1960s and late 1980s respectively, it became clear that the vast majority of cirrhosis/end stage CLD and associated hepatocellular carcinoma (HCC) around the world are caused by these two viruses. Later, during the last two decades it has been revealed that a good proportion of currently encountered cirrhosis result from a form of CLD named as nonalcoholic fatty liver disease (NAFLD), which is presently sweeping the world in epidemic form.

Thus, at the present time alcohol, HBV, HCV and NAFLD constitute the major causes of cirrhosis/end stage CLD. Interestingly, a relatively benign noncirrhotic CLD, known by several names including noncirrhotic portal fibrosis (NCPF) has recently been shown to progress and contribute to a small proportion of end stage CLD. As a result of these accumulated data, causes of the vast majority of cirrhosis/end stage CLD in adults are now known and the segment of so-called ‘cryptogenic’ CLD of unknown etiology has shrunken considerably to about 3 per cent of all CLD. With the information available and likely to be available in near future on preventive and therapeutic measures against HBV and HCV infections and on the global socio-cultural practices, some predictions can be made on the prevalence dynamics of different etiologic types of cirrhosis/end stage CLD over the next few decades. HBV and HCV related diseases will gradually decline, faster in developed countries than in developing ones while alcohol and NAFLD related diseases are likely to increase and comprise the major etiologic types of cirrhosis. In young children a unique form of cirrhosis/end stage CLD manifests with hepatic copper overload in active stage of disease. Of unknown etiology, this disease used to be endemic only in some geographic regions but is apparently fast declining.

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Chapter 4

Hepatocellular Carcinoma Screening, Diagnosis and Management in the Cirrhotic Patient: A Western Review and Recommendations

V. Sundaram[1], K. Chopra[1] and T.C. Gamblin[2,3*]
University of Pittsburgh Medical Center
[1]Division of Gastroenterology, Hepatology & Nutrition, Department of Medicine
[2]Division of Transplantation, Department of Surgery
[3]Division of Surgical Oncology, Department of Surgery

Epidemiology

Hepatocellular carcinoma (HCC) accounts for 85– 90% of all primary liver cancers [1] . It is the sixth most common cancer overall, and the third most common etiology of cancer-related deaths worldwide [2], accounting for nearly 600,000 new diagnoses annually and approximately 600,000 related deaths. There is wide geographic variation of prevalence of HCC, with Asia and Africa having 40 times more cases than other parts of the world [2-4]. However, the incidence of HCC has been rising throughout the world, with particularly large increases seen in industrialized nations such as Japan, the United States, and Denmark[5, 6]. In the United States, this rise in incidence, has led to increasing mortality due to HCC from 1.54 to 2.58 per 100,000 between 1980 and 1990 [7, 8].

Both incidence and mortality rates are higher in male subjects with a mean male to female ratio of 2–4:1. The possibility of a hormonal cause of HCC has been raised due to male predominance, but experimental data and the lack of efficacy of hormonal manipulation

* Corresponding Author:
T. Clark Gamblin, MD, MS UPMC Liver Cancer Center,
3459 Fifth Avenue, Pittsburgh, PA 15213, USA
gamblintc@upmc.edu, phone 412-692-2001, fax 412-692-2002

suggest that other factors are likely involved[8-10]. Ethnicity may also be a risk factor in HCC development. In the United States, substantial variation has been noted among different ethnic groups, with the highest rates among those of Asian, Hispanic, and African Americans [11, 12]. In one study utilizing the SEER registry, an increased occurrence of HCC has been documented among African-Americans compared to Caucasians, with prevalence of 25% for white males (6.7% females) and 40% for black males (13% females). Similarly, the incidence rate in the United States is lowest for whites (male 2.3 and female 1.1 per 100,000) (4).

Risk Factors

Cirrhosis

The largest single risk factor for the development of HCC is liver cirrhosis, which is present in 70–90% of HCC patients. Furthermore, the etiology of hepatic cirrhosis also affects the risk of developing HCC [13]. Involved in the pathogenesis of liver cirrhosis is chronic inflammation, which leads to a cycle cell necrosis and regeneration. Oxidative stress also occurs and leads to genomic damage alterations [14]. Additionally, liver fibrosis leads to the loss of cell-cell contacts and cell-matrix interactions, which further disrupts the normal process of hepatocyte regeneration.

HBV

Viral hepatitis accompanies up to 80% of all HCC, with hepatitis B virus (HBV) infection being responsible for two thirds of all viral hepatitis associated cases. Worldwide, cirrhosis as consequence of HBV infection is the major cause of death related to HCC [15]. Geographic variability exists regarding the epidemiology and natural history of HBV induced liver cancer. In developed countries most HBV infections are acquired in adolescence or adulthood through blood transfusion, invasive medical procedures or through sexual contacts. However, in high-risk regions such as Asia and Africa, vertical transmission of the infection through the birth canal is the dominant mode of acquiring the disease, causing the majority of cases to occur in infancy or childhood. Therefore, 60% of HCC in Africa and Asia is attributable to HBV infection, while in Europe and the United States, only 20% of cases result from HBV [4]. Though 70–90% of HBV-associated HCC develops in the setting of liver cirrhosis, HBV is still an important risk factor even in the absence of cirrhosis [1].

Several factors influence the progression to HCC in HBV-induced cirrhosis. For instance, more advanced stage and longer duration of cirrhosis lead to increased risk of developing HCC [16]. Activity of HBV is also a risk factor for HCC occurrence. Suppression of HBV replication or loss of HBsAg in the serum results in a low risk to develop HCC, while high HBV viral load or hepatitis B e antigen (HBeAG) are risk factors in HCC development [17-19]. Among the eight HBV genotypes (A–H) several studies have shown infection with HBV genotype C confers higher risk of developing HCC [20-22], and that HBV genotype C is associated with an increased risk to develop HCC independently of the serum HBV DNA

level [23]. A superinfection with hepatitis delta virus further increases the risk of malignancy in cirrhotic patients with HBV infection by as much as 3-fold [24, 25].

HCV

Infection with hepatitis C virus (HCV) leads to chronic inflammation that may result in fibrosis and ultimately liver cirrhosis. Additionally, viral infection may lead to carcinogenesis by the induction of chromosomal mutations and malignant transformation of hepatocytes, due to generation of reactive oxygen species [26]. Once cirrhosis has developed, the annual incidence of HCC ranges from 2–5% [27]. In patients with only fibrosis due to HCV, the incidence of HCC is significantly lower than in patients with cirrhosis, but increases with the stage of fibrosis [28, 29].

A prior meta-analysis by Donato et al., regarding HBV/HCV co-infection indicates a synerginistic effect of the two infections in development of HCC, with an OR of 22.5 (95% CI 19.5–26.0) for HBV infection, an OR of 17.3 (95% CI 13.9–21.6) for HCV positivity, and an OR of 165 (95% CI 81.2–374) for infection with both diseases [30].

Co-infection with HIV

Patients who are co-infected with HBV or HCV and human immunodeficiency virus (HIV) may have faster progression towards cirrhosis and higher risk of developing HCC at the time cirrhosis is reached [31]. The MORTAVIC study, which sought to determine mortality due to cirrhosis in a cohort of HIV-infected patients 5 years after the introduction of highly active antretroviral therapy (HAART) and to compare this with mortality before and during the early years of HAART, indicated that HCC was responsible for 25% of all liver deaths in the post-HAART era [32, 33]. There is no difference in screening criteria for patients co-infected with HIV compared to those with HBV or HCV alone.

Alcohol

In the Western world, alcohol consumption is one of the major risk factors for developing liver cirrhosis, and subsequently HCC. Alcohol consumption is typically associated with a 2-fold increased risk for HCC development [34], but can be as high as 5- to 7-fold in those who consume more than 80 gm/day for over 10 years [35]. The annual incidence of HCC in the setting of alcoholic cirrhosis is 1-2%/year [35, 36].

Although no direct carcinogenic effect of alcohol has been reported, a synergistic effect of alcohol intake and viral hepatitis has been demonstrated. Donato et al. reported a linear increase in HCC risk with a daily intake of 60 g alcohol/day, with this risk being even doubled in the presence of HCV infection [37]. Excess alcohol intake may also increase the risk associated with aflatoxin B1, a toxin which is also metabolized by CYP2E1, and an alcohol-related increase of the mutation rate of the p53 tumor-suppressor gene, induced by aflatoxin B1, has been demonstrated [31].

Obesity, Diabetes, and NASH

Obesity, defined as a body mass index (BMI) > 30 kg/m2, is associated with an increased risk of HCC, especially in men [38-40]. Regarding diabetes as a risk factor for HCC, the largest study evaluating this compared 2,061 HCC patients with 6,183 controls and showed a significantly greater prevalence of diabetes in the HCC group compared to the control group (43% vs. 19%) [41]. Diabetes was further associated with a 3-fold increased risk to develop HCC in multiple logistic regression analysis (OR 2.87, 95% CI 2.49–3.30) [41]. Several subsequent case-control studies confirmed the increased risk for HCC in diabetic patients with OR's ranging from 3.12 [42] to 3.7 [38]. Additionally, a meta-analysis of thirteen studies demonstrated a pooled OR of 2.5 (95% CI 1.8–3.5) [43]. Furthermore, presence of diabetes also increases the risk of developing HCC in patients with cirrhosis secondary to alcoholism or viral hepatitis [44, 45].

Non-alcoholic steatohepatitis (NASH), which ranges in disease severity from of non-alcoholic fatty liver disease (NAFLD) to steatohepatitis and finally cirrhosis, may be a risk factor towards HCC development, particularly in those who develop cirrhosis. A recent study by Malik et al demonstrated the prevalence of HCC in U.S. patients with NASH cirrhosis to be 17%, similar to that of patients with alcohol and HCV induced cirrhosis [46].

Hemochromatosis, Iron Overload and HCC

Hepatic iron overload, either as a consequence of hereditary hemochromatosis or from secondary causes, may lead to liver damage and cirrhosis. Furthermore, HCC has been diagnosed in patients with hemochromatosis both in the presence and absence of cirrhosis, leading to the belief that iron itself may be carcinogenic [47, 48]. Support for this hypothesis comes from studies in animal models, demonstrating rats fed a diet high in iron developed HCC in the absence of fibrosis or cirrhotics [49]. Alcohol intake may act synergistically with the mutagenic effects of iron [50]. Among patients with hemochromatosis, homozygosity for the C282Y mutation yields the highest risk for HCC development, even in the absence of cirrhosis [12, 51, 52]. However, whether mutations in the HFE gene alone lead to the development of HCC is not well established, as one study found a higher prevalence of HFE gene mutations in patients with liver cirrhosis and HCC compared those with cirrhosis but without HCC (12.4 vs. 3.7%) [53], other studies were unable to confirm these findings [54]. Another prospective study on 162 patients with alcoholic cirrhosis and 139 patients with HCV induced cirrhosis found mutations of the C282Y gene as an independent risk factor for HCC in patients with alcoholic cirrhosis but not in cirrhosis secondary to HCV [55].

Aflatoxins

Aflatoxin B1 has been classified as carcinogenic by the International Agency for Research on Cancer because of its strong hepatocarcinogenic potential. It is produced by fungi of the Aspergillus species, primarily in Asia and Africa, where it may be a contaminant of grain, nuts and vegetables. The effect of Aflatoxin B1 is a mutation in the p53 tumor-

suppressor gene, which has been demonstrated in 30–60% of HCCs diagnosed in areas which are endemic to aflatoxin [12]. The carcinogenic effect of aflatoxin may be potentiated by HBV or alcohol intake

Surveillance

There are two types of tests used for HCC screening: serological and imaging. Of available serological, alpha-fetoprotein (AFP) has been the most studied and is the most often clinically utilized [56-59]. Trevisani et al. examined the optimal cutoff AFP level, utilizing receiver operating curve analysis, and found a value of about 20 ng/mL provides the greatest area under the receiver operating curver [56]. However, at this level the sensitivity is only 60%, a level generally considered inadequate as a screening test. Therefore, AFP alone is considered an inadequate screening test. However, AFP in conjunction with imaging still has a role in the surveillance and diagnosis of HCC, as a cirrhotic patient with a liver mass and a serum AFP level greater than 200ng/mL has a very high positive predictive value for HCC [56]. Additionally, studies have shown that a persistently elevated AFP is a risk factor for HCC [60].

Another serological test used to diagnose HCC is des-gamma-carboxy prothrombin (DGCP), also known as Prothrombin Induced by Vitamin K Absence II (PIVKA II) [59, 61-64]. Although there are reports of its use in a surveillance mode, these do not yet provide sufficient justification for routine use of this marker. There are also reports that DGCP is a marker for portal vein invasion by tumor [65]. Additional tests that have been evaluated include glycosylated AFP (L3 fraction), [66-71] alpha fucosidase [72, 73] and Glypican-3 [74, 75]. None of these has been adequately investigated and cannot be recommended as a screening test [76].

The radiological test most widely used worldwide for surveillance is ultrasonography [77]. The appearance of HCC on ultrasound may vary. Certain lesions may be echogenic because of the presence of fat in the cells. Other lesions may be hypoechoic or show a "target lesion" appearance. However, none of these appearances is specific for HCC. This modality has a sensitivity of 65% to 80% and specificity greater than 90%, although these characteristics have not been as well defined in cirrhotic livers [78, 79]. The primary drawback regarding the use of ultrasound is that it is operator dependent and visualization may be impaired with large body habitus. The combined use of AFP and ultrasonography increases detection rates and is the primary mode of screening [80].

High-risk groups in whom HCC screening and surveillance is recommended include [81]:

- Hepatitis B carriers
  - Asian males ≥ 40 years
  - Asian females ≥ 50 years
  - All cirrhotic HBV carriers
  - Family history of HCC
  - Africans > 20 years
- Non-hepatitis B cirrhosis
  - Hepatitis C

  - Alcoholic cirrhosis
  - Genetic hemochromatosis
  - Primary biliary cirrhosis
- No recommendations for or against surveillance
  - α-1 Antitrypsin deficiency
  - Nonalcoholic steatohepatitis
  - Autoimmune hepatitis

We recommend a contrast enhanced MRI or CT scan at the time of diagnosis of liver disease regardless of the laboratory values.

Surveillance Interval

According to the most recent guidelines from the American Association for Study of Liver Diseases, the ideal surveillance interval is not known, though an interval of 6-12 months has been proposed based on tumor doubling times [81]. Although most hepatologists use a 6-month interval, there is no evidence that demonstrates a 6-month interval is superior to a yearly interval.

Our clinical practice is to obtain a serum AFP every six months along with abdominal imaging every six months – a reasonable approach would be an abdominal ultrasound alternating with a contrast enhanced CT or MRI every six months.

We favor an annual contrast enhanced CT or MRI provided no suspicious lesions exist. If suspicious lesions are present, findings are correlated with serum AFP and short interval follow-up scanning is pursued. A second form of contrast imaging is often sought if CT or MRI reveals a questionable lesion.

Diagnosis

Imaging

Computed tomography (CT) has become a mainstay for diagnosis of HCC, with triphasic examination of the liver utilizing an arterial (arterial-dominant), portovenous , and delayed phase scan regarded as the standard technique [82]. Magnetic resonance imaging (MRI) with gadolinium based contrast also is a reliable test for the evaluation of patients with suspected HCC. In both CT and MRI, the characteristic behavior of HCC is that of hypervascularity in the arterial-dominant phase and washout in the portovenous or delayed phase. Washout can be depicted with higher accuracy in delayed phases [82, 83]. Both the presence of hypervascularity and pathological washout allows the definite noninvasive diagnosis of a HCC [81]. However, these criteria for HCC diagnosis may lead to confusion with other lesions such as arteriovenous shunts or dysplastic nodules, as well as false-negative findings from well-differentiated HCC without arterial hyperenhancement.

The detection rates of HCC from CT or MRI are variable. One trial, which compared triphasic CT with whole-liver explant showed a sensitivity of 61%, a specificity of 66% and a negative predictive value of 30% for the detection of HCC by means of triphasic CT [84]. This study further demonstrated MRI to show superior results to CT, with a 76% sensitivity for MRI vs. 61% for CT. Specifically, for HCC lesions > 2 cm, MRI and CT detected 100%. However, for HCC between 1 and 2 cm MRI and CT showed a detection rate of 89 and 65% ($p = 0.03$) respectively, and for HCC < 1 cm a detection rate of 34% and 10% ($p = 0.06$) were respectively shown [84]. Additional support for these findings comes from subsequent trials comparing ultrasound, CT, and MRI. Some of these reports have found MRI to be superior to the other modalities [85-88]. MRI may be more specific than CT, particularly when differentiating HCC from regenerative nodules or high-grade dysplastic nodules [84, 89, 90]. The recent introduction in the U.S. of Eovist, a contrast agent approved by the FDA, may provide important imaging qualities. CT exposes the patient to radiation and has become a source of concern regarding longterm effects. MRI also carries disadvantages as it requires more time to perform, may require an enclosed space and is not available to patients with metal implants.

Liver Biopsy

Although it is believed that good-quality, contrast-enhanced CT and MRI are appropriate for the diagnosis of liver tumors, the accuracy of diagnosing small malignant liver tumors (< 2 cm) especially in cirrhotic livers, may be difficult from imaging alone [91]. In these cases, biopsy is the next test needed for diagnosis if the serum AFP is not diagnostic.

Macroscopically, HCC appearance can be variable with either single or multicentric tumor nodules, which are either well demarcated from the surrounding liver or with infiltrative growth into the liver parenchyma. The tumor nodules may frequently show areas of hemorrhage and necrosis.

Microscopically, HCC may resemble normal liver tissue depending on the degree of differentiation. Patterns seen include trabecular, acinar, or scirrhous patterns [92]. Cytologically, the tumor cells of HCCs can be distinguished from normal hepatocytes by a higher nuclear/cytoplasmic ratio, abundant granular eosinophilic cytoplasm, prominent nucleoli, and intranuclear cytoplasmic invaginations [93].

Management

Surgical Resection

The optimal treatment for HCC in patients without liver cirrhosis is surgical resection, although this group accounts for less than 5% of patients in Western countries [77]. Patients with HCC and cirrhosis are not often suitable for resection because of the potential for hepatic decompensation after surgical resection, and selection of candidates for resection who have cirrhosis, requires careful deliberation. Historically, selection of appropriate candidates for surgical resection was based on Childs-Turcotte-Pugh score. However, a recent study has

demonstrated that normal serum bilirubin level and absence of significant portal hypertension, as indicated by presence of varices, platelet levels, or hepatic venous pressure gradient measurement, are better predictors of short- and long-term survival [94]. The 5-year survival following liver resection is less than 30% in patients with bilirubin >1 mg/dL and presence of portal hypertension.

In addition to the post-surgical morbidity and mortality for tumor resection in patients with cirrhosis, one must also be aware of tumor recurrence. Studies have demonstrated the 5-year recurrence rates following resection in cirrhotics exceeds 50% [77, 95], with 60% to 70% of recurrences corresponding to intrahepatic metastases, while 30% to 40% are de novo tumors [96-98]. Several variables are known to affect the risk of recurrence following resection, including tumor size, number of tumors, vascular invasion, and the width of the resection margin. Regarding tumor size, there is a significant difference in the 5-year recurrence rates for tumors >5 cm compared to those <5 cm (43% vs 32%, respectively) [99]. Additionally, multinodular tumors have been determined to have an increased tendency to recur, with one large cohort study demonstrating the 5-year survival after resection of single tumors to be 57%, as compared to 26% for 3 or more nodules [100, 101]. When performing surgical resection, the appropriate tumor margin may vary due to the need to balance adequate margins with preservation of hepatic function. One prospective study which compared wide (2 cm) to narrow (1 cm) resection margins demonstrated high recurrence rates in both groups, but greater survival rates for the wide margin group [102]. Laparoscopic liver resection plays a select role in the surgical management of HCC and recent technologic advances have made this approach a feasible option [103, 104].

We recommend surgical resection for patients who are Childs A cirrhotics and anatomically amenable to resection. We also carefully select early Childs B patients beyond Milan criteria or who do not qualify for liver transplant due to current substance abuse or other prohibitive risk factors for liver resection. We do not rountinely study portal pressures as a screening tool for liver resection.

Liver Transplantation

In cirrhotic patients, liver transplantation is the ideal therapy for HCC, as it minimizes risk of recurrence as well as treats the underlying cirrhosis to circumvent post-operative hepatic decompensation. Currently, the recommended United Network for Organ Sharing (UNOS) criteria is based on the Milan scoring system, defined as a single lesion ≤5 cm or maximum of 3 lesions each less than 3 cm in diameter. These critieria have been evaluated multiple times and have shown 5-year survival rates of >70% and recurrence rates of <15% [105-107]. An additional set of expanded criteria (UCSF criteria), defined as a single lesion up to 6.5 cm in diameter, or up to 3 lesions, each of which are up to 4.0 cm, with a maximum combined tumor bulk of ≤8.0 cm, has been proposed by the University of California in San Francisco [108]. Studies evaluating these criteria have shown similar outcomes to the Milan criteria [109, 110].

In the setting of liver transplant listing, patients with HCC may be awarded Model for End stage Liver Disease (MELD) exception points, based on the 3-month pre-transplantation mortality rates. The criteria to qualify include presence of a solitary lesions ≥2 cm and <5 cm,

or up to 3 lesions, each <3 cm. Patients who meet these criteria receive a MELD score upgrade of 22, unless their native MELD score is otherwise greater. For each 3-month interval that they remain on the waiting list, an additional 3 exception points are awarded based on an expected increase of 10% for the 3-month mortality rate [81].

For patients with HCC, with tumor size and tumor burden outside of the Milan criteria, several options are available. One possibility is downstaging of tumors with locoregional bridge therapy, to reduce tumor size and tumor burden. This may be achieved through using radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), or trans-arterial chemoembolization (TACE). Studies have shown that successful downsizing can be achieved in up to 70% of the patients treated with one or more of these modalities, and successful liver transplantation was subsequently accomplished in nearly half of these patients [111, 112]. Though locoregional therapy such as RFA or TACE may be used to downsize patients and potentially offer liver transplantation, the survival of those patients was not significantly different from those who presented with a stage of HCC eligible for immediate transplantation. [113]. Another possibility is salvage liver transplantation after initial resection of HCC, though the outcomes for this approach have been sub-optimal compared to primary liver transplantation for HCC [114]. At a recent UNOS consensus meeting, there was agreement that the allocation policy for liver transplantation should result in similar risks of removal from the waiting list and similar transplant rates for HCC and non-HCC candidates. In addition, the allocation policy should select HCC candidates so that there are similar posttransplant outcomes for HCC and non-HCC recipients. There was a general consensus for the development of a calculated continuous HCC priority score for ranking HCC candidates on the list that would incorporate the calculated MELD score, alpha-fetoprotein, tumor size, and rate of tumor growth. Only candidates with at least stage T2 tumors would receive additional HCC priority points [115].

We recommend liver transplantation to those patients with Childs B or C cirrhosis and HCC within Milan criteria. Patients beyond Milan criteria are treated with loco-regional therapy in an attempt to down-stage the tumor. Patients with successful down-staging are offered the option of liver transplantation – living-donor liver transplantation [LDLT] or deceased-donor liver transplantation [DDLT].

Percutaneous Ablation

Minimally invasive percutaneous treatments are the best treatment alternatives for HCC patients who are not eligible for surgical resection or transplantation. The most widely utilized methods to induce tumor necrosis are PEI and RFA. PEI is a procedure performed under ultrasound guidance, which consists of injecting absolute ethanol directly into the HCC lesions, to achieve necrosis of the tumor. Studies have demonstrated complete tumor necrosis in 70%–80% of solitary HCC ≤3 cm [116] and in almost 100% in tumors less than 2 cm. However, successful treatment is less likely in larger tumors, as studies have demonstrated approximately 70% necrosis for tumors between 2 and 3 cm and 50% those between 3 and 5 cm [117-119]. In Ryu et al's study of 3225 patients with solitary tumors <3 cm, there were no significant differences in survival between surgical resection and PEI. Furthermore, survival after PEI was greatest for those with tumors <3 cm and <3 lesions [120]. Therefore, it appears

that PEI is best reserved for patients with HCC lesions <3 cm. PEI is less expensive to administer than RFA but routinely requires repeat administration.

RFA provides more complete ablation with fewer sessions than PEI [121, 122] and is particularly more efficacious in treating tumors >2 cm. Several recent randomized trials compared RFA and PEI in treating patients with small HCC <4 cm and demonstrated the superiority of RFA in terms of lower local recurrence and longer overall and disease-free survival [122-124]. Additionally, treatment required a mean of 1.1–2.1 sessions for RFA, compared to 4.8–6.5 for ethanol injection. Adverse events were similar for both groups. Local recurrence rates were significantly lower among those receiving RFA, ranging from 8% to 14%, compared to 22%–34% in those treated with PEI. Overall survival rates for patients treated with RFA were 100% and 98% for 1 and 2 years, respectively, compared to 96% and 88%, respectively, in the PEI group [124]. Based on these findings, RFA is preferred over PEI for local ablation of tumors <4 cm. RFA may be performed laparascopically and has a local recurrence rate of 18% in primary HCC over a mean follow-up time of 17 months [125-127]. Laparascopic RFA can be used for unresectable HCC with median survival of 25.3 months and 3 and 5-year survival rates of 21% and 8.3%, respectively [128].

We recommend RFA for single lesions less than 4 cm. If the patient is eligible for transplant, we rountinely list the patient prior to RFA in case of hepatic decompensation. If the patient is a Childs B or C cirrhotic, RFA is not pursued as transplant is the treatment of choice.

Transarterial Embolization/Chemoembolization

TACE is considered the first-line therapy for patients who exceed the criteria for liver transplantation, and is also the first-line therapy for downstaging tumors that exceed the criteria for transplantation [112]. The goal of embolization is to induce ischemic tumor necrosis through arterial occlusion. Embolization may be done alone or in combination with certain chemotherapeutic agents such as doxorubicin, mitomycin, or cisplatin, and the contrast agent, lipiodol. Adverse events from this procedure occur in approximately 10% of patients, and include ischemic cholecystitis, nausea, vomiting, abdominal pain, and bone marrow suppression [129]. A postembolization syndrome has also been reported to occur in >50% of patients treated with TACE, of which symptoms include fever, abdominal pain, and fatigue. Treatment-related mortality is less than 5%. Furthermore, TACE can be performed in patients in whom RFA cannot be performed due to of tumor location near the gallbladder, biliary tree, or vasculature. However, use of TACE is limited to patients with acceptable preserved liver function [129].

TACE induces tumor necrosis in 30% to 50% of treated patients [130]. A recent meta-analysis of 7 randomized controlled trials comparing arterial embolization/TACE to conservative management demonstrated that arterial embolization improved 2-year survival compared with that in control subjects (odds ratio, 0.53; 95% CI: 0.32–0.89) [131]. In another large cohort, prospective study of 8510 patients who received TACE for unresectable HCC, the median survival was 34 months, with 3- and 5- year survivals of 47% and 26%, respectively [132]. In a randomized controlled trial comparing TACE to conservative therapy for unresectable HCC, survival was significantly higher in those receiving TACE at 1, 2, and

3 years (p=0.002), with a relative risk of mortality of 0.49 [133]. A randomized controlled trial by Llovet et al, demonstrated similar findings regarding significantly greater survival in those undergoing TACE, with a hazard ratio for mortality of 0.47 [130].

Recent Korean studies have reported the use of TACE in patients with portal vein thrombosis. TACE must be administered in a highly selective manner in these cases as the hepatic artery is the sole blood supply to the parenchyma.

We recommend TACE be used in patients which unresectable HCC with acceptable liver function and performance status. We treated patients with a total bilirubin less than 3.0 and an ECOG performance status less than 2.

Yttrium 90 Radioembolization

Therapy with Yttrium 90 radioembolization (^{90}Y-RE) consists of delivering implantable radioactive microspheres into the arteries that feed the tumors, to provide a high dose of radiation to tumor nodules while preserving the non-tumoral liver tissue from receiving a harmful level of radiation. Yttrium (MDS Nordion) is approved by the FDA under a humanitarian device exemption (HDE). ^{90}Y serves as a source of radiation with a half-life of 64.2 h and tissue penetration mean of 2.5 mm and maximum of 10 mm; usually, within 11 days of implantation, 95% of the radiation is delivered [134].

^{90}Y-RE is a procedure that requires a multidisciplinary team including hepatologists or oncologists, interventional radiologists, and nuclear medicine specialists. To avoid toxicity, a thorough angiographic evaluation is needed in order to identify the extrahepatic vessels that may feed the tumors and to occlude any collateral vessels that arise from the hepatic artery and that may subsequently carry microspheres to extrahepatic organs [134, 135]. The absolute contraindications for ^{90}Y-RE include: a hepatopulmonary shunt that would result in substantial radiation being delivered to the lungs, the inability to prevent embolization of microspheres into the gastrointestinal tract, or a history of prior external irradiation to the liver. Relative contraindications include a compromised pulmonary function and an inadequate liver reserve [135].

Side effects are not common after ^{90}Y-RE, with most patients experiencing mild pain or nausea as the only adverse reactions. Since a macroembolic effect is not observed after ^{90}Y-RE [136], a true post-embolization syndrome is rarely seen [137]. Mild to moderate lymphopenia is usually seen after ^{90}Y-RE but not associated with increased susceptibility to infections [138]. However, ^{90}Y-RE may produce toxic effects as a result of radiation of non-target organs including cholecystitis [138], gastrointestinal ulceration [139], and liver toxicity. Liver damage after ^{90}Y-RE typically occurs 4–8 weeks after microsphere injection, presenting as jaundice, mild ascites and a moderate increase in alkaline phosphatase, with mild or absent changes in transaminases [140]. Long-term outcome is diverse, but pathological changes consistent with veno-occlusive disease have been observed in severe cases. The incidence is difficult to establish, with some series reporting incidences between 0 and 11% [139] and others reporting elevations in aminotransferases in as much as 38% of patients [138]. The main risk factors for radiation induced liver disease is the amount of liver that is targeted, the tumor burden, and the how healthy the non-malignant liver parenchyma is [140]. In a more recent report, patients with Child–Pugh A disease, with or without portal

vein thrombosis PVT, benefited most from treatment. Patients with Child–Pugh B disease who had PVT had poor outcomes [141]

We recommend Y90 for advanced unresectable HCC and use it preferentially over TACE if portal vein thrombosis is present. This is due to the smaller degree of embolization with the radiation delivery.

Chemotherapy

During the last few years, several molecular targeted agents have been evaluated in clinical trials in advanced HCC. Specifically in advanced HCC, Sorafenib, which has been extensively investigated in phase I–III trials.

Sorafenib (Nexavar) is an oral multikinase inhibitor, which blocks tumor cell proliferation and angiogenesis by targeting the serine/threonine kinases Raf-1/B-Raf and the tyrosine kinases of VEGFR-2/-3 and PDGFR [142, 143]. In a phase I study conducted in 69 patients with advanced refractory solid tumors, sorafenib demonstrated preliminary antitumor activity, particularly among patients with renal cell carcinoma and HCC [144]. In a phase II trial of 137 patients with advanced HCC, treatment with sorafenib 400 mg twice daily resulted in a median survival of 9.2 months and a median time to progression of 4.2 months [145]. There were no significant pharmacokinetic differences between Child-Pugh A and B patients. Furthermore, patients with higher phosphorylated ERK (pERK) staining in tumor biopsies had a significant longer time to progression (178 vs. 46 days), suggesting raf inhibition as an important mechanism of action of sorafenib and pERK as a potential marker of response [145].

Based on this data, a large randomized, double-blind, placebo-controlled phase III trial, known as the SHARP trial, was conducted to further assess the efficacy and safety of sorafenib 400 mg twice daily. The study was performed in 602 patients with advanced HCC and and well compensated cirrhosis [146]. A partial response was seen in 2.3% of patients, and 71% of patients had disease stabilization. Median survival time was 10.7 months with sorafenib and 7.9 months with placebo ($p< 0.001$). In addition, median time to progression was 5.5 months compared to 2.8 months in controls ($p<0.001$). The primary side effects were diarrhea, hand-foot skin reaction, weight loss and hypophosphatemia.

Sorafenib was also tested in combination with conventional systemic chemotherapy. A phase I study evaluated sorafenib in combination with doxorubicin in 18 patients.

Grade 3/4 neutropenia occurred in 61% of patients, though the toxicity profile was manageable without clear evidence of synergistic toxicity. The response rate was 6% and 63% of patients achieved disease stabilization, with median duration of 17.2 weeks [147]. The results of a randomized, double-blind phase II study of sorafenib plus doxorubicin versus placebo plus doxorubicin, in 96 patients with advanced HCC, also showed encouraging results, with median time to progression of 8.6 months versus 4.8 months in the controls, and survival of 13.7 months versus 6.5 months [148]. Although sorafenib has significantly improved the treatment of patients with advanced HCC, several challenges remain. Survival benefit from sorafenib is only moderate and median survival on sorafenib therapy in clinical practice is often lower [149-151] than reported in the SHARP trial. Therefore, there is need for further evaluation. Currently, an ongoing, prospective, multi-center trial (STORM trial) is

being conducted to evaluate whether sorafenib in the adjuvant setting, after tumor resection or ablation, delays time to tumor recurrence and increases median survival time.

Both safety and efficacy of sorafenib in patients with more advanced cirhosis (Child-Pugh B and C) are still unknown, because most patients enrolled in the SHARP trial were Child-Pugh A (97%) [146]. Pinter et al. reported their findings regarding using sorafenib in HCC patients with different stages of liver cirrhosis, and found the median survival time in patients with Child-Pugh A, B, and C was 8.3, 4.3, and 1.5 months, respectively [149].

We recommend Sorafenib for patients with HCC whose ECOG performance status is ≥ 2 or in the presence of extrahepatic disease.

Molecular Markers

High-dimensional array technology provides a unique tool to determining predictive molecular markers characterizing tumors with regard to metastatic potential. Over 300 studies with use of DNA microarray technology have been published, in regards to characterizing HCC in terms of diagnosis, recurrence, or metastasis.

Molecular Markers for Diagnosis

Diagnostic markers for HCC have been lacking, as AFP is the only widely available tool and does not have adequate sensitivity or specificity to make a diagnosis. Jia et al's study, utilized a microarray technique to determine the expression profiles of 218 HCC specimens from patients with either high or low serum AFP. From the microarray study, five candidate genes (i.e., GPC3, PEG10, MDK, SERPINI1, and QP-C) were found to be overexpressed in HCC. A significant increase in the expression of the five candidate genes could be detected in most of the HCC samples, including those with normal serum AFP and small tumors. Furthermore, a significant increase in serum midkine, encoded by the MDK gene, was associated with HCC patients, regardless of serum AFP [152]. Microarray analysis may therefore accurately identify HCC versus non-cancerous hepatic cells.

Markers for Early Recurrence after Resection and Transplant

Hoshida et al's study aimed to demonstrate the feasibility of gene-expression profiling of more than 6,000 human genes in formalin-fixed, paraffin-embedded (FFPE) tissues, which in contrast to frozen tissues, can be easily archived and transported [153]. They performed this on 307 specimens with HCC and found a gene-expression signature associated with survival. They also demonstrated a reproducible gene expression signature that correlates with survival is present in liver tissue adjacent to the tumor [153]. In a study by Dvorchik et al, tissue samples from the explants of 183 patients at two centers were evaluated using a technique of fractional allelic imbalance (FAI) rate [154]. This was determined by selecting a total of 9 tumor-suppressor gene markers and dividing the number of mutated markers over the total number of allelic markers. Based on their findings, the authors created a prognostic model based on FAI rate and presence or absence of macrovascular invasion.

Marker for Metastasis

The occurrence of intrahepatic metastasis or multicentric carcinogenesis can worsen the prognosis of HCC. One study utilized microarray analysis in the diagnosis of these two forms of HCC and aimed to identify genes associated with intrahepatic metastasis [155]. The findings demonstrated that microarray analysis could identify genes, which lead to liver metastasis. However, studies on HCC predictors have only been retrospective studies, limiting their status for clinical use, until larger prospective studies have been performed.

Summary

In summary, HCC represents a lethal disease with an increasing incidence in Western centers. We advocate contrast imaging [CT or MRI] at diagnosis of liver disease to rule out HCC and follow an annual contrast enhanced image or abdominal ultrasound alternating with a contrast enhanced CT or MRI every six months and six monthly serum AFP in patients with cirrhosis or high risk patients and no questionable lesion. Patients with an indeterminant lesion and/or elevations in serum AFP are imaged with a second modality and followed more closely.

Surgical resection is offered to selected Childs A and B patients while transplantation plays a vital role in advanced cirrhotics. Techniques such as TACE or RFA are rountinely administered for patients with adequate liver function as method to bridge a patient to liver transplantation.

TACE and Y90 are also important tools for patients with advanced unresectable HCC and with the recent introduction of Sorafenib therapy now exists for patients with poor performance status, extrahepatic disease and gross vascular invasion.

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Chapter 5

Hepatic Myofibroblasts: Origin and Role in Liver Fibrogenesis

Erica Novo[1,2], Chiara Busletta[1,2], Claudia Paternostro[1,2], Davide Povero[1,2], Stefania Cannito[1,2] and Maurizio Parola[1,2]
[1] Department of Experimental Medicine and Oncology
[2] Interuniversity Centre for Liver Pathophysiology, University of Torino,
Corso Raffaello 30, 10125, Torino

Abstract

The definition of hepatic myofibroblasts is currently attributed to a rather heterogenous population of cells that sustain liver fibrogenesis and then fibrotic progression of chronic liver diseases of different aetiology to the common advanced-stage of cirrhosis. These highly proliferative and contractile myofibroblasts actively participate to the progression of the chronic disease by means of their multiple phenotypic responses, including excess deposition of extracellular matrix components and its altered remodelling as well as the synthesis and the release in a paracrine/autocrine way of a number of critical growth factor which sustain and perpetuate fibrogenesis, chronic inflammatory response and neoangiogenesis. According to current literature hepatic myofibroblasts, which are mostly α-smooth muscle actin (α-SMA) - positive cells, mainly originate from hepatic stellate cells or from fibroblasts of portal areas through a process of activation and trans-differentiation. Hepatic myofibroblasts have been reported to originate also from bone marrow – derived stem cells, including mesenchymal stem cells or circulating fibrocytes, able to engraft chronically injured liver. It is currently debated whether myofibroblasts may also originate from hepatocytes and cholangiocytes through a process of epithelial to mesenchymal transition. Hepatic myofibroblasts have been reported to play additional crucial roles, including modulation of immune responses in the chronically injured liver and the cross talk with hepatic progenitor (stem) cells as well as with malignant cells of either primary hepatocellular carcinomas or of metastatic cancers.

1. Introduction

Chronic liver diseases (CLDs) can be envisaged as conditions characterized by the reiteration of hepatocyte injury that are usually induced by chronic infection by hepatotropic viruses (mainly hepatitis B and C viruses), autoimmune injury, as well as by metabolic and toxic/drug – induced causes, with chronic alcohol consumption being predominant in western countries. Reiterated liver injury then results in chronic activation of inflammatory response and of the wound healing response that, together with other mechanisms including oxidative stress, derangement of epithelial-mesenchymal interactions and epithelial to mesenchymal transition, sustain persistent liver fibrogenesis (i.e., the process) and represents a major driving force for liver fibrosis (i.e., the result) (Parola and Robino, 2001; Friedman, 2000, 2003, 2004; Bataller and Brenner, 2005; Friedman 2008b; Novo and Parola, 2008; Parola et al., 2008).

Liver fibrogenesis is currently considered as a dynamic and highly integrated molecular, tissue and cellular process that leads to the progressive accumulation of extracellular matrix (ECM) components in an attempt to limit hepatic damage in a chronic liver disease (CLD), irrespective of the aetiology. Persistent fibrogenesis is responsible for the progression of any CLD to the end-points of liver cirrhosis and hepatic failure, with cirrhosis being currently defined as an advanced stage of fibrosis, characterized by the formation of regenerative nodules of parenchyma surrounded and separated by fibrotic septa, and associated with significant changes in organ vascular architecture, development of portal hypertension and related complications (variceal bleeding, hepatic encephalopathy, ascites and hepatorenal syndrome) (Friedman, 2003, 2004; Pinzani and Rombouts, 2004; Bataller and Brenner, 2005; Friedman 2008b;).

Fibrotic progression of CLDs has been described to proceed through at least four distinct patterns of fibrosis that are intimately related to the underlying cause of CLD. The main fibrotic patterns are also related to the "topographic site" of tissue injury and, pertinent to this review, to the involvement of different populations of MFs and the predominant pro-fibrogenic mechanism (Cassiman and Roskams, 2002; Pinzani and Rombouts, 2004; Parola et al., 2008; Parola and Pinzani, 2009).

The first pattern of ECM deposition and septa formation is that of **bridging fibrosis**, which is typically described mainly in the liver of patients carrying HBV- or HCV-related chronic hepatitis. This pattern is characterized, as a result of portal-central bridging necrosis, by the development of fibrotic septa that connect portal areas with the area of central vein (i.e, portal-central septa) or different portal areas (portal-portal septa), as well as of blind septa in the parenchyma. To this pattern belongs those classic histopathological images of fibrotic septa leading to the obliteration of central veins and of early changes in vascular architecture and connections with the portal system, which eventually favor the development of portal hypertension. Chronic activation of wound healing is believed to represent the major pathogenic mechanism driving this pattern of fibrosis progression. As we will see later in this review, fibrogenesis in these settings can be sustained by hepatic populations of pro-fibrogenic myofibroblasts (MFs) that can originate either from hepatic stellate cells (HSCs), portal fibroblasts or even from bone marrow - derived stem cells. However, oxidative stress and reactive oxygen species (ROS) have also been described to offer a significant pathogenic contribution.

A second relevant pattern of fibrosis has been defined as **perisinusoidal/pericellular fibrosis**. This pattern has been described in CLDs which follow either excess alcohol consumption (ASH or alcoholic steatohepatitis) or metabolic derangement and then progress from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH). In these clinical settings excess deposition of ECM components is first described in the space of Disse and leads to the peculiar "chicken-wire" pattern. MFs in these conditions are believed to derive mostly from the activation of hepatic stellate cells (HSC/MFs), with ROS and oxidative stress playing a predominant pro-fibrogenic and pathogenic role.

A third pattern is that of **biliary fibrosis**. This definition is used in those conditions affecting the biliary tree that are characterized by a characteristic scenario of concomitant proliferation of reactive bile ductules and periductular MFs (here mainly derived from periportal fibroblasts or, possibly, by EMT transition of cholangiocytes). This scenario is dominated by the formation of portal-portal septa that for a long time do not significantly affect vascular connections with the portal system. For this pattern, either significant alterations in the interactions between cholangiocytes and mesenchymal cells or cholangiocyte transition into MF-like phenotype, as well as oxidative stress, have been proposed as major mechanisms.

The last pattern commonly included in the classification is that of **centrilobular fibrosis.** However, centrilobular fibrosis is unrelated to CLDs but rather is typically described in patients affected by chronic heart failure in which a significant alteration of venous outflow is realized. In these patients fibrotic septa develop among central vein areas (central-central septa) and lead to the unique scenario often defined as "reversed lobulation".

2. Hepatic Myofibroblasts: Definition and Origin

Irrespective of the specific aetiology of a CLD and of the prevalent pattern of fibrosis, liver fibrogenesis is sustained by hepatic myofibroblasts (MFs), a heterogenous population of pro-fibrogenic cells, mostly positive for α–smooth muscle actin (αSMA, see Figure 1), that are mainly found in chronically injured livers (i.e., fibrotic and or cirrhotic) (Cassiman et al., 2002; Friedman, 2008a, 2008b; Parola et al., 2008; Parola and Pinzani, 2009). Hepatic MFs are highly proliferative and contractile cells that actively participate to the progression of the chronic liver disease by means of their multiple phenotypic responses, including excess deposition of extracellular matrix components and its altered remodelling as well as the synthesis and the release in a paracrine/autocrine way of a number of critical growth factor which sustain and perpetuate fibrogenesis, chronic inflammatory response and neoangiogenesis.

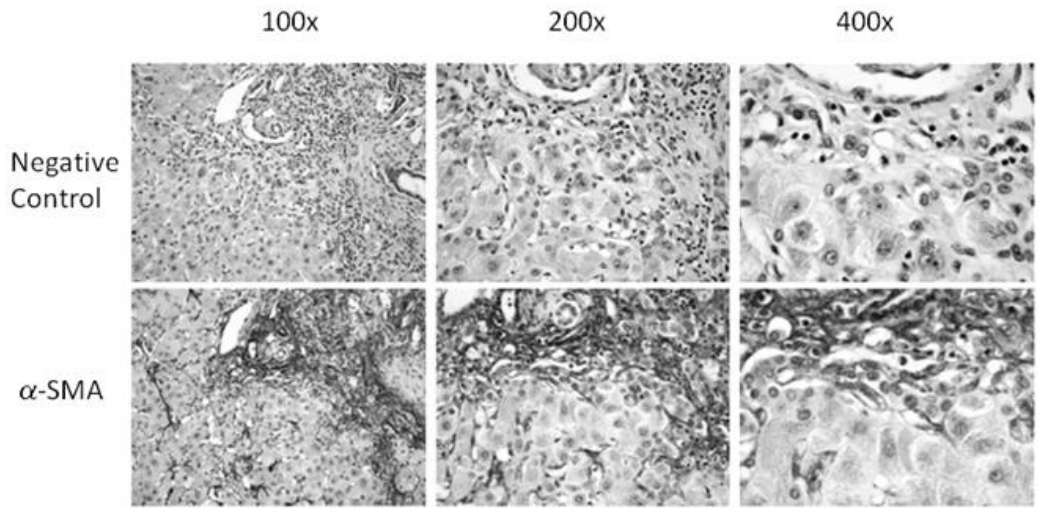

Figure 1. Serial liver sections from a cirrhotic HCV patient with hepatic MFs positively stained for α-SMA.

Although other liver cell populations will of course offer a significant contribution to fibrogenesis (injured hepatocytes, activated Kupffer and sinusoidal endothelial cells), in all CLDs hepatic MFs represent a unique and crucial cellular crossroad where incoming paracrine and autocrine signals (ROS from injured cells, growth factors, inflammatory and angiogenic signals, chemokines, adipokines, etc.) are integrated in order to "operate" all those phenotypic responses designed to sustain fibrogenesis and the progression of CLDs to the end-points of cirrhosis and hepatic failure. Accordingly, MFs-dependent progressive fibrogenesis is sustained by at least three main pro-fibrogenic mechanisms (Parola et al., 2008): a) chronic activation of the wound healing response (apply to any CLDs, predominates in chronic injury by viral agents or autoimmunity; b) ROS and other oxidative stress – related reactive mediators (apply to all CLDs, mainly associated to metabolic or alcoholic aetiology); c) derangement of epithelial-mesenchymal interactions and EMT, detected in chronic cholangiopaties.

Clinical and experimental evidence suggests that different populations of MFs exist and that these populations may be recognized according to their antigen profile or their tissue localization. On these basis Cassiman et al. (2002) have proposed the following terminology to be applied to MFs detected in chronically injured livers:

1) **portal/septal MFs** (PS/MFs), which are MFs displaying an overlapping antigen repertoire, that are commonly found in the expanded connective tissue around portal tracts (portal MFs) or in the inner part of fibrotic septa (septal MFs); it is believed that these MFs derive through a process of activation/transdifferentiation from portal fibroblasts (PFs).
2) **interface MFs** (IF/MFs), found at the edge between fibrotic septa and the surrounding parenchyma (i.e., where active fibrogenesis occurs);
3) **activated, myofibroblast - like, hepatic stellate cells** (HSC-MFs), αSMA -positive cells found primarily in or around capillarised sinusoids of fibrotic/cirrhotic livers.

A fascinating and still incompletely resolved issue is represented by the "in vivo" origin of hepatic MFs. According to current literature and prevalent views, hepatic myofibroblasts mainly originate from hepatic stellate cells or from fibroblasts of portal areas through a process of activation and trans-differentiation. Hepatic myofibroblasts have been reported to originate also from bone marrow – derived stem cells, including mesenchymal stem cells or circulating fibrocytes, able to engraft chronically injured liver. It is currently debated whether myofibroblasts may also originate from hepatocytes and cholangiocytes through a process of epithelial to mesenchymal transition. The issue of the intra- and extra-hepatic origin of liver MFs will be addressed in the following paragraphs. The reader should note from the beginning that, apart from their pro-fibrogenic role, hepatic myofibroblasts have been reported to play additional crucial roles, including modulation of immune responses in the chronically injured liver and the cross talk with hepatic progenitor (stem) cells as well as with malignant cells either of primary hepatocellular carcinomas or of metastatic cancers.

3. Hepatic Stellate Cells as a Source of Hepatic MFs

Hepatic stellate cells (HSC) in normal liver are perisinusoidal cells of still uncertain embryological origin, responsible for the synthesis of basal membrane like – ECM components of the subendothelial space of Disse and for storage and metabolism of vitamin A and retinoids. HSC have been also proposed to act as "liver specific pericytes" and to significantly contribute to hepatic development and regeneration (Friedman 2008a). HSC have been the first cell source of pro-fibrogenic MF-like cells to be identified and HSC/MFs still remain the most investigated cell population for which the process of activation and pro-fibrogenic mechanisms are best characterized (Friedman, 2008a). HSC-MFs are likely to be involved in most of clinical conditions of CLDs, with a prevailing involvement in the pattern of fibrosis progression defined as "perisinusoidal/pericellular fibrosis", recognising a metabolic or alcoholic aetiology. HSC may contribute also to the origin of interface MFs and to the pattern of "bridging fibrosis" found in patients affected by chronic viral hepatitis (Parola et al., 2008). HSC are indeed a fascinating cell type in both normal and pathologic liver and the interested reader will find more exhaustive details on HSC and HSC/MFs (i.e., MFs originated from HSC) in the excellent review by Friedman (Friedman, 2008a). Here it is sufficient to state that research on HSC and HSC/MFs has been fundamental for our present knowledge concerning liver fibrogenesis and related molecular mechanisms. Pioneer studies have revealed that under conditions of chronic liver injury quiescent HSC located in the perisinusoidal space of Disse can undergo a peculiar process of activation. This process has been first outlined by carefully following changes in morphology and phenotypical responses observed in HSC, obtained from either human or rat liver, when cultured on plastic substrate. HSC in these conditions undergo a trans-differentiation from the original "storing or quiescent phenotype" to the one of activated MFs, classically including the following relevant features (Friedman, 2000; 2008a; 2008b; Pinzani and Marra, 2001; Pinzani and Rombouts, 2004; Bataller and Brenner, 2005): a) high proliferative attitude; b) increased synthesis of ECM components, particularly fibrillar collagens, as well as of factors involved in ECM remodeling; c) ability to migrate (chemotactic response); d) increased synthesis of growth

factors (autocrine loops) and pro-inflammatory cytokines; e) contractility in response to vasoactive compounds. Although this scenario proposed on the basis of "in vitro" studies may represent an oversimplification, it is still considered as reasonably similar to the one that is likely to occur "in vivo". Moreover, it is believed that all pro-fibrogenic MFs, whatever the origin, may share a common pattern of phenotypic responses as well as to undergo a process of activation/transdifferentiation somewhat similar to the one described for HSC and HSC/MFs (Friedman 2008b; Parola et al., 2008; Parola and Pinzani 2009). We will expand these concepts later on in this chapter since the process of activation/transdifferentiation of HSCs into HSC/MFs is, as already mentioned, by far the best characterised.

4. Portal Fibroblasts and Portal Myofibroblasts

Portal fibroblasts (PFs) in the normal liver are similar for morphology and antigen repertoire to other fibroblasts and, unlike HSCs, express the highly specific fibroblast marker TE7 (Dranoff and Wells, 2010). Other markers considered specific for PFs include elastin, IL-6, fibulin 2 and the ecto-ATPase nucleoside triphosphate diphopshohydrolase-2 (NTPD2). The origin of PFs, as for HSCs, is still debated and uncompletely resolved. PFs as well as vascular smooth muscle cells are likely to originate from α-SMA positive cells of the ductal plate during human embryogenesis but a recent murine studies has suggested that HSCs and PFs may derive from a common putative precursor in the early embryo (Asahina et al., 2009).

Similar to what described for HSC, PFs can differentiate into α-SMA positive MFs (P/MFs) both "in vivo" under conditions of chronic liver injury as well as "in vitro" when cultured on plastic or glass. However, it should be noted immediately that P/MFs may theoretically originate not only by PFs but also from other sources, including vascular smooth muscle cells of the wall of hepatic artery or portal vein (Dranoff and Wells, 2010).

Convincing evidence for a major role of both PFs and P/MFs in liver fibrogenesis is actually available. There is little doubt that these pro-fibrogenic cells have a primary role in biliary fibrosis although most of our knowledge comes from experimental models of cholestatic injury, particularly the model of bile duct ligation in rodents (reviewed in Dranoff and Wells, 2010). The available evidence supporting a role for PFs and P/MFs in biliary fibrosis mostly relies on the knowledge that the injury to bile duct epithelial cells (BDEC) is a prerequisite for the diffentiation of PFs into P/MFs. The hypothesis is that, once damaged, BDEC express TGFβ2 and release a number of growth factors and pro-inflammatory mediators (including for example PDGF-BB, IL-6 and MCP-1) that are likely to be responsible for the initiation of the myofibroblastic differentiation of PFs that express related receptors. Similarly to what described for HSCs, this is then followed by an autocrine perpetuation by P/MFs. However, a number of studies indicate that P/MFs may also significantly contribute to CLD progression in other clinical conditions of different aetiology characterised by bridging fibrosis. As recently pointed out (Dranoff and Wells, 2010), the critical point is likely to be represented by the cross-talk between damaged and/or activated BDEC and PFs rather than the specific aetiology. Several investigators have shown the existence of a direct correlation between the intensity of the so-called ductular reaction (a peculiar form of hyperplastic response of BDEC) and the severity of fibrosis in either animal models as well as human liver diseases of different aetiologies, including chronic HCV and

NAFLD/NASH (Beaussier et al., 2007; Clouston et al., 2005; Fabris et al., 2007; Richardson et al., 2007). Moreover, as a final comment for the issue of PFs and P/MFs in liver fibrogenesis, it has been proposed (Dranoff and Wells, 2010) that PFs may be as multifunctional as activated HSCs or HSC/MFs, including a role in the liver progenitor cell niche and then in hepatic progenitor cell expansion and differentiation (see the paragraph dedicated to the roles attributed to hepatic MFs for more details).

5. Bone Marrow – Derived Cells and Hepatic MFs

Although current research in the field of regenerative medicine applied to liver diseases is actively exploring the therapeutic efficiency of autologous stem cell transplantation (including stem cells from bone marrow), clinical and experimental evidence indicates that under conditions of chronic liver injury, pro-fibrogenic MFs (mainly IF/MFs and, possibly, some P/MFs) can also originate from progressive recruitment of bone marrow derived cells (Kallis et al., 2007; Henderson and Forbes, 2008; Friedman, 2008b; Parola et al. 2008). The first evidence for a bone marrow and then extra-hepatic origin of MFs was provided by Forbes and co-workers (Forbes et al., 2004) which analysed fibrotic liver either of male patients (affected by CLDs of different aetiology) that had received liver transplants from female donors and subsequently developed CLD, or of a female that received bone marrow transplant from a male donor and afterward developed HCV-related cirrhosis. By employing fluorescence in situ hybridization (FISH) for the Y chromosome together immune-histochemistry for MFs specific antigens this group showed that a significant numbers of Y chromosome in fibrotic areas were found in the nuclei of α-SMA positive cells having a myofibroblast phenotype. In the liver transplant cases, a percentage of α-SMA positive MFs ranging from 6.8% to 22.2% contained the Y chromosome whereas in the female recipient of a bone marrow transplant from a male donor, 12.4% of the myofibroblasts were Y chromosome positive. This already very relevant study was followed by a number of carefully designed experimental studies that confirmed the concept (i.e., MFs may originate from circulating cells derived from bone marrow recruited into chronically injured liver) and were instrumental to identify at least two distinct populations of MFs precursors, mesenchymal stem cells (MSC; Russo et al., 2006; Valfrè di Bonzo et al., 2008) and circulating fibrocytes (Kisseleva et al., 2006).

Russo and coworkers (Russo et al., 2006) employed **f**emale mice which were first lethally irradiated, then received a transplant of whole bone marrow or cell population enriched in MSC from mice male donors and were finally submitted to different protocols of fibrosis induction. BM-derived cells were tracked through FISH analysis for the Y chromosome and results obtained indicated unequivocally that the bone marrow contributed significantly to hepatic stellate cell and MFs populations. Moreover, these bone marrow - derived MFs were able to actively synthesize collagen type 1 and originated largely from the mesenchymal stem cells. In a subsequent study (Valfrè di Bonzo et al., 2008), NOD-SCID mice were sub-lethally irradiated, transplanted with highly purified populations of ex-vivo expanded human MSC and then submitted to a protocol of chronic injury in order to induce fibrosis. When chimeric livers were then analyzed for expression of human transcripts and antigens it was found that a significant number of cells of human origin (identified by expression of HLA class I antigens)

exhibited a myofibroblast-like morphology. Interestingly, human MSC in their (myo)-fibroblastic phenotype were shown to respond by proliferation and or migration to the same cytokines and chemokines effective on HSC/MFs, including in particular PDGF-BB and MCP-1, indirectly suggesting that the pattern of polypeptide mediators known to be generated in CLDs may have a role in the hepatic recruitment/engraftment of MSC.

In a further study, Kisseleva and coworkers offered an additional contribute by performing experiments in which chimeric mice, transplanted with donor bone marrow from collagen alpha1(I)-GFP+ reporter mice, were subjected to the bile duct ligation (BDL)-induced liver injury. In response to injury, bone marrow-derived collagen-expressing GFP+ cells were detected in liver tissues of chimeric mice. These bone marrow – derived cells, that were negative for α-SMA and desmin (then distinct from HSC/MFs), were found to co-express collagen-GFP+ and CD45+, suggesting that these cells represent a unique population of circulating fibrocytes that, in addition, increased numerically in bone marrow and spleen of chimeric mice in response to injury. Moreover, these authors showed that these fibrocytes cultured in the presence of TGF-β1 differentiated into α-SMA and desmin positive collagen-producing MFs, potentially able to contribute to liver fibrosis.

Although a single report (Higashiyama et al., 2009), perhaps quite controversial (see Kallis and Forbes, 2009), has questioned the real relevance of the phenomenon, the overall scenario emerging from available data suggests that MFs derived from bone marrow – derived cells recruited to chronically injured liver can offer a significant additional contribution to liver fibrogenesis. Such a contribution, from a quantitative point of view, is likely to vary depending on both the aetiology and the progression rate of the single CLD.

6. Epithelial to Mesenchymal Transition, Hepatic MFs and Liver Fibrogenesis

Epithelial to mesenchymal transition (EMT) is a fundamental biological process, paradigmatic of the concept of cell plasticity, that leads epithelial cells to lose their polarization and specialized junctional structures, to undergo cytoskeleton reorganization, and to acquire morphological and functional features of mesenchymal-like cells, including the ability to migrate and to produce and secrete components of the extracellular matrix (Cannito et al., 2010). Although EMT and the related opposite process of MET (mesenchymal to epithelial transition) have been originally described in embryonic development, where cell migration and tissue remodeling have a primary role in regulating morphogenesis in multicellular organisms, recent literature has provided evidence suggesting that the EMT process is a more general process that may have a significant role in several pathophysiological conditions, including cancer progression and, pertinent to the subject of this chapter, organ fibrosis (Thiery and Sleeman, 2006; Acloque et al., 2009; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009; Cannito et al., 2010).

Where organ fibrosis is concerned, several laboratories have proposed that EMT may have a pathogenic role particularly in those conditions in which fibrosis may result from chronic and uncontrolled activation of wound-healing response, as it can be appreciated in a number of fibro- proliferative diseases in which progressive fibrogenesis (i.e., the process) with the time can result in the progressive accumulation of ECM components, derangement of

tissue and vascular architecture and eventually organ failure. Indeed (see specific discussion in Cannito et al., 2010), some of the most compelling data suggesting the involvement of EMT in organ fibrosis were generated in kidney fibrosis studies (Iwano et al., 2002; Yang et al., 2002; Zeisberg and Kalluri, 2004) in which, by using several approaches, including bone marrow chimaeras and transgenic reporter mice (Iwano et al., 2002), it was shown that a significant number of pro-fibrogenic kidney fibroblasts were positive for FSP1 (fibroblast specific protein 1), an antigen believed to be specific for fibroblast-like cells derived from local EMT.

More recently, several studies (reviewed in Choi and Diehl, 2009; Cannito et al., 2010) have proposed that pro-fibrogenic cells may originate in CLDs through EMT involving either cholangiocytes (BDEC) or hepatocytes. Where hepatocytes are concerned, although typical EMT changes were reported "in vitro" for either primary hepatocytes or non tumorigenic hepatocytic cell lines in culture, only recently (Zeisberg et al., 2007) it was described a progressive appearance in the injured livers of FSP-1 positive cells, although less than 10% of FSP-1+ cells were shown to co-express the typical and widely accepted MFs marker α-SMA. In the same study these authors also performed lineage-tracing experiments using *AlbCre.*R26Rstop*lacZ* double transgenic mice in order to investigate whether hepatocytes undergoing EMT may contribute significantly to fibrosis induced by chronic treatment with the hepatotoxin CCl_4. They reported that approx. 15% of hepatic cells were FSP-1 positive at the time of severe fibrosis and that approx. 5% of the hepatic cells were co-expressing either FSP-1 and albumin or FSP-1 and β-gal, suggestive of EMT. Moreover, these authors also performed experiments showing that BMP-7, which is known to antagonize TGFβ1 signalling, significantly inhibited progression of fibrosis and almost abolished putative EMT-derived fibroblasts. Similar results, once again suggesting a hepatocyte contribution to fibrosis by a TGFβ1 – dependent induction of EMT, were described by another group that employed a transgenic mouse model of Smad7 over expression in hepatocytes to counteract CCl_4 – induced fibrosis (Dooley et al; 2008). The latter study also reported preliminary morphological evidence for "in vivo" EMT in biopsies from chronic HBV patients in terms of positive hepatocyte nuclear staining for SNAI1 and phospho-Smad2 (the latter a sign of activation of TGFβ1 signalling).

Where BDEC are concerned, several "in vivo" and "in vitro" data have been published related to EMT of BDEC in either experimental and clinical conditions associated with a form of biliary fibrosis, which represent approx. less than 10% of CLDs in western countries. From an historical point of view, the first report suggesting a BDEC origin of portal MFs was published in 2006 (Xia et al., 2006). This study provided evidence suggesting that BDEC undergoing the process of bile ductular reaction in the rat model of secondary biliary fibrosis due to bile duct ligation were co-expressing α-SMA and cytokeratin 19 (CK-19, a BDEC marker that also stain hepatic progenitor cells or HPCs). Such an EMT scenario was reproduced "in vitro" by treating BDEC with TGFβ1 (followed by up-regulation of α-SMA and fibronectin, with down-regulation of CK19) and prevented, both in vivo and in vitro, by pre-treatment with HGF. The experimental scenario was confirmed in the same model of BDL (rat and murine) by a series of elegant studies from the group of Anna Mae Diehl (reviewed in Choi and Diehl, 2009), the most relevant (Omenetti et al., 2008a, 2008b, and reference therein) being able to describe an apparently clear cause-effect relationships among EMT of BDEC, appearance of portal MFs and biliary fibrosis as well as the closely related major involvement of Hedgehog signalling pathway. Moreover, the same group as well as

other groups described morphological evidence for EMT of BDEC (following co-expression of markers like CK19, α-SMA, HSP-47, phospho-Smad2/3, FSP-1, etc) also in liver biopsies from human patients affected by primary biliary cirrhosis (PBC, Jung et al., 2007; Robertson et al., 2007; Omenetti et al., 2008b), primary sclerosing cholangitis (Kirby et al., 2008) and biliary atresia (Diaz et al., 2008). Moreover, (Robertson et al., 2007), EMT of BDEC was also described in post-transplantation recurrence of PBC, and the relevance of Hedgehog and TGFβ1-Smad2/3 signalling was reported also in human patients (Jung et al., 2007; Omenetti et al., 2008b; Robertson et al., 2007; Rygiel et al., 2008). At present, two reports (Jung et al., 2007, 2008) have also provided the first evidence for EMT in BDEC and/or HPCs in biopsies from patients affected by alcoholic liver disease, with again Hedgehog and TGFβ1-Smad2/3 signalling having a major role.

Although the previously mentioned studies seem to support the view that EMT of either hepatocytes or BDEC may significantly contribute to liver fibrogenesis, a number of issues have very recently questioned whether EMT may be really involved in chronic liver diseases. A first cautionary issue is represented by actual re-evaluation of the specificity of FSP1 (also known as S100A4) as a marker of EMT which comes from carefully performed experiments in kidney. Some studies have revealed that FSP1 is not a marker for fibroblasts but rather for leukocytes and other non-fibroblastic cell types (Le Hir et al., 2005; Lin et al., 2008), raising obvious general questions on whether the involvement of EMT based on FSP1 immune-staining may be considered as appropriate. In addition, a recent elegant fate tracing study (using Cre/Lox techniques) has clearly shown that although genetically labelled primary proximal epithelial cells exposed in culture to TGFβ undergo apparent EMT becoming MF-like cells, no "in vivo" evidence was detected that epithelial cells may migrate outside of the tubular basement membrane and differentiate into interstitial MFs in a model of kidney fibrosis (Humphreys et al., 2010). By contrast, the same authors provided evidence suggesting that interstitial pericytes were the most likely direct precursors of α-SMA positive kidney MFs, a scenario that is indeed reminiscent of the "hepatic" one where HSCs are considered as liver specific pericytes.

Even more relevant, the group of Brenner has very recently published two elegant experimental studies that are challenging the involvement of EMT of either hepatocytes or cholangiocytes (or BDEC) as a pathogenic mechanism in liver fibrogenesis. In the first study (Taura et al., 2010) authors employed triple transgenic mice expressing ROSA26 stop beta-galactosidase (beta-gal), albumin Cre, and collagen alpha1(I) green fluorescent protein (GFP), in order to have hepatocyte-derived cells permanently labeled by beta-gal and type I collagen-expressing cells labeled by GFP. These engineered hepatocytes underwent changes towards a fibroblast morphology when cultured in the presence of TGFβ1 but when authors isolated hepatic cells from the liver of triple transgenic mice after induction of fibrosis (carbon tetrachloride chronic model) they could not find cells showing double-positivity for GFP and beta-gal. All beta-gal-positive cells exhibited the typical morphology of hepatocytes and did not expressed mesenchymal markers like α-SMA, FSP-1, desmin, or vimentin, whereas GFP-positive areas in fibrotic livers were coincident with fibrotic septa but never overlapped with X-gal-positive areas. Taura and coworkers then concluded that type I collagen-producing cells do not originate from hepatocytes.

In the second study that has been very recently become available (Scholten et al., 2010) EMT was again investigated with Cre/LoxP system in order to map cell fate CK-19 positive BDEC in CK-19(YFP) or FSP-1(YFP) mice that were generated by crossing tamoxifen-

inducible CK-19(CreERT) mice or FSP-1(Cre) mice with Rosa26(f/f-YFP) mice. MET of GFAP(+) HSCs was studied in GFAP(GFP) mice. Transgenic mice were then subjected to bile duct ligation or chronic carbon tetrachloride treatment. When the livers of fibrotic transgenic mice were analyzed specific immunostaining of CK-19(YFP) cholangiocytes showed no expression of EMT markers such as α-SMA, desmin, or FSP-1. Moreover, cells genetically labeled by FSP-1(YFP) expression did not coexpress neither the cholangiocyte marker CK-19 nor E-cadherin. Finally, genetically labeled GFAP(GFP) HSCs did not express epithelial or liver progenitor markers in response to liver injury. These results led authors to two major conclusions: a) EMT of BDEC does not contribute to liver fibrogenesis in murine models; b) HSCs do not apparently co-express epithelial markers suggesting that these cells do not apparently undergo MET in response to fibrogenic liver injury, an hypothesis raised recently by some authors (Yang et al., 2008).

As a take-home message, at present the real involvement of EMT as a pathogenic mechanism contributing to liver fibrogenesis in CLDs is still controversial and represents the subject of an intense debate. The interested reader may find excellent discussion "pro" and contra" in two recently published editorials (Wells, 2010; Popov and Schuppan, 2010), including the caution in accepting as unequivocal data coming from transgenic models. As a matter of fact, as nicely pointed out by Popov and Schuppan, (2010), Cre/Lox system has a major limitation: it is an excellent tool to demonstrate that lineage conversion does occur but suffers of the fact that efficiency of Cre-mediated recombination in vivo is never total. For example, in their paper Scholten and coworkers indicated just a partial (i.e., 40%) efficiency of Cre recombination and this does not allow to unequivocally rule out that a significant subset of cholangiocytes may have not been traced.

7. Role of Hepatic MFs in Liver Fibrogenesis snd Chronic Liver Diseases

As previously suggested, hepatic MFs may originate from different cell sources through a process of activation/transdifferentiation that may involve common mediators, mechanisms and signalling pathways, but our present knowledge mostly derives from "in vivo" and "in vitro" studies performed on activated human or rodent HSC (Friedman, 2008a). Accordingly, here we will mainly refer to activation of HSC to HSC/MFs and to main responses operated by HSC/MFs.

Based on actual knowledge, activation of HSC, following chronic liver injury, progress in sequential stages of initiation and perpetuation (Friedman, 2008a; 2008b). Initiation should be considered as an early response stimulated by several paracrine signals, leading to a transient and potentially reversible contractile and profibrogenic phenotype. This "transient" phenotype is characterized by a rapid induction of platelet-derived growth factor (PDGF) β receptor expression and is primed to respond to several growth factors and mediators that will be crucial in eliciting phenotypic responses operated by fully activated MF-like phenotype (perpetuation), including those already cited of proliferation, migration/chemotaxis, contractility, excess deposition and altered remodelling of ECM and much more (see Figure 2). As previously mentioned, it is likely that at least portal fibroblasts (Dranoff and Wells,

2010) and, possibly, circulating bone marrow -derived cells recruited in the chronically injured liver may follow a similar process of activation and differentiation.

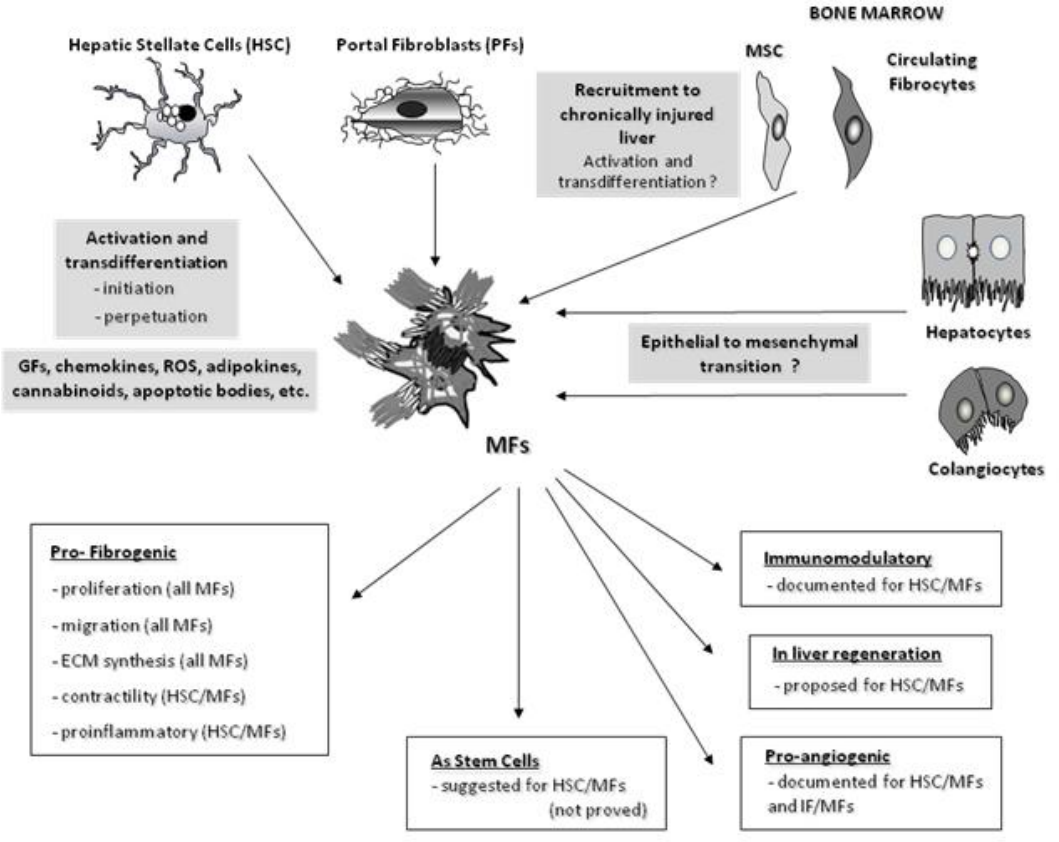

Figure 2. MFs: origin and role fibrogensis.

7.1. The Phenotypic Responses of HSC/MFs that May Be Common to All Hepatic MFs

Proliferation of activated MF-like cells is elicited by a number of mitogens, including PDGF, bFGF, Angiotensin II, VEGF and thrombin (Pinzani and Marra, 2001; Friedman, 2008b; Parola et al., 2008). However, proliferation of hepatic MFs is mainly sustained by homo- and heterodimeric PDGF isoforms, either released by activated kupffer cells, sinusoidal endothelial cells, platelets or by activated MFs in an autocrine pathway, as well as by sustained expression of related receptors. PDGF-dependent down-stream signalling involves Ras/ERK pathway, phosphatidyl-inositol 3-kinase (PI-3K), ERK5 and others (Pinzani and Marra, 2001; Friedman, 2008b). In particular, the homodimer PDGF-BB also represents the best characterized and most potent chemoattractant for HSC/MFs (Pinzani and Marra, 2001) and MF-like cells from human MSC (Valfrè di Bonzo et al., 2008), with migration / chemotaxis being an essential feature of MFs in order to reach the site of injury

and to align with both nascent and established fibrotic septa. Migration/chemotaxis of hepatic MFs is also elicited by monocyte chemoattractant protein 1 (MCP-1), angiotensin II, vascular endothelial growth factor (VEGF), angiopoietin-1, reactive oxygen species (ROS), CXCR3 ligands, all polypeptides and mediators involving primarily Ras/ERK signalling (Novo et al., 2007; Friedman, 2008; Novo and Parola, 2008).

Being the most relevant pro-fibrogenic cells, hepatic MFs are of course the main responsible for excess deposition of fibrillar matrix (mainly collagen type I and III), a hallmark of fibrotic and cirrhotic livers. Hepatic MFs synthesize ECM components primarily as a response to TGFβ1 released by Kupffer cells as well as, when CLD is established, by HSC/MFs (paracrine and autocrine sources), through downstream signalling involving Smads 2 and 3. Connective tissue growth factor (CTGF) and cannabinoids have been also identified as potent profibrogenic signals for HSC/MFs (Friedman, 2008b).

In addition, HSC/MFs show features of smooth muscle cells and contractility; by responding to opposing vasoactive mediators such as endothelin-1 and nitric oxide, they contribute to increased portal resistance during early stages of fibrosis whereas the late and persistent increase in portal pressure found in the cirrhotic liver is largely due to the distortion of hepatic angioarchitecture.

7.2. Hepatic MFs, Matrix Degradation/Remodelling and Resolution of Fibrosis

Progressive fibrogenesis is typically characterized by replacement of low-density basement membrane of the subendothelial space of Disse with fibril-forming matrix, a scenario that negatively affect differentiated cell functions (mainly of hepatocytes). This scenario is believed to result primarily from a disequilibrium between excess deposition of fibrillar collagens as well as of other ECM components and a reduced/altered degradation and remodelling of fibrotic ECM. Accordingly, literature data have unequivocally shown that HSC/MFs mainly express metallo-proteinases (MMPs) able to degrade basement membrane (MMP-2, MMP-9, MMP3 or stromelysin) that are less efficient to degrade fibrillar matrix, with a low expression of MMP-1 (interstitial collagenase). HSC/MFs and, more generally, MFs also overexpress tissue inhibitor of metalloproteinase type 1 (TIMP-1) that inhibit interstitial collagenases and act as anti-apoptotic for HSC/MFs. Deposition of fibrillar matrix and formation of fibrotic septa is also favoured by the fact that HSC/MFs and, likely, all MFs, develop resistance to induction of apoptosis (El-Sharkawy et al., 2005; Novo et al., 2006; Friedman, 2008b; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010). Related to this concept are findings of the last decade suggesting that (Iredale, 2007; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010) liver fibrosis and, possibly, initial stages of cirrhosis are potentially reversible in the presence of effective therapy and/or aetiology eradication. Regression of histopathology develops as a result of increased apoptosis of HSC/MFs and MFs and is paralled by increased expression of interstitial collagenases by hepatic macrophages. However, it should be noted that based on the absence of any unequivocal clinical finding, most researchers believe that advanced human cirrhosis is unlikely to regress (Iredale, 2007; Henderson and Iredale, 2007; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010).

7.3. HSC/MFs, Inflammatory Signalling and Regulation of Immune Response

Persisting inflammatory response in a CLD is considered as one of the major "driving forces" sustaining fibrogenesis. HSC/MF and, likely, all MFs behave like target cells for inflammatory cytokines and other pro-inflammatory signals, including: a) ROS and other oxidative stress - related mediators like 4-hydroxy-2,3-nonenal or HNE, generated as a consequence of hepatocyte injury and necrosis; b) apoptotic bodies (engulfing and activating); c) bacterial endotoxin or other endogenous activators of Toll Like Receptor 4 (TLR4) of innate immunity displayed by HSC/MFs. On the other hand, HSC/MFs have been unequivocally shown to represent the cell source (even in an autocrine manner) of a number of pro-inflammatory molecules, including TLR ligands, MCP-1 and other chemoattractants and chemokines (Bataller & Brenner, 2005; Friedman, 2008b).

Unexpectedly, some years ago activated HSC have emerged as significant mediators and modulators of hepatic immunoregulation (reviewed in Friedman, 2008a; 2008b), a scenario that may be relevant also for fibrogenesis progression. By expanding the concept previously introduced, HSC/MFs are able to produce and release several chemokines including not only MCP-1 but also RANTES, CCL21 and CCR5, being able to amplify the inflammatory response by favouring the infiltration of either mononuclear or polimorphonuclear leukocytes. Moreover, they also express TLRs and then can respond to the presence of endotoxin (LPS). TLRs, which are pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs) to discriminate the products of microorganisms from the host, are expressed on Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, hepatic stellate cells, and hepatocytes in the liver. TLR signaling can induce potent innate immune responses in these cell types and this is relevant since the liver is constantly exposed to PAMPs, such as LPS and bacterial DNA through bacterial translocation from intestine. Recent evidence demonstrates the role of TLRs in the activation of hepatic immune cells and stellate cells during liver fibrosis. Activated human HSC/MFs express not only TLR4 but also CD14 and MD2, forming the LPS receptor; LPS then lead to activation of NF-kB and JNK isoforms resulting in the synthesis and release of chemokines and adhesion molecules. Moreover, crosstalk between TLR4 signaling and TGF-beta signaling in hepatic stellate cells has been reported, suggesting an additional pro-fibrogenic mechanism (Aoyama et al., 2010).

Concerning the immune modulatory action, a number of relevant issues should be outlined for HSCs and MFs (the interested reader may refer to excellent comprehensive reviews including Friedman, 2008a; 2008b; Unanue, 2007):

1) they can act as professional antigen presenting cells (reviewed in Unanue, 2007), able to stimulate either lymphocyte proliferation or apoptosis;
2) these cells can regulate leukocyte behaviour and are affected by specific lymphocyte populations, with CD8 lymphocytes, being more pro-fibrogenic towards HSC/MFs than CD4 cells;
3) these cells can induce locally immunotolerance throughout T cell suppression;
4) natural killer cells (NK cells) seems able to selectively kill HSC/MFs (stimulated by interferon and inhibited by ethanol).

7.4. HSC/MFs, hepatic MFs and Liver Angiogenesis

HSC/MFs and at least IF/MFs have an additional dual role in pathological angiogenesis that accompanies progression of CLDs. Here, a brief analysis of major findings and concepts supporting the emerging pathogenic role of angiogenesis in CLDs is offered, with a specific emphasis on the crucial role of hypoxic conditions and hepatic stellate cells (HSCs), particularly when activated to the myofibroblast - like pro-fibrogenic phenotype. The interested reader can refer to recent more comprehensive reviews for more details (Medina et al., 2004; Fernandez et al., 2009; Valfrè di Bonzo et al., 2009). The following major concepts and findings correlating MFs, fibrogenesis and angiogenesis should be outlined.

1) Angiogenesis and up-regulation of VEGF expression has been documented in different experimental models of acute and chronic liver injury as well as in specimens from human fibrotic/cirrhotic liver, including chronic infection by HBV and HCV, and autoimmune diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).
2) In both experimental and clinical conditions angiogenesis and fibrogenesis develop in parallel and a the strict relationships between hypoxia, angiogenesis, VEGF expression and fibrogenesis has been observed in liver specimens from fibrotic/cirrhotic rodent and human livers.
3) VEGF expression, as detected in either experimental and clinical conditions, is mostly limited to hepatocytes and to HSC/MFs and, possibly, other hepatic myofibroblasts.
4) Other polypeptides have been involved in hepatic angiogenesis associated with the fibrogenic progression process in CLDs, including, in particular, leptin and also Hedgehog (Hh) ligands.
5) Where hepatic MFs are concerned, these cells have also been reported to play a significant pro-angiogenic role. This is particularly true for hepatic stellate cells even in physiological angiogenesis because of their role as liver specific pericytes, connected to their strategic location in the space of Disse and intimate contact with sinusoidal ECs (Friedman 2008a). As for recent literature data, HSC/MFs should be considered as a hypoxia – sensitive, cyto- and chemokine-modulated cellular crossroad between necro-inflammation, pathological angiogenesis and fibrogenesis on the basis of the following major concepts (reviewed in Fernandez et al., 2009; Valfrè di Bonzo et al., 2009) : a) HSC and HSC/MFs react to conditions of hypoxia and leptin by up-regulating transcription and synthesis of VEGF, Angiopoietin 1, as well as of their related receptor VEGFR-2 and Tie2; b) HSC/MFs respond to the action of VEGF and Angiopoietin 1 in terms of proliferation, increased deposition of ECM components and increased migration and chemotaxis (Novo et al., 2007).

   This is a scenario that is likely to be relevant for the *in vivo* progression of a CLD. In both human and rat fibrotic/cirrhotic livers (Novo et al., 2007) α-SMA-positive MFs able to express concomitantly VEGF, Ang-1 or the related receptors VEGFR-2 and Tie-2, are found at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa. This distribution may reflect the existence of an early phase of CLD, occurring in developing septa, in which fibrogenesis and

angiogenesis may be driven/modulated by HSC/MFs, and of a later phase occurring in larger and more mature fibrotic septa where the chronic wound healing is less active and fibrogenic transformation more established. In the late setting pro-angiogenic factors are expressed only by endothelial cells, a scenario that is likely to favour the stabilization of the newly formed vessels.

6) Angiogenesis is now seen as a potential therapeutic target in the treatment of CLDs (reviewed in Fernandez et al., 2009; Valfrè di Bonzo et al., 2009). The bulk of available experimental data indeed indicate that antiangiogenic therapy is effective in preventing progressive fibrogenesis. Moreover, most of these treatments were also able to significantly inhibit the development of portal hypertension, porto-systemic collateral vessels and hyperdynamic splanchnic circulation. This putative new therapeutic strategy is at present debated and requires properly designed clinical trials.

7.5. Hepatic MFs in Liver Regeneration and Cancer

The role of hepatic MFs in liver regeneration has been originally limited to the notion that these cellular elements, particularly HSC/MFs, may promote liver regeneration by producing growth factors for either mature epithelial cells or oval cells, bipotent progenitors of hepatocytes and cholangiocytes (Pinzani & Marra, 2001, Friedman 2008a, 2008b). However two recent studies have significantly modified this scenario adding fascinating hypotheses.

In a first study HSC/MFs, that are known to contribute to liver stem cell niche, were shown to express also the stem cell marker CD133 (Kordes et al., 2007) and it has proposed that they may then directly differentiate into stem or precursor cells. As recently discussed by Friedman (2008b), this is a fascinating hypothesis for liver regeneration but it may also offer a possible explanation for the fact that fibrosis is a "near-absolute" requirement for the development of hepatocellular carcinoma (HCC). Related facts and hypothesis are the following: neoplastic cells may derive either from hepatic progenitor cells (HPCs) or adult and DNA-damaged hepatocytes sustained by paracrine or survival factors released by MFs or directly from HSC/MFs through a process of mesenchymal to epithelial transition into HPCs (speculative, but supported by the notion that in HSC/MFs operate hedgehog and Wnt signalling, commonly implicated in stem cell differentiation and cancer). This still speculative but fascinating scenario related to HCC may include the reduced tumour surveillance and decrease in NK cells number and function detected particularly in HCV patients.

Along these lines, it is worth mentioning a second study (Yang et al., 2008) in which authors evaluated more directly the hypothesis that HSC may represent a type of oval cell and, thus, being capable to generate hepatocytes to repopulate injured livers. In this elegant study, since quiescent HSC express glial fibrillary acidic protein (GFAP), mice in which GFAP promoter elements regulated Cre-recombinase were crossed with ROSA-loxP-stop-loxP-green fluorescent protein (GFP) mice to generate GFAP-Cre/GFP double-transgenic mice. These mice were fed methionine choline-deficient, ethionine-supplemented diets to activate and expand HSC and oval cell populations. GFP-positive progeny of GFAP-expressing precursor cells were then characterized by immunohistochemistry. Images clearly indicated that when activated by liver injury or culture, HSC downregulated expression of

GFAP but remained GFP(+); they became highly proliferative and began to coexpress markers of mesenchymal and oval cells. These transitional cells apparently disappeared as GFP-expressing hepatocytes emerged, began to express albumin, and eventually repopulated large areas of the hepatic parenchyma. These findings led Authors to suggest that HSC are a type of oval cell transiting through a mesenchymal phase before to differentiate into hepatocytes during liver regeneration.

Conclusion

Hepatic MFs represent a rather heterogenous population of cells that sustain liver fibrogenesis and then fibrotic progression of chronic liver diseases of different aetiology to the common advanced-stage of cirrhosis. These highly proliferative and contractile myofibroblasts actively contribute to the fibrogenic progression of the chronic liver disease, irrespective of the aetiology, by means of their multiple phenotypic responses. The hallmarks of this fascinating phenotype include excess deposition of extracellular matrix components and its altered remodelling as well as the synthesis and the release in a paracrine/autocrine way of a number of critical growth factor which sustain and perpetuate fibrogenesis, chronic inflammatory response and neoangiogenesis. According to current literature hepatic MFs, which are mostly α-smooth muscle actin (α-SMA) - positive cells, mainly originate from hepatic stellate cells or from fibroblasts of portal areas through a process of activation and trans-differentiation, with some MFs also reported to originate from bone marrow – derived stem cells such as mesenchymal stem cells or circulating fibrocytes, able to engraft chronically injured liver. It is currently debated whether myofibroblasts may also originate from hepatocytes and cholangiocytes through a process of epithelial to mesenchymal transition. Hepatic myofibroblasts have been reported to play additional crucial roles, including modulation of immune responses in the chronically injured liver and the cross talk with hepatic progenitor (stem) cells as well as with malignant cells either of primary hepatocellular carcinomas or of metastatic cancers.

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Chapter 6

Cirrhosis of Liver Due to Hepatic Vena Cava Disease

Santosh Man Shrestha*
Liver Foundation, Nepal

Introduction

Liver cirrhosis (LC) is not a single disease. It is the outcome of different diseases that are associated with chronic loss of hepatocytes and its replacement by fibrosis and formation of regenerative nodules. The process distorts the architecture of liver parenchyma resulting in development of porto-systemic shunting of blood and impairment of hepatic function. Chronic alcohol abuse, chronic hepatitis B virus (HBV) and chronic hepatitis C virus (HCV) infections are major causes of liver cirrhosis in the world. There are other minor causes of LC (Table 1). Prevalence of the type of LC in any country or community is determined by geo-cultural factors. Whereas HBV infection is the major cause cirrhosis in Asia and Africa, HCV infection is the predominant cause in Japan [1, 2]. Alcohol and now emerging HCV infection are important causes of cirrhosis in the West [3, 4]. Among minor causes of cirrhosis hepatic vena cava disease (HVD) is reported only from developing countries [5-10]. The reported incidence of LC in HVD (Table 2) varied from 71 to 100% [11-14].

* Address for correspondence: Dr Santosh Man Shrestha MBBS, DTM&H, FRCPE, Liver Foundation Nepal, Sitapaela Height, PO Box 3439, Kathmandu, Nepal, Email: liver@ mail.com.np

Table 1. Causes of Liver Cirrhosis

I Common
Alcohol
Chronic hepatitis B
Chronic hepatitis C
II Uncommon
Hepatic vena cava disease
Autoimmune chronic hepatitis
Primary biliary cirrhosis
Wilson's disease
Hemochromatosis
III Rare
Hepatic vein thrombosis (Budd-Chiari syndrome)
Sinusoidal obstruction syndrome (Veno-occlusive disease)
Glycogen storage disease
Alpha 1-antitrypsine deficiency
Galactosemia
Tyrosinemia

Table 2. Prevalence of LC and HCC in patients with HVD from literature

Authors/Country	No. of patients	LC (%)	HCC (%)	Ref No
With IVCO				
Nakamura et al				
1968, Japan	7	7 (100%)	2 (28.5%)	2
Nakamura et al				
1968 Japanese Literature (100%) (43.7%)	64*	64	28	2
Takeuchi et al				
1971, Japan	7	5 (71%)	1 (14%)	3
Nakamura & Takezawa				
1982, Japan (100%)	13**	13	6 (46%)	5
Simson				
1982, South Africa (43.7%)	101*		48	11
Rector et al				
1985, USA	10		2 (20%)	14
Kew et al				
1989, South Africa	15		6 (47.5%)	12
Okuda et al				
1995, Japan	148		10 (6.7%)	18
Shrestha et al				
1995, Nepal	150		7 (4.6%)	9
Dilawari et al				
1999, India	115		9 (7.8%)	7
Shrestha				
2009, Nepal (78.5%)	56**	44	6 (10.7%)	

Table 3. Classification of Hepatic Venous Outflow tract Obstruction

Particulars	Sinusoidal Obstruction Syndrome (Veno-occlusive Disease)	Hepatic Vein Thrombosis (Budd-Chiari Syndrome)	Hepatic Vena Cava Disease (IVC Obstruction)
Primary site of lesion Secondary site	Sinusoidal endothelium -	Hepatic Veins PV/IVC	Hepatic portion of IVC HV/ Radicals of HV/PV
Etiology	Toxic injury - Pyrrolizidine alkaloids - Immunosuppressive drug Azothioprine	Thrombosis due to -Hypercoagulable state Myeloproliferative disease Coagulation defect	Thrombophlebitis -Bacterial infection
Epidemiology	Sporadic in person with organ transplantation Outbreak: contamination of wheat with seeds of crotaleria, senachio etc	Sporadic- rare	Endemic in developing countries
Clinical	Acute: hepatomegaly, ascites, jaundice or chronic Mild/ moderate/ severe	Acute/subacute acute exacerbation Rarely fulminant	Chronic with recurrent Rarely acute
Outcome	Spontaneous recovery LC -rare	Acute liver failure-rare LC- rare	LC- common & HCC- moderate

IVC= Inferior vena cava, HV= Hepatic vein, PV=Portal vein, LC=Liver cirrhosis, HCC=hepatocellular carcinoma

Table 4. Incidence of Hepatocellular Carcinoma in various types of Cirrhosis

Incidence of HCC	Types of LC
High	Hemochromatosis (20-30%) Alpha 1-antitrypsin deficiency (30%) Tyrosenemia (40%)
Moderate	Hepatic vena cava disease (10-14%) Alcohol (5-10%)
Low	Autoimmune chronic hepatitis Wilson's disease Primary biliary cirrhosis

Hepatic vena cava disease is a form of hepatic venous outflow obstruction (HVOO) that results in hepatic venous congestion and portal hypertension (Table 3). Previously it was grouped together with hepatic vein thrombosis due to prothrombotic disorders like myeloproliferative disorder and Factor V Lyden deficiency under Budd-Chiari syndrome (BCS) [15, 16]. Factor V Lyden deficiency frequently also causes thrombosis of IVC that transforms into a membrane on resolution [17]. Though HVT and HVD share some common anatomical sites of involvement in the hepatic outflow tract, the epidemiology, clinical features and natural history of the two diseases are different [14, 15, 18, 19]. HVD is a primary disease of the hepatic portion of the IVC caused by bacterial infection [20]. Initial thrombophlebitis typically involves the posterior wall of the IVC opposite the site of the entry

of hepatic veins (Fig 1). This site in the IVC is prone to microscopic endothelial damage as it is the junction between the upper part that is fixed in the groove of the liver and diaphragm and the distal part that is free [21]. At this site hepatic blood flow rich in coagulant factors joins the IVC at right angle. Damaged intima invites infection and thrombosis. High incidence of gram negative bacteremia in developing countries [22] predisposes this site to thrombophlebitis [20]. Hepatic vein ostia which are anatomically part of the IVC are commonly involved in the process. If the initial lesion is large (Fig 2) it may cause symptoms of acute HVOO or caval obstruction [23]. If the lesion is small (Fig 3) the event may go unnoticed. Resolution of the lesion results in localized stenosis or rarely complete obstruction (Fig 4). Cavo-caval collateral anastomosis develops in an attempt to restore the hemodynamic balance. The damaged part of the IVC becomes susceptible to re-infection whenever bacteremia occurs (Fig 5), as it happens in damaged heart valve in subacute bacterial endocarditis. Re-infection is associated with deposition of fresh thrombus at the site of the lesion and results in acute exacerbation (AE) of the disease [24].

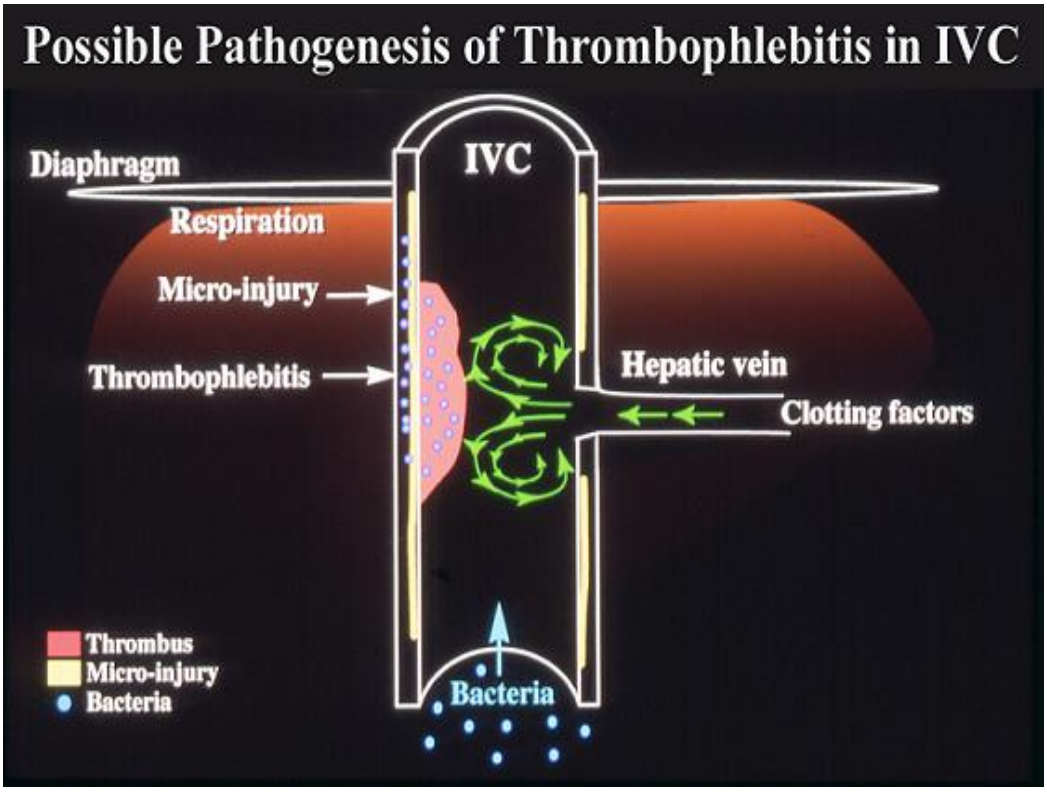


Figure 1. Pathogenesis of Hepatic Vena Cava Disease.

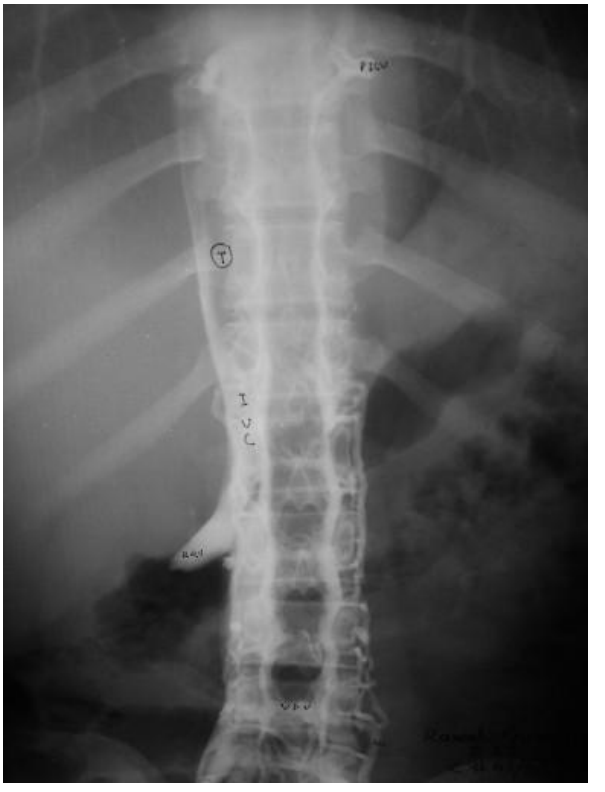


Figure2. Cavogram of a patient with acute HVD: showing a large thrombophlebitis (reprinted with permission from J Gastroentrology & Hepatology).

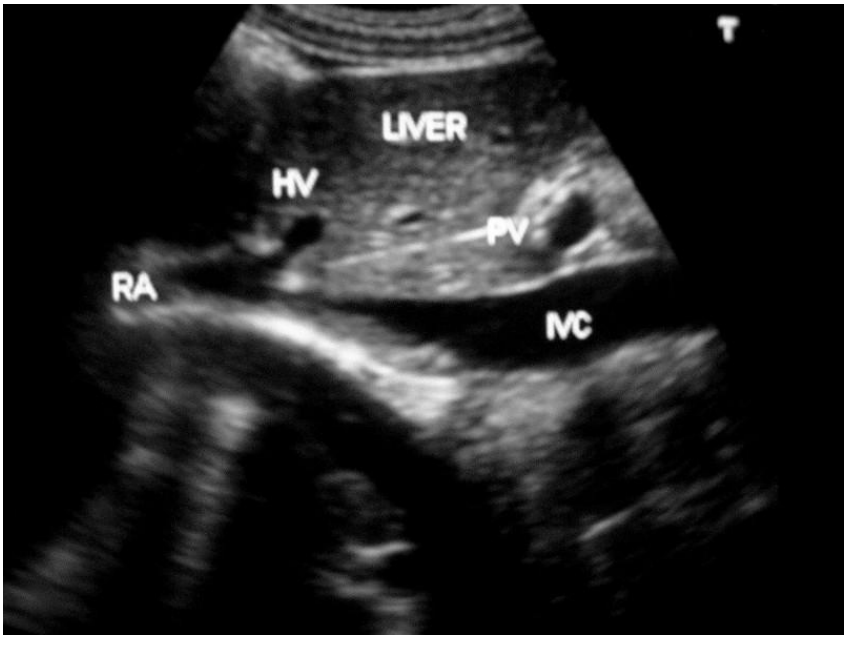


Figure 3. US of a patient with sub-acute HVD: shows localized narrowing of the IVC near cavo-atrial junction with thick echoic posterior wall and a thin deposit of thrombus.

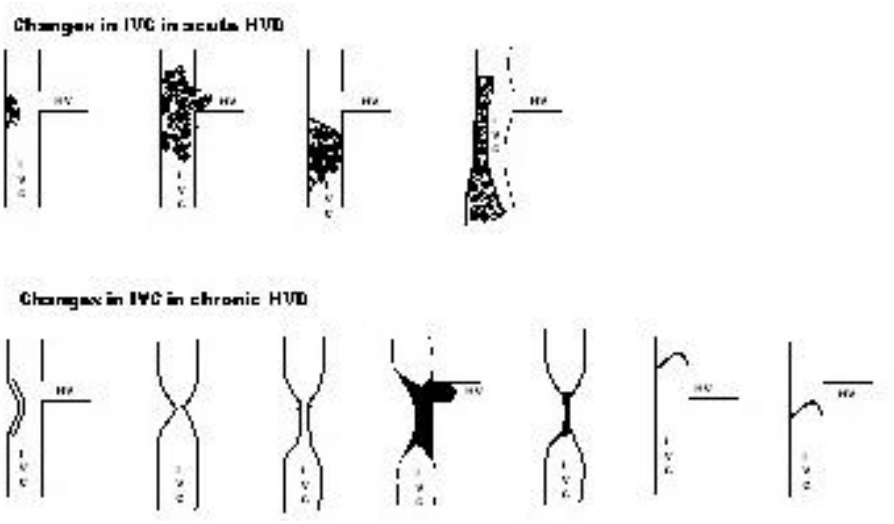


Figure 4. Acute and chronic changes in the IVC in HVD

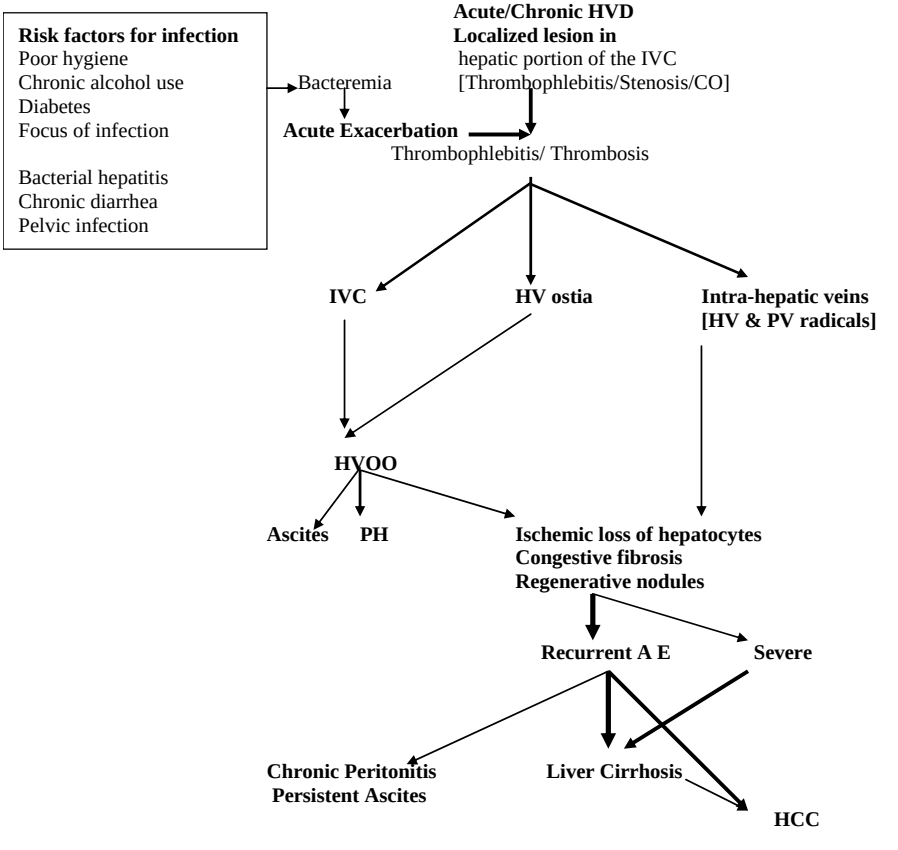


Figure 5. Pathogenesis of Acute Exacerbations.

Hepatic vena cava disease in the past was considered a congenital vascular malformation [25] and it was variously described as Budd-Chiari syndrome affecting the hepatic portion of the IVC, membranous obstruction of the IVC, coarctation of the IVC, chronic Budd-Chiari syndrome, Asian-African type of BCS or obliterative hepatocavopathy. Sometime HVD and HVT are not differentiated and HVD was sometime described under hepatic vein occlusion [26] or under BCS [27]. The proposed name "Hepatic Vena Cava Disease" is considered appropriate as it is a primary disease of the hepatic portion of the IVC where the lesion in IVC takes various forms from a localized thrombophlebitis in acute stage to mild to severe stenosis or thick fibrous obliteration in chronic stage [20, 28].

Hepatic vena cava disease was considered a rare disease. But as Wang in China found out with increasing awareness of the syndrome and the use of imaging techniques number of cases of the IVC obstruction (IVCO) detected in developing countries have increased [9]. Recognition of early stage of the disease [23] and its easy diagnosis by ultrasound and color Doppler has further widened the diagnostic net. The disease is endemic in China, India and Nepal [5-10]. It is a common cause of hospital admission of patients with liver disease and an important cause of LC and hepatocellular carcinoma in Nepal (HCC) [10, 29, 30].

Historical Background

William Osler [31] described a case of HVD in 1878 in his paper 'Case of obliteration of vena cava inferior with great stenosis of orifices of hepatic veins'. The clinical features and autopsy findings mentioned in the paper represents a classical description of HVD. The disease however was recognized much earlier. According to Pleasants [32] of Johns Hopkins University who described 14 cases and reviewed 314 cases from the literature in 1911, the credit for the first autopsy description of the disease goes to Schenks of Lyons (1644) and for recognition of collateral circulation that developed following IVCO to many that included Langhanns (1747), Haller (1756), Sappy-Dumontpallier (1862). There were several reports of the disease in the past [33-36]. However, as Dixon Mann and Walker Hall [37] had commented while reporting a case in Edinburgh Medical Journal in 1904- "although not of exceptionally rare occurrence, obstruction of the inferior vena cava is rarely described in the ordinary text-book of medicine" (pp 56). As such the disease lost its separate identity and became less recognized till attention to it was drawn again by many reports from Japan in the first half of the 20^{th} century [11-13, 38-41]. Nakamura et al [11] who in 1968 reviewed the published Japanese literature found that the disease was complicated by a very high incidence of cirrhosis (90%) and HCC (41%). Similar high incidence of HCC in the disease was reported from South Africa by Simson [42] and Kew [43]. Occurrence of cirrhosis in the HVD was mentioned earlier by Willcocks from UK in 1896 [34] and that of cirrhosis and hepatocellular carcinoma by Rosenblatt (1867) from Germany [44] and Hutchinson and Simpson (1930) from UK [45]. With the improvement in hygiene the disease has disappeared from the West and Japan. It is now confined to developing countries.

Epidemiology of HVD

Epidemiology of hepatic vena cava disease is not well established, because in the past the differential diagnosis between HVD and HVT was not clear and most reports with large number of cases did not separate the two disorders [26]. Further the diagnosis of HVD was then based on findings of complete membranous obstruction of the IVC either at autopsy or by imaging techniques [43]. Complete obstruction of the IVC is the end stage of the pathological process of the lesion in IVC seen only in a few [14]. Stenosis (Fig 6a, 6b) is more common which led Madangopalan and colleagues in India to name it 'coarctation of inferior vena cava' [46]. The narrowing of the hepatic portion of the IVC seen in cavogram was earlier thought to be due to compression by enlarged caudate lobe of the liver as it occurs in HVT [47]. In HVD the narrowing of this part of the IVC is due to stenosis [20, 26, 46, 48] and is not associated with caudate lobe enlargement.

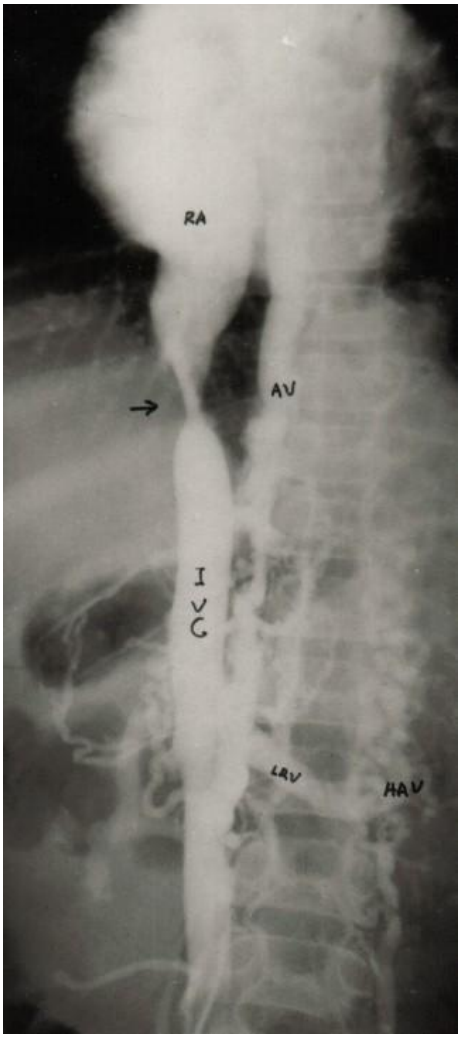


Figure 6a. Cavogram of a young boy with IVC Stenosis. Also shows deep collaterals Azygos and Hemi-Azygos veins.

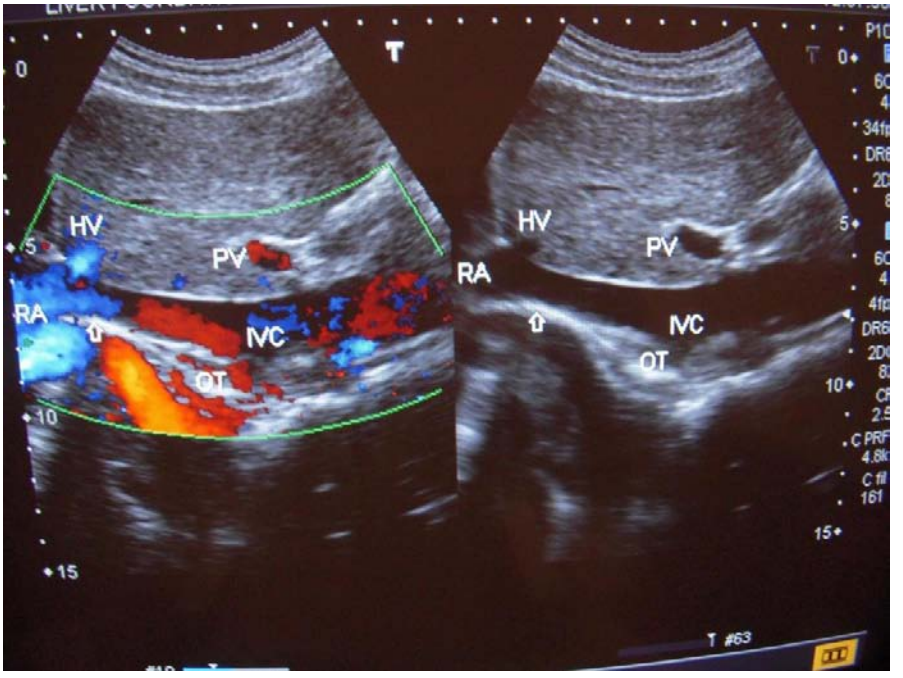


Figure 6b. US showing Stenosis of the IVC near cavo-atrial junction. Attached to the proximal end of the posterior wall of the stenosed segment are layers of organizing thrombus.

Congestive changes in the liver biopsy corroborated with cavographic evidence of IVCO [42] and its incidence in liver biopsies gives an estimate of the prevalence of the disease. The incidence of congestive cirrhosis and IVCO among 110 autopsied cases of HCC in Japan in 1921 was 8% [49] which dropped to 0.09% in 1980s [quoted in [50]. Epidemiological study of the disease carried out in Japan in 1990 showed existence of about 300 cases in the country (prevalence of 2.4/million) with 20 new cases occurring every year [50]. The incidence of chronic congestive fibrosis among a total of 1675 liver biopsy in South Africa was 4.9% [42], indicating to the possibility of a large number of the cases in that country.

Review of the reported cases in the world literature in 1980 led Okuda [51] to conclude that incidence of the disease in different countries was inversely related to the level of general hygienic state. HVD is a disease associated with poor hygiene and sanitation [52]. It's prevalence in developing countries is likely to be high. However it was reported only from a few countries and from a few centers in these countries indicating that its incidence probably depended on the level of awareness about the disease among institutions. Ultrasonogram is specific and sensitive for diagnostic of HVD [53, 54]. The procedure is available in all developing countries. It is cheap, may be repeated and is convenient for follow-up studies [14]. Routine use of US and color Doppler of IVC and liver in patients with acute and chronic liver diseases as well in those with prolonged bacterial infection have increased detection of HVD in Nepal. High incidence of HVD in Nepal and report of 2,677 cases from four hospitals in China during the period 1981 to 2003, of which 95% underwent surgery or intervention procedures [55] suggested that the disease at present is probably under diagnosed in many developing countries [56]. The cause of LC and HCC in 25 to 55% and 42-46% respectively in developing countries is unknown [57-62]. It is possible some of these may be due to HVD.

Natural History of HVD [14]

Hepatic vena cava disease occurs in both sexes and in all age groups from infancy to old age but has a high incidence in male in second decade. It is a chronic disease with insidious onset and long asymptomatic period. Two important features of the disease are development of vascular collateral anastomosis and occurrence of recurrent AE. AE are precipitated by bacterial infection. Symptoms of AE include fever, jaundice, ascites and hepatomegaly, edema of legs or pleural effusion. However diagnosis of HVD requires high index of suspicion, otherwise it may be missed or miss-diagnosed as other diseases such as acute hepatitis, acalculus cholecystitis, or TB peritonitis or pleural effusion.

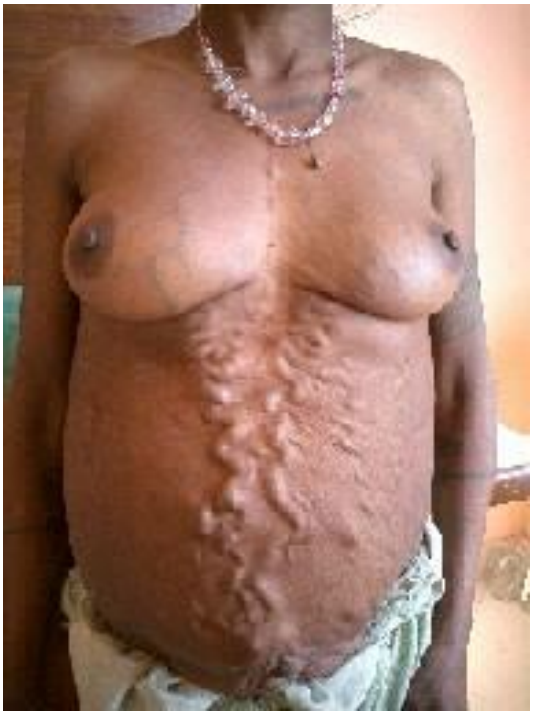


Figure 7a. Superficial collaterals in a patient with LC due to HVD: dilated superficial veins in abdomen in a female patient with complete IVC obstruction. The collaterals developed long before the development of LC.

Chronic disease may be associated with dilated superficial veins in the body trunk with upward flow (Fig 7a, 7b), varicose veins in legs, intermittent leg swelling, hepatomegaly or splenomegaly or sterility. It first clinically manifest as AE with fever, jaundice or ascites or mild to moderate elevation of ALT. Aerobic microorganism may be isolated from culture of the blood, pleural or ascitic fluid during AE. Gastrointestinal bleeding from esophageal varices may occur and is commonly mild and temporary. Esophageal varices regress or disappear after successful treatment of AE with antibiotic. Ascites and varices that disappear with treatment indicated to the occurrence of transient HVOO and transient PH in the disease. AE is recurrent and in between episodes patient often remains well. Patients with advance

disease may have evidence of poor venous circulation in legs as indicated by - cold legs with increase pigmentation or poorly healing ulcers and dilated superficial capillaries (Fig 8).

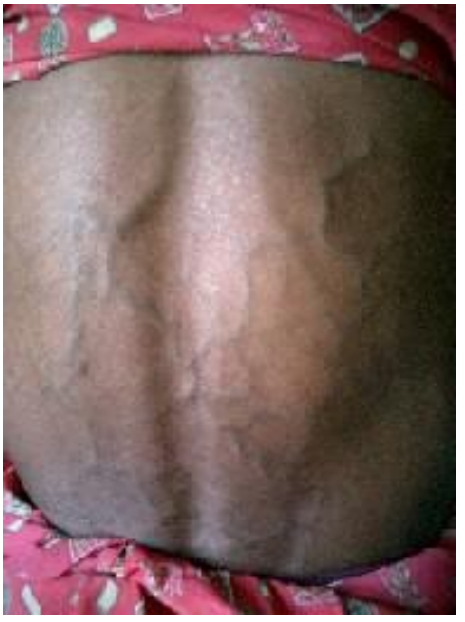


Figure 7b. Superficial collaterals: plexus of prominent superficial vein in the back at lumbar region of the same patient.

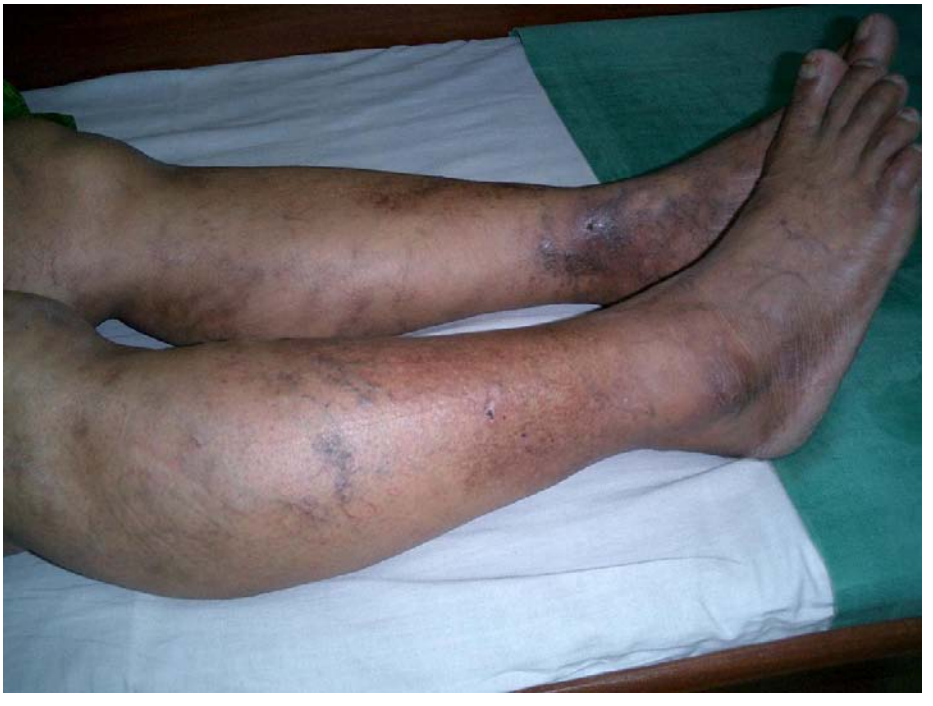


Figure 8. Changes in leg due to chronic venous congestion in a patient with long standing chronic HVD. This patient developed with LC and HCC.

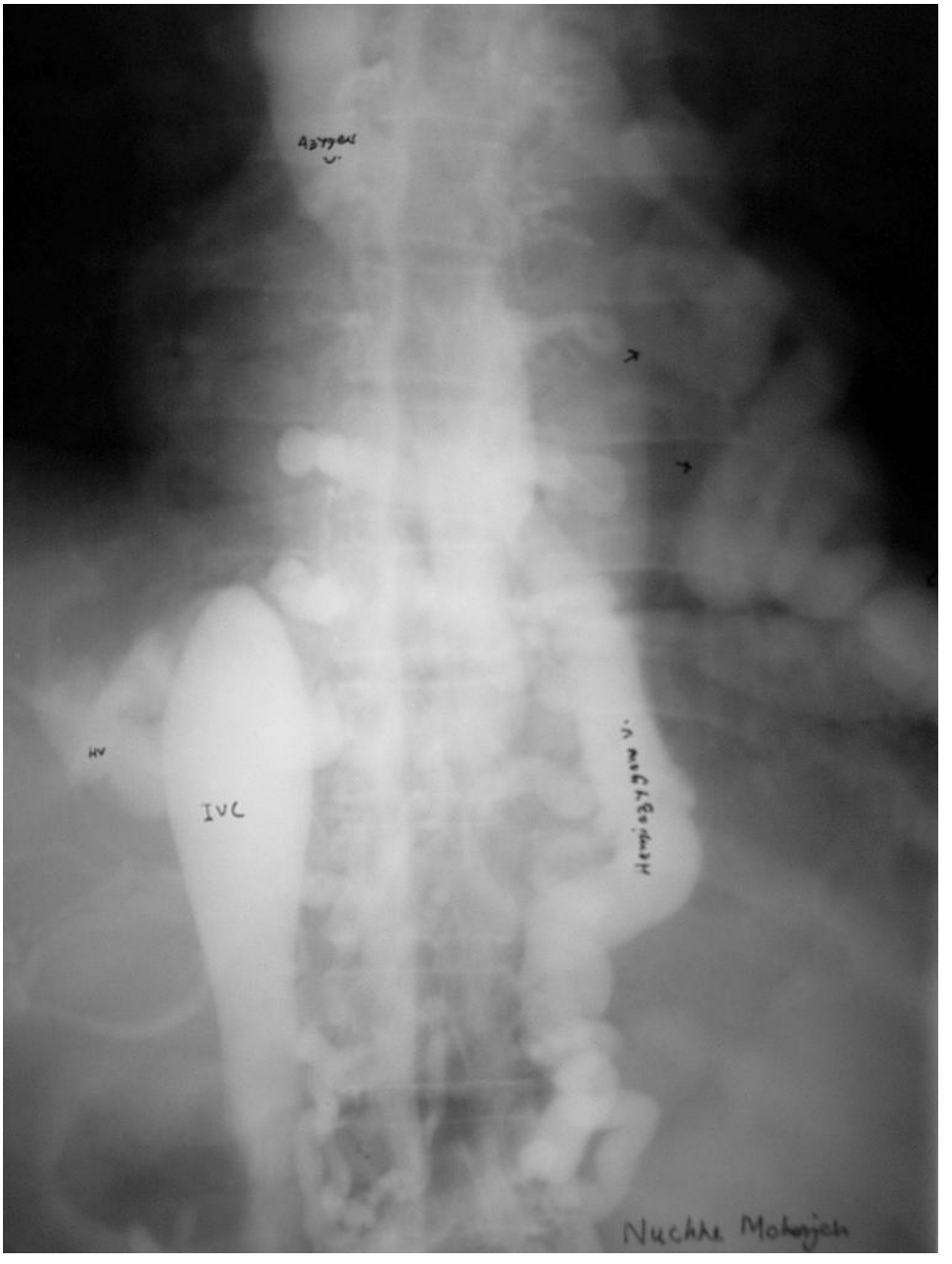


Figure 9a. Cavogram of a patient with chronic HVD who developed LC: shows complete IVCO and deep collaterals azygos, hemiazygos and left paracardiac veins.

HVD patients have long survival on conservative management [14, 32, 63]. It is probably due to development of extensive cavo-caval anastomosis and collateral circulation in and around the liver (Fig 9a, 9b, 9c,9d) that attempts to reestablish the circulatory balance and the absence of persistent liver damage as indicated by absence necro-inflammation in liver biopsy [14, 32, 42]. Liver damage in HVD is episodic and is related to AE which is preventable [14]. Prevention or adequate treatment of AE improves survival. Early death in the disease is from

septicemia related to AE and late date is from HCC or natural cause. Long term follow-up of HVD patients showed that 78 % developed cirrhosis and 11% developed HCC. Average age of the patients who died from AE was 45 years and that from HCC was 57 years [14].

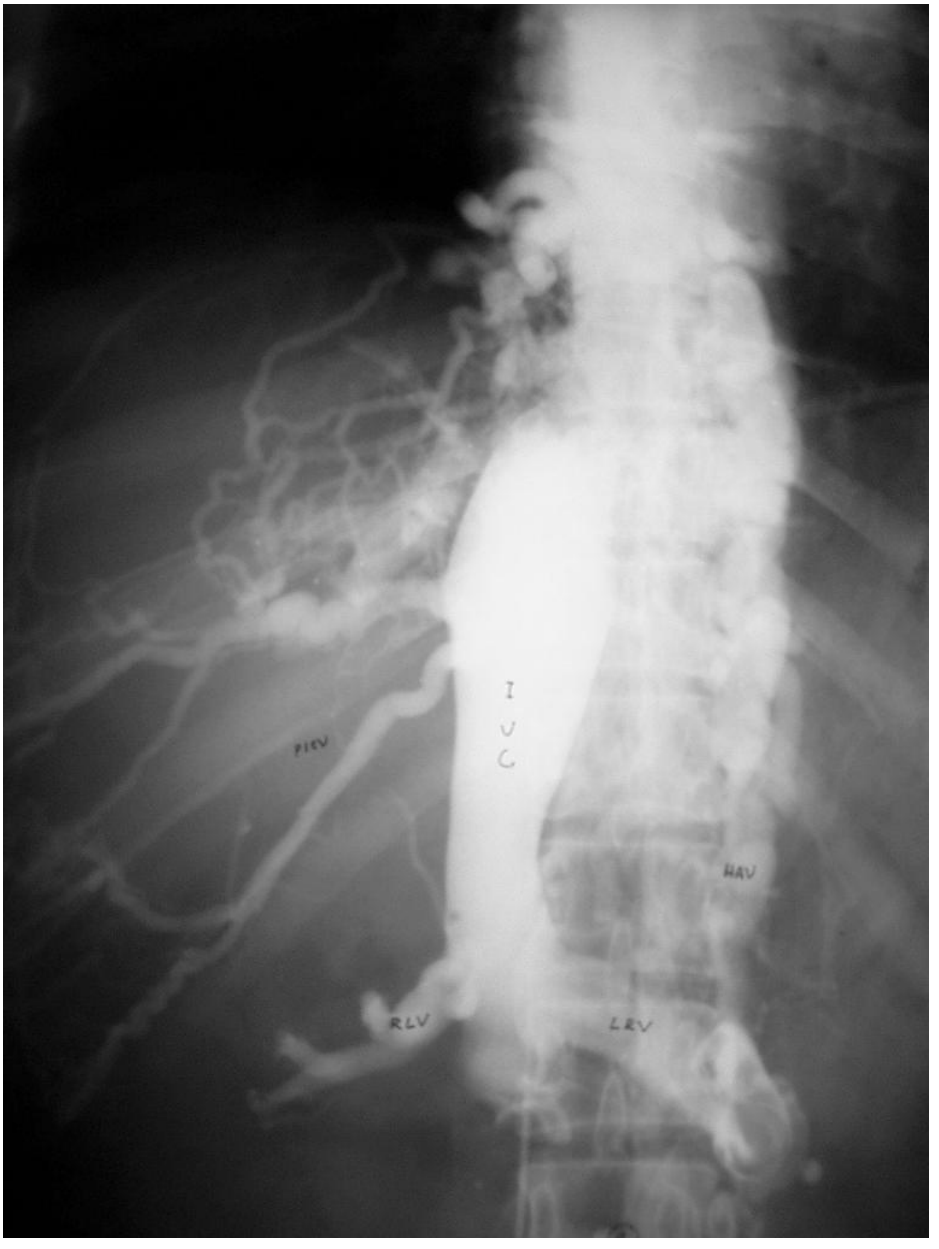


Figure 9b. Cavogram of a patient with chronic HVD who developed LC: shows complete IVCO and collaterals at the site of obstruction and around the liver.

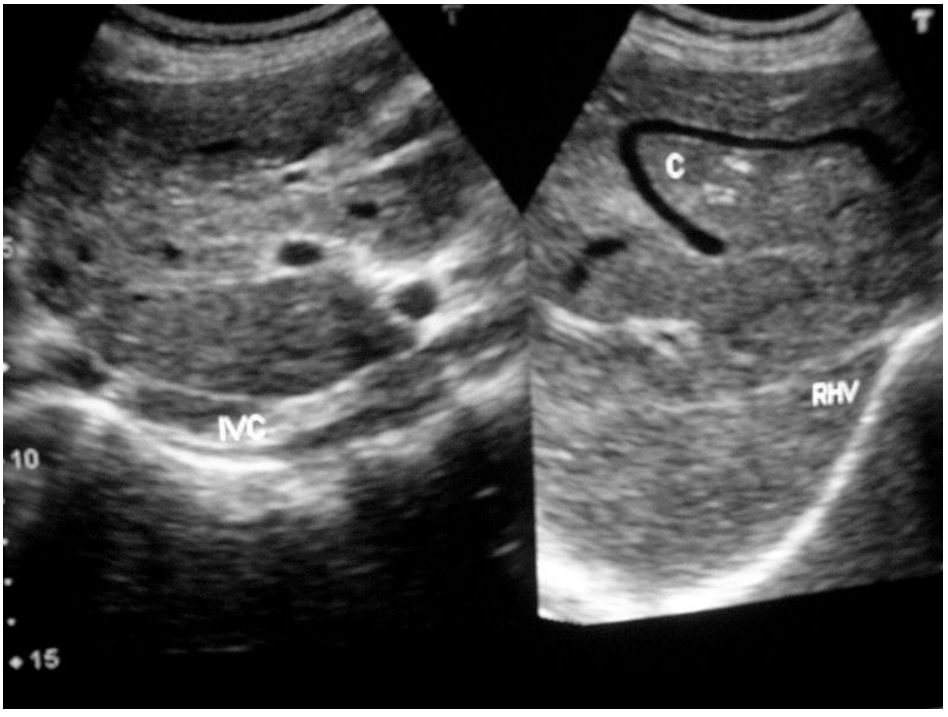


Figure 9c. US of a young lady showing complete obstruction of the IVC with thrombus of different ages, right hepatic vein completely thrombosed and a prominent collateral vein is seen in liver with evidence of chronic fibrosis.

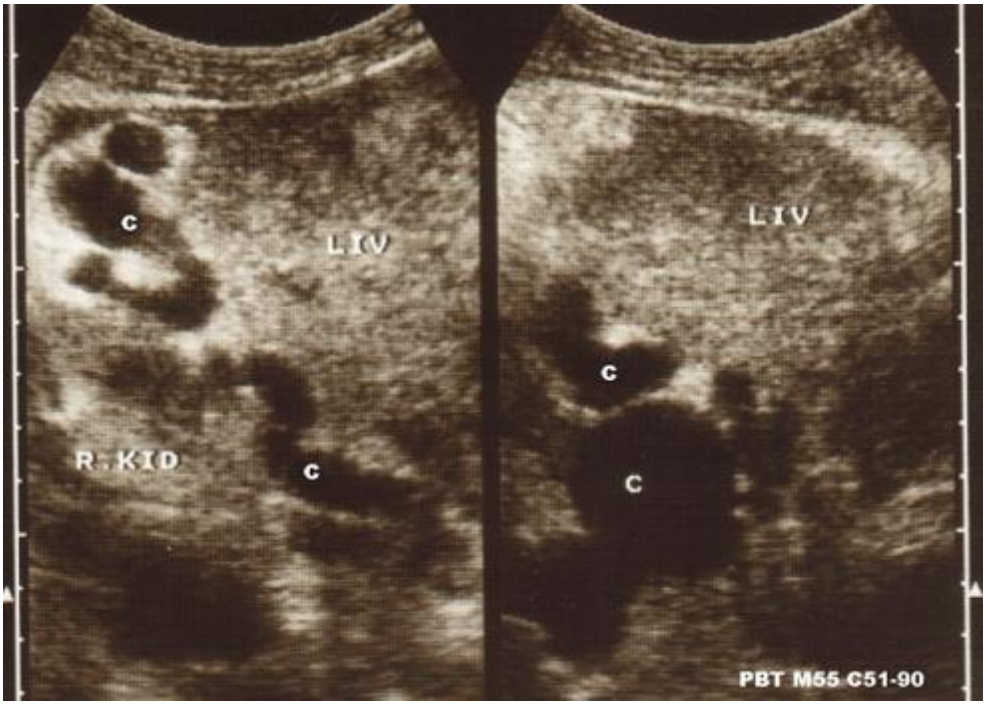


Figure 9d. US: collaterals around liver in a patient with LC and HCC with chronic HVD .

Pathogenesis of LC In HVD

Prolonged congestion due to HVOO was considered the possible mechanism of development of LC in HVD [52]. Prolonged congestion results in ischemic loss of hepatocytes around central veins. With continued high sinusoidal pressure hepatocytes fails to regenerate and is replaced by fibrous tissue. Centrilobular fibrosis and formation of regenerative nodules commonly at periportal area led to development of venocentric or reversed lobulation cirrhosis [42].

Study of the natural history of the disease indicated to the possibility of other mechanisms of development of cirrhosis in HVD [14]. Ascites, a clinical marker of increased sinusoidal pressure did not develop in 50% of the patients who developed cirrhosis. And the incidence of ascites in those who developed cirrhosis and those who did not was not different. Further, serial liver biopsy done in two patients who developed cirrhosis within 6 months and 6 years showed no histological evidence of persistent venous congestion.

Development of cirrhosis in HVD was directly related to severity and frequency of acute exacerbations (P=0.017) and not to the duration of the disease or to the type of the caval lesion or the occurrence of ascites [30]. Acute exacerbation was precipitated by apparent or in-apparent bacterial infection. It was associated with deposition of fresh thrombus at the site of IVC lesion (Fig 10a, 10b) and thrombotic obstruction of radicals of hepatic and portal veins. Caval thrombosis may result in recurrent HVOO, centrilobular congestion and ascites. Intra-hepatic venous thrombosis results in ischemic necrosis of the hepatocytes of the area drained by the obstructed veins. Development of advanced congestive cirrhosis (Fig 11a, 11b) within 9 months in a patient with acute HVD who had thrombosis of sublobular hepatic vein with massive hepatocellular necrosis was documented by serial liver biopsy [30].

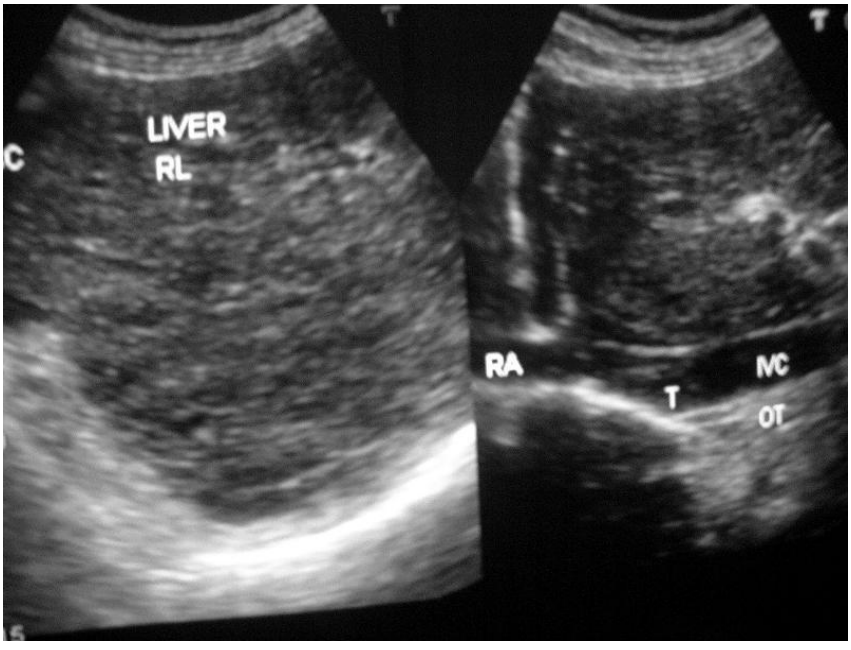


Figure 10a. AE in HVD: US of a patient with LC with stenosis of the IVC near cavo-atrial junction with layers of organized on posterior wall and presence of 'fresh' thrombus.

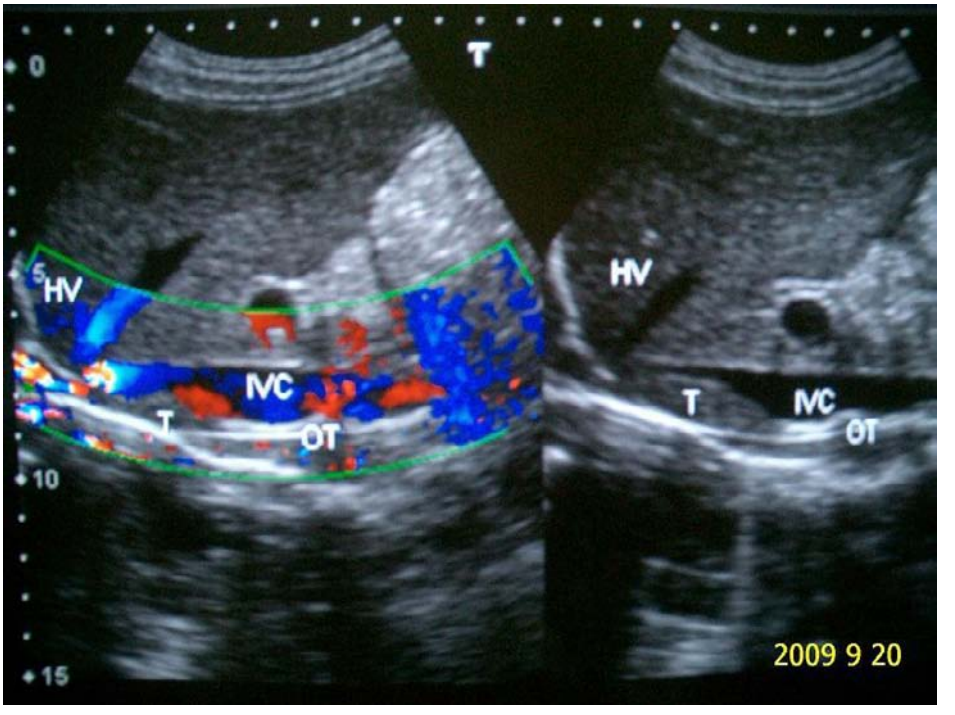


Figure 10b. AE in HVD: US showing recent and layers of old organized thrombus.

Figure 11a. Acute massive necrosis of hepatocytes in a patient with acute HVD associated with thrombosis of sublobular hepatic vein.

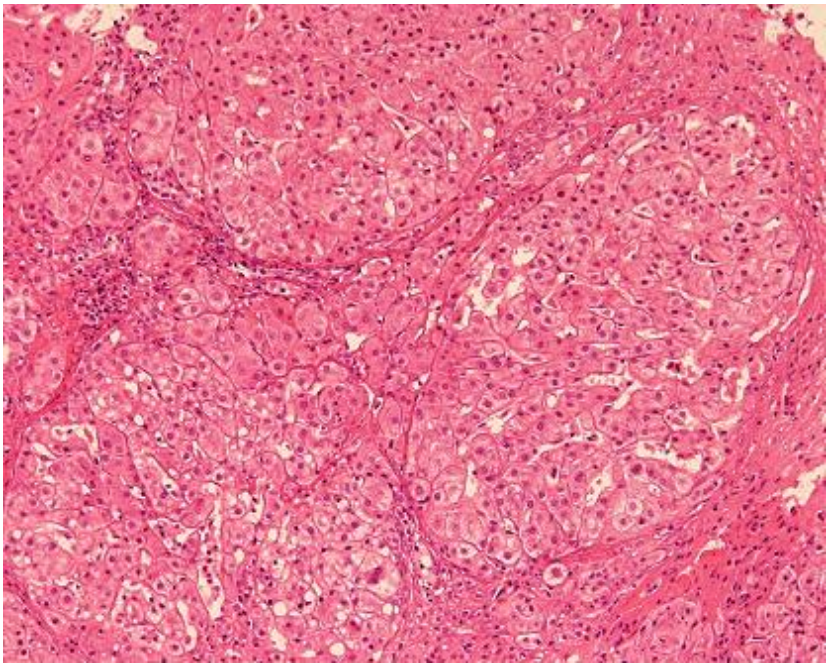


Figure 11b. Development of advanced congestive cirrhosis in the same patient within 9 months of acute HVD.

Occurrence of AE in HVD was documented by many earlier workers who observed thrombus of different ages in the IVC and/ hepatic vein ostia in autopsy studies [32-37, 45, 64] and correlated it to acute clinical exacerbations [12, 45]. Earlier autopsy studies also documented occurrence of thrombotic obstruction of radicals of hepatic and portal veins in HVD [25, 64]. Parker in a large autopsy study [26] observed thrombotic obstruction of radicals of portal vein in about 25% of cases. Mann and Hall described occurrence of congestive necrosis of the hepatocytes related to thrombotic obstruction of hepatic and portal vein radicals in an autopsy study of a patient with HVD [37].

Presence of thrombus of different ages or layers of organized thrombus at the site of the lesion in IVC (Fig 12), thrombotic obstruction or replacement of a long segment of right hepatic vein near its ostia by 'cord' like structure or echogenicity of intra-hepatic veins with no flow in color Doppler study are common among HVD patients. These observations indicated to occurrence of thrombus in IVC and intra-hepatic veins during AE. It is postulated that acute massive or recurrent ischemic loss of hepatocytes related to recurrent AE may be an important mechanism of development of cirrhosis in HVD.

Development of congestive cirrhosis even in chronic heart failure is considered by Wanless and colleagues to be due to intrahepatic venous thrombosis rather than to generalize venous congestion [65]. In chronic heart failure thrombus begins in sinusoids and propagate to hepatic veins and portal vein and results in ischemic parenchymal extinction and fibrosis. In HVD, however, thrombosis of small hepatic and portal veins occurred during acute stage and AE and was related bacterial infection.

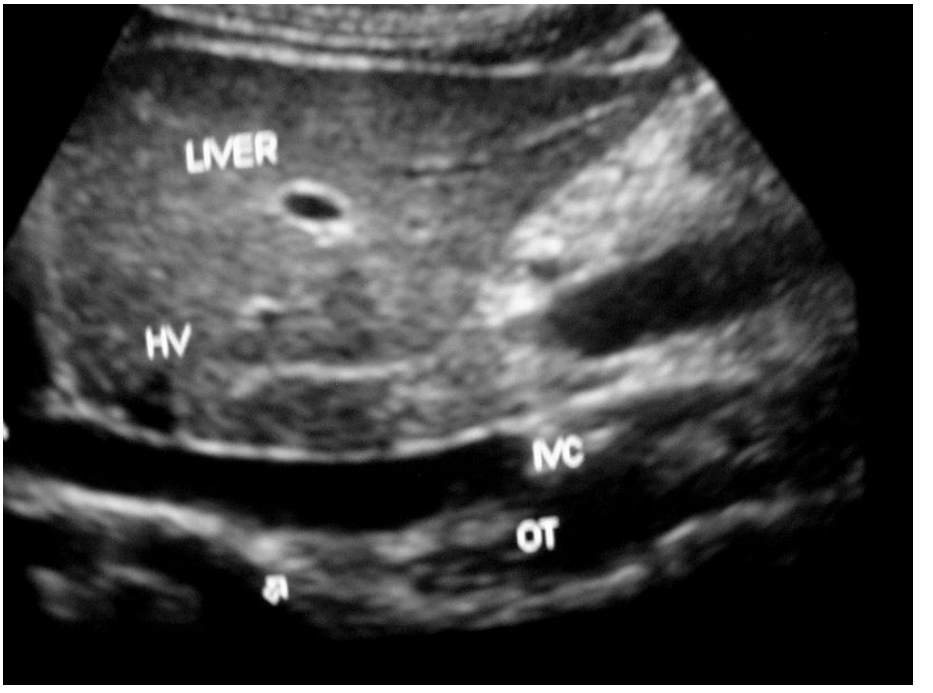


Figure 12a. Recurrent AE: US shows organized thrombus in the posterior wall of the IVC attached to the proximal end of stenosed segment of the IVC (narrowed segment with thick echoic posterior wall near cavo-atrial junction).

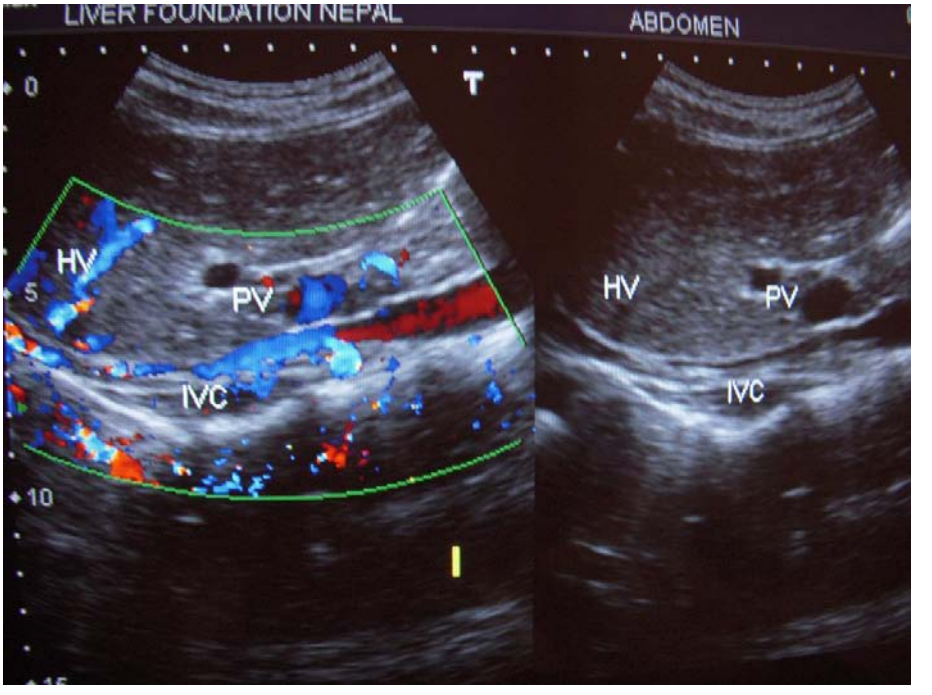


Figure 12b. Recurrent AE: US showing thrombus of different ages in the IVC.

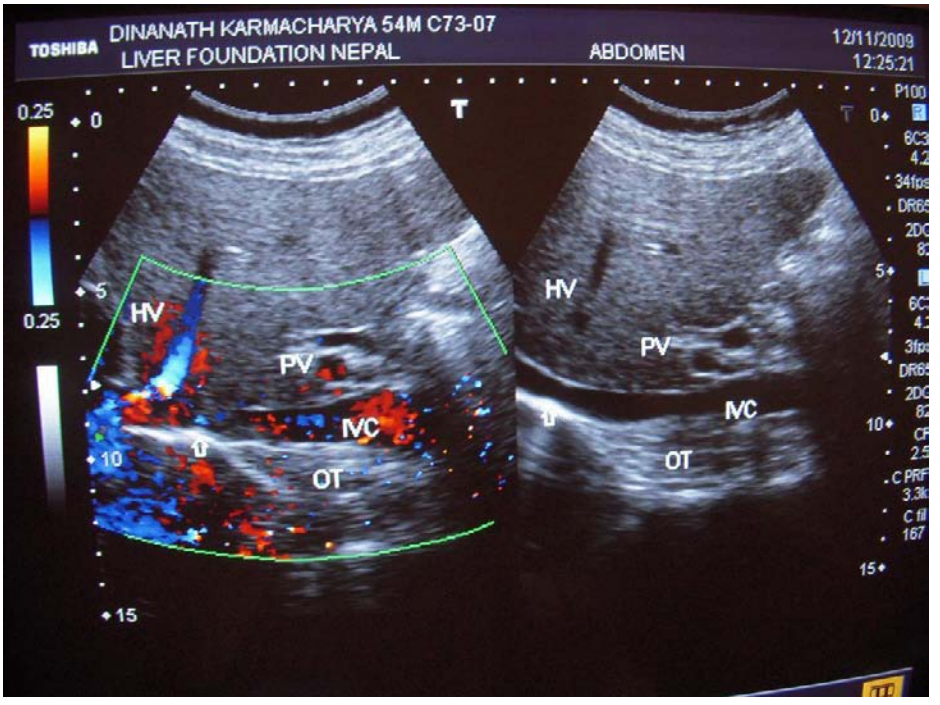


Figure 12c. Recurrent AE: IVC stenosis near cavo-atrial junction and layers of organized thrombus along the posterior wall of the IVC narrowing the lumen of the IVC.

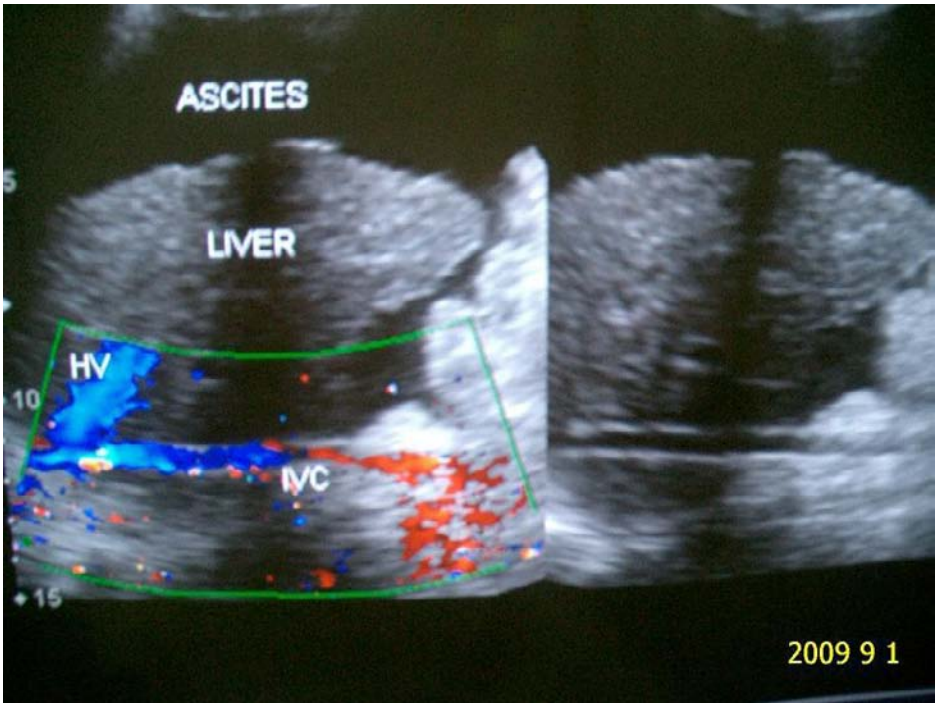


Figure 12d. IVC in a patient with LC shows narrowed IVC lumen due to layers of old organized thrombus.

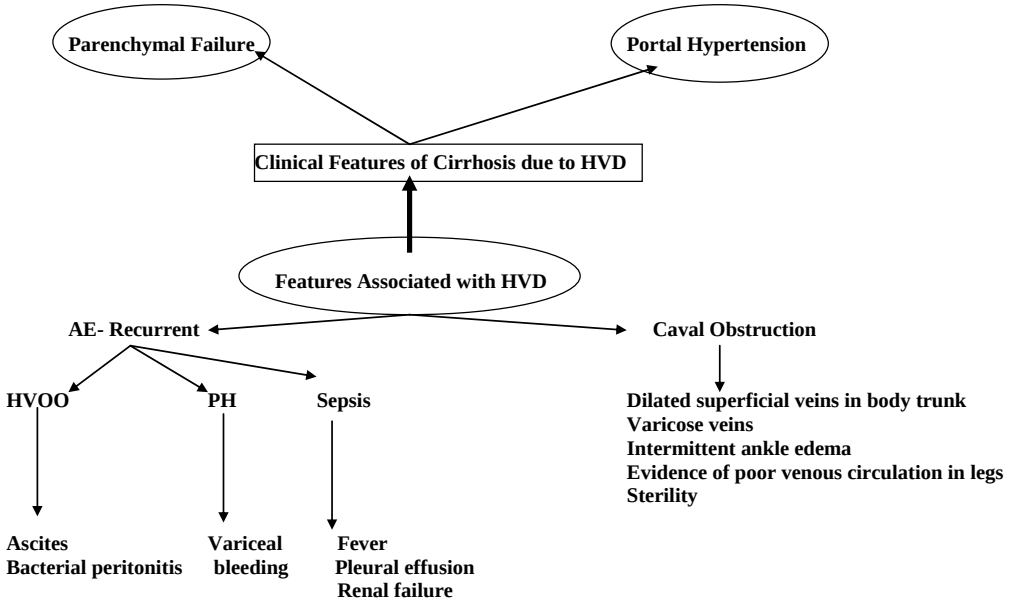
LC in HVD is probably caused by the following mechanisms:

- Prolonged hepatic outflow obstruction causing massive ischemic necrosis of hepatocytes.
- Acute thrombotic obstruction of large or medium sized hepatic and/or portal veins causing ischemic loss of hepatocytes.
- Recurrent HVOO or recurrent thrombotic obstruction of small radicals of hepatic and portal veins causing recurrent ischemic loss of hepatocytes.

The first two mechanisms would result in rapid development of cirrhosis within a few months and third mechanism would result in cirrhosis over a period of some years.

Clinical Features of Cirrhosis in HVD

Clinical features of cirrhosis are usually determined by three vectors- (i) parenchymal failure, (ii) portal hypertension (iii) the features of the original disease that caused cirrhosis (Fig 13). Relative importance of these three vectors differed in different diseases causing cirrhosis and even in the same disease at different stages of the cirrhosis. In alcoholic cirrhosis symptoms due to parenchymal failure or PH dominate whereas in HVD clinical features related to the disease like occurrence of AE are prominent [18].



HVD= hepatic vena cava disease, AE= acute exacerbation, HVOO= hepatic outflow obstruction, PH= portal hypertension

Figuire 13. Clinical features in LC due to HVD

Cirrhosis in HVD is associated with mild to moderate hepatomegaly in 86%, splenomegaly in 34%. Symptoms like recurrent ascites, edema of legs and jaundice dominate the clinical picture. Ascites occur late and carries bad prognosis in cirrhosis due to other causes like chronic hepatitis B or C as its presence indicates severe hepatocellular damage. However in HVD ascites and jaundice are early features of the disease and are related to AE and do not carry the same bad prognosis. Ascites in HVD has high protein content and high incidence of bacterial peritonitis. As bacterial peritonitis in HVD is related to presence of intra-abdominal source of infection- the infected IVC it is not called spontaneous bacterial peritonitis [24]. Dilated superficial veins in the abdomen, chest and back and splenomegaly or sterility also occur early in the course of the disease before the development of cirrhosis. Signs of hepatocellular failure like coagulopathy, spider nevi, palmer erythema, gynaecomastia, testicular atrophy, cyanosis, clubbing, hyperkinetic circulation or encephalopathy are uncommon in cirrhosis due to HVD.

Long term follow-up of HVD patients showed that onset of cirrhosis is commonly asymptomatic and is recognized on ultrasound by presence of increased coarse echo-texture of the liver parenchyma and in a few by development of persistent low WBC or platelet counts. Ultrasound features of cirrhosis developed a few years after the histological confirmation [14].

AE is prevented by adoption of good hygiene and early treatment of bacterial infection. HVD patients with cirrhosis has better prognosis if bacterial infection is prevented or adequately treated (Fig 14a, 14b, 14c). AE is treated with high dose antibiotic for 4 to 8 weeks. Patients with ascites are managed with sodium restriction in diet along with diuretics when needed.

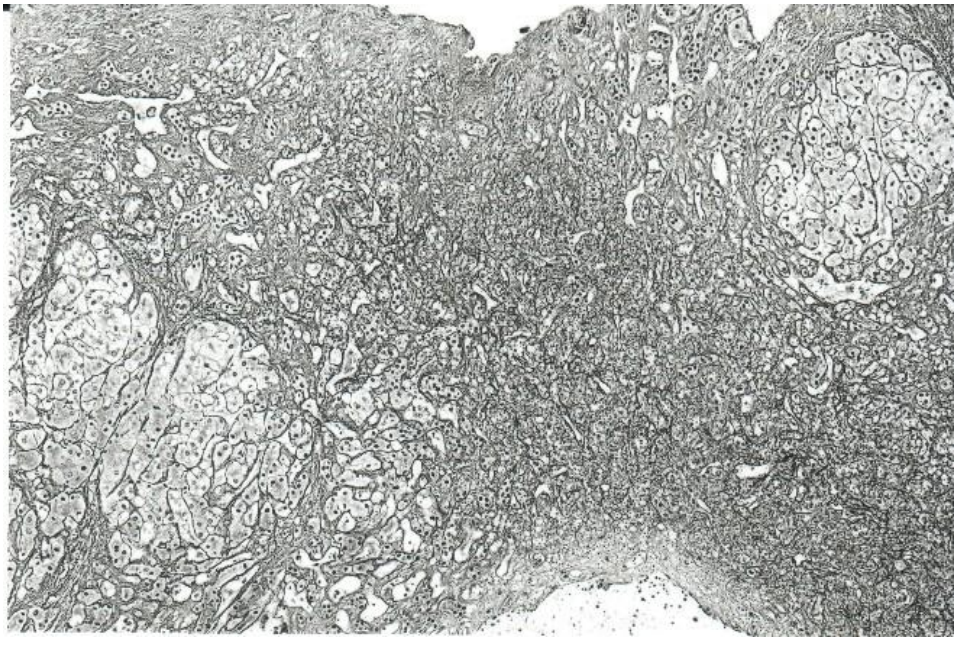


Figure 14a. Liver biopsy in 1991showing LC in a patient with history of recurrent episodes of fever and mild well tolerated g-i bleeding from esophageal varices of several years duration.

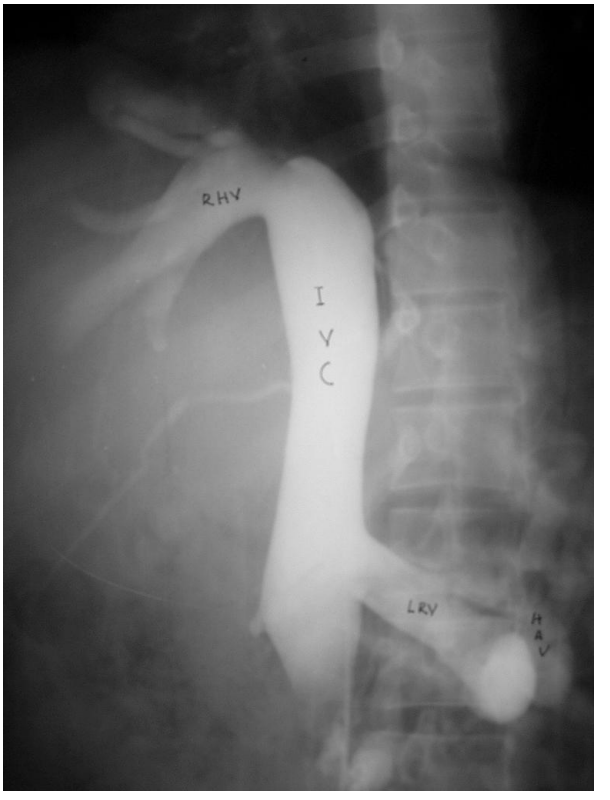


Figure 14b. Cavogram of the same patient showing complete obstruction of the IVC and ostia of middle and left hepatic veins. Right hepatic vein is dilated.

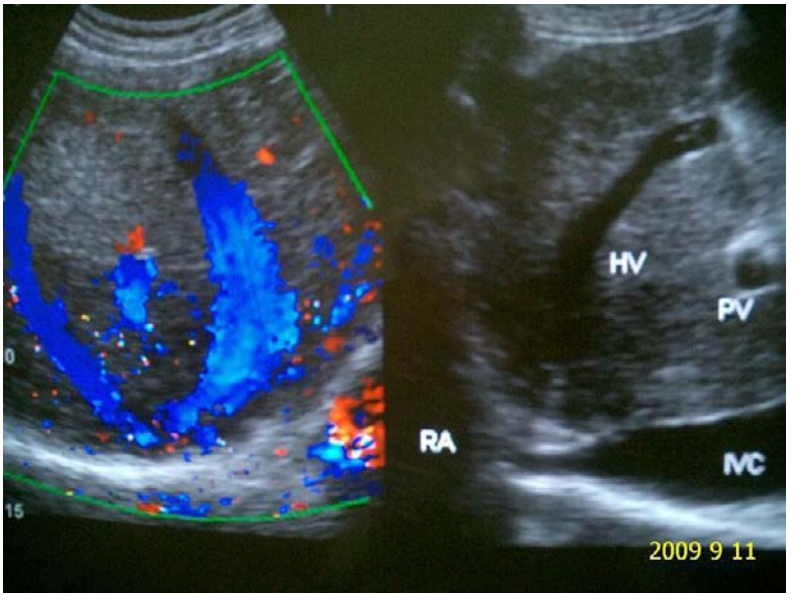


Figure 14c. US of the same patient showing IVC obstruction and LC with dilated hepatic veins. This patient on regular follow-up has remained well with for last 18 years. Had received advise about hygiene of the food and water and immediately treated recurrent infections.

Diagnosis of HVD in LC

HVD should be considered in the differential diagnosis of the etiology of LC in patients from developing countries. Clinical features that indicate to HVD are history of AE- recurrent ascites or jaundice or fever, dilated superficial veins in the body trunk with upwards flow and bacterial peritonitis in the presence of high protein content ascites. Cirrhosis among females in Nepal was more commonly due to HVD than to other causes (37% vs 6%) (P=0.0404) [29].

Ultrasonogram combined with color Doppler study of IVC is diagnostic of HVD. Ultrasound is sensitive, cheap, and easily available and should be used as the first line of diagnosis of HVD. Presence of complete obstruction of IVC is not necessary for the diagnosis of HVD. Occurrence of mild to moderate stenosis as indicated by narrowing of the IVC near cavo-atrial junction with thick echoic posterior wall is common (Fig 10a, Fig 15). Presence of thrombus of different ages or layers of old organized thrombus along the posterior wall of the IVC attached to the proximal end of the stenosed segment is diagnostic of recurrent AE. Cavogram, MRI or liver biopsy may be used to corroborate the ultrasound finding in the diagnosis of HVD.

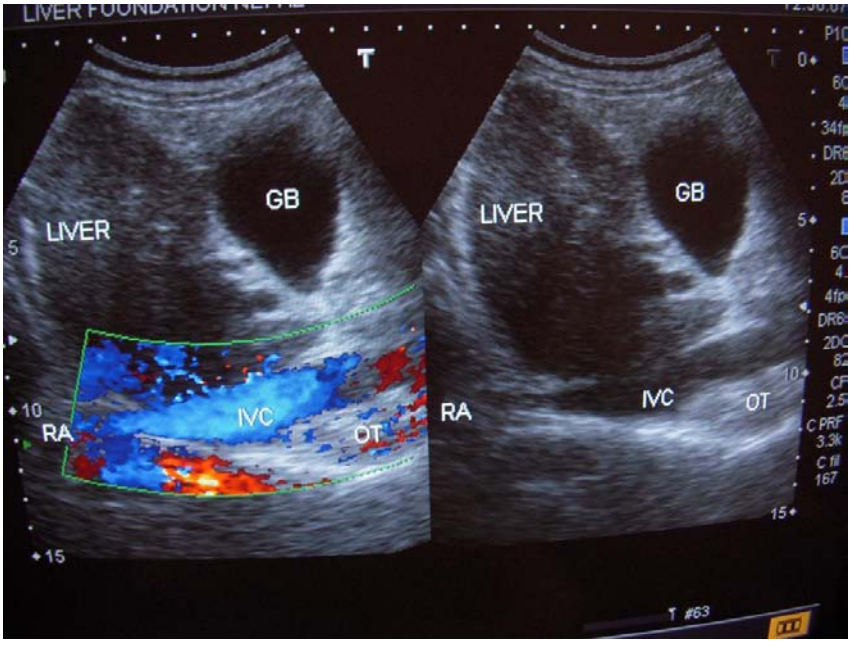


Figure 15. US Shows LC, IVC stenosis near cavo-atrial junction and layers of organized thrombus on posterior wall. First consulted doctor when he developed fever and jaundice that lasted for some months. Was noted to have LC and HBsAg (+). He is HBeAg (-), anti-HBe(+) and has low level of HBV DNA. This patient had used alcohol (< 100g/ daily) regularly for some years. Is LC due to HBV infection or alcohol or HVD?

Ultrasound also helps in the recognition of cirrhosis due to HVD. Presence of increased coarse echo-texture of the liver parenchyma with rounded edge and irregular surface indicate to cirrhosis. Unlike in cirrhosis due to other causes where hepatic veins are attenuated and often not visible in HVD these may be prominent (Fig 14c). Presence of prominent veins,

intra-hepatic collaterals, obliterated or thrombosed intra-hepatic veins, prominent right inferior hepatic vein or collaterals around the liver in LC are diagnostic of HVD. Ascites, portal hypertension or HCC when present are recognized. Other US features associated with chronic HVD are thick peritoneal wall, thick echoic capsule of the liver- indicative of perihepatitis and very thick GB wall.

Co-Morbid Conditions in LC Due to HVD

Patients with HVD may have associated co-morbid conditions like chronic alcohol use, and chronic HBV and HCV infections that cause LC and HCC [29]. In Nepal patients with LC and HCC commonly have associated HVD and HBV infection. Among 70 patients with cirrhosis IVCO was detected in 77%, HBV DNA and HCV RNA in 47% and 10% respectively and history of significant alcohol use ($\geq$ 80g /day at least for 5 years) in 26%. Among 54 LC patients with IVCO HVB DNA was positive in 23 (43%), HCV RNA was positive in 6 (11%) and 14 (26%) gave history of significant alcohol use. These observations suggest that in developing countries possibility of HVD should be considered in the etiology of cirrhosis of unknown causes as well as in those with known causes. The predominant cause of HCV infection in Nepal is intravenous drug abuse (IDA). Many of them also suffered from bacteremia and develop HVD. Jaundice and ascites observed in IDA patients in Nepal during early period of HCV infection is related to HVD.

HVD also causes fluctuating mild ALT elevation due to AE. So in patients with HVD and other co-morbid condition it becomes important to decide the relative role of the different diseases in the pathogenesis of LC. Patient with HBsAg+ for > 6 months but is HBeAg negative, anti-HBe positive with serum HBV DNA <2,000 IU/ml is considered inactive HBsAg carrier and ALT elevation or LC in these patient may be due to associated HVD (Fig 15). Similarly LC in patient with chronic alcohol use of < 40 g/ day for <5 years or anti-HCV positive but HCV RNA negative may be due to associated HVD.

Incidence and Pathogenesis of HCC in HVD

The reported incidence of HCC in HVD (Fig 16) varied from 4.6% to 47% [8-14, 42, 43, 50, 51]. Higher incidence was reported in series that included autopsied cases [11, 42]. Though most of the HCC in HVD occur in patients with LC, it can also occur in those with out cirrhosis [11, 12, 42, 43]. Over all incidence of HCC in HVD patients that have been followed for long term was 10%. It was 14% in 44 HVD patients with LC followed for 14.8 $\pm$ 9 years [29].

Pathogenesis of HCC in HVD is not clear. Regenerative activity of liver cells, which has long been linked to hepatocarcinogenesis, is generally low in congestive liver disease and in cirrhosis due to chronic congestion [50]. Kew [43] who observed high incidence of HCC in HVD patients from Transvaal province compared to other geographic regions of South Africa concluded that individuals with IVCO are rendered susceptible to specific environmental hepatocarcinogens. He proposed that centrilobular congestion, cell necrosis, regeneration and fibrosis that result from chronic hepatic outflow obstruction rendered hepatocytes susceptible to one or more environmental carcinogens. HBV infection that sometime occurs together with

HVD was considered by a few as a possible cause of HCC in the disease [66]. Simson [42], Okuda [45] and Kew [43] who studied the prevalence of HCC in HVD arrived at the conclusion that HBV infection is not related to development of HCC in the disease. Reported incidence of HCC in HVD was similar in Japan and South Africa the two countries with four fold differences in the prevalence of HBsAg carrier rate [50]. In Simson's series of 101 cases of HVD with 47% incidence of HCC, HBsAg was positive in 20% compared to its prevalence of 8%in the population and 68% in HCC associated with macronodular cirrhosis [42]. None of the HVD cases with HCC reported by Kew were HBsAg positive [43]. Though HVD patient positive for HBV DNA had significantly high incidence of HCC compared to patients with HVD only (80% vs 43%) (p= 0.036) [29], only one of our patient out of six who developed HCC on long term follow was HBV DNA positive [30]. The incidence of positive HCV RNA and significant alcohol use among HVD patients who developed HCC was low indicating that HVD alone independent of HBV or HCV infection or alcohol was a risk factor for HCC [30]. Similarly Kew also had ruled out the possibility of alcohol or cigarettes or HBV infection as possible co-carcinogen in HCC in IVCO [43]. Thus it appears HVD is an independent and direct risk factor for HCC [30].

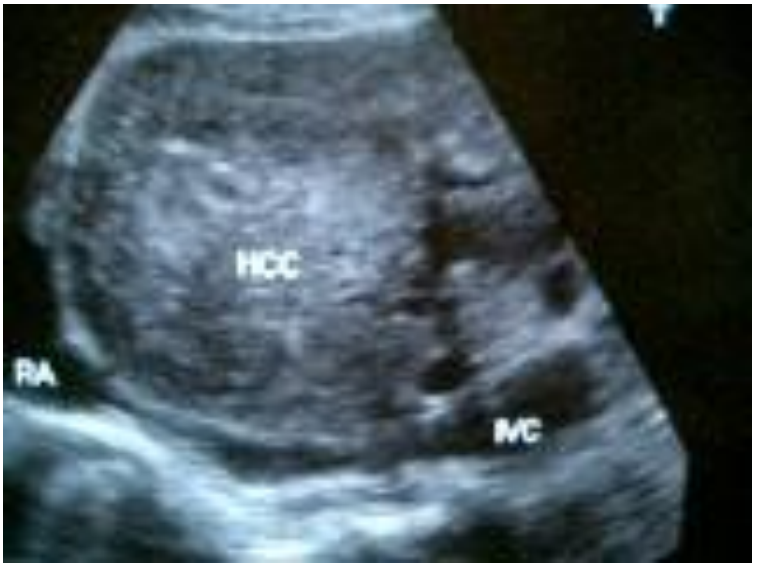


Figure 16. Pathogenesis of HCC in HVD.

Absence of necro-inflammation in HVD indicated that hepatocellular damage in this disease is not continuous as in chronic HBV or HCV infections. What then is the pathogenesis of HCC in HVD? HVD is clinically characterized by apparent or inapparent AE precipitated by bacterial infection. AE is characterized by deposition of fresh thrombus in the IVC at the site of obstruction and thrombosis of intra-hepatic veins resulting in episodic congestive necrosis of hepatocytes followed by fibrosis. Occurrence of LC and HCC in HVD is related to the frequency of AE and not to the duration of the disease or the type of the obstructive lesions (P= 0.017) [30]. Longitudinal study of six patients who developed HCC showed that all had suffered from frequent episodes of AE [30]. Regenerative activities that

follow the recurrent loss of hepatocytes caused by recurrent AE thus appeared to be a possible cause of hepatocarcinogenesis in HVD (Fig 5, Fig 17).

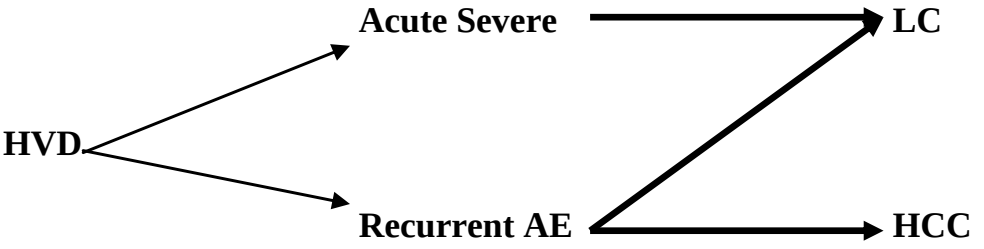


Figure 17. LC and HCC in HVD.

Conclusion

Hepatic vena cava disease occurs in developing countries, where it is still an under-diagnosed entity. It is caused by bacterial infection of the hepatic portion of the IVC at the site where hepatic vein joins it. The initial thrombophlebitis later converts into stenosis or complete obstruction. It is a chronic disease characterized by development of cavo-caval, intra-hepatic and extra-hepatic collateral anastomosis and clinical manifestation associated with recurrent AE precipitated by apparent or inapparent bacterial infection. Ascites and variceal bleeding in cirrhosis due to HVD is often related to HVOO and has better prognosis compared to cirrhosis due to other causes like alcohol. HVD is complicated by high incidence of LC and moderate incidence of HCC.

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Chapter 7

The Role of Surgery in Patients with Liver Cirrhosis

Georgios Tsoulfas [*] ***and Polyxeni Agorastou***
[1] Department of Surgery, Aristoteleion University of Thessaloniki, Greece
[2] Department of Gastroenterology, Aristoteleion University of Thessaloniki, Greece

Abstract

This chapter will present the critical interplay between liver cirrhosis and surgery. First, the question of patients with cirrhosis needing to undergo a surgical procedure will be explored. Specifically, the different ways that the level and severity of cirrhosis can be estimated will be examined, and how these could determine the safety of proceeding with a surgery, as well as the prognosis of these patients, according to the different surgical procedures. The second issue to be examined is the role of surgery as therapy for portal hypertension, one of the more life-threatening complications of cirrhosis. This will be achieved by analyzing the pathophysiology of portal hypertension in cirrhosis and presenting the different surgical procedures to treat portal hypertension and its complications, with special emphasis on the indications and contraindications for each. Finally, an established danger of cirrhosis is the development of hepatocellular carcinoma. This chapter will present the pathophysiology that leads to this progression, as well as the role of surgery as treatment of hepatocellular carcinoma in a cirrhotic patient with the two main alternatives being surgical resection versus transplantation. The aim would be to identify the groups of patients that would be best served by each alternative.

[*] Correspondence: Georgios Tsoulfas
66 Tsimiski Street, Thessaloniki 54622, Greece
Tel: 306971895190. Fax: 302310342094. Email: tsoulfasg@msn.com

Introduction

It has been well-documented that patients with liver cirrhosis in need of surgery are at an increased risk, with high morbidity and mortality. This presents the medical/surgical team with a significant challenge when faced with the decision of whether a patient would benefit from a surgical procedure, or whether the underlying risks given the presence of cirrhosis, are prohibitive. To be able to make that decision, it is necessary to enlist the help of a multidisciplinary team, consisting of a surgeon, a hepatologist, an anesthesiologist and an intensivist. More importantly, it is useful to have a framework for risk assessment. This chapter will attempt to do that, as well as address the role of surgery in the treatment of portal hypertension and hepatocellular carcinoma, two problems encountered in high frequency in patients with cirrhosis.

Risk Assessment and Management of Patients with Cirrhosis for Non-Hepatic Surgery

In order to evaluate the risk involved in non-hepatic surgery in patients with cirrhosis and decide on the best management, this section of the chapter will analyze some of the determining factors, such as the role of anesthesia, the type of surgery, ways to evaluate the degree of liver dysfunction, the need for surgery while on the waiting list for a liver transplant, and some algorithms useful in making these decisions.

Anesthesia and Liver Disease

Factors that can and will determine the success of anesthesia in a cirrhotic patient are the effect of cirrhosis on the patient's hemodynamics, and the effect of the liver dysfunction on the use of the different anesthetic medications.

Regarding the effect of anesthesia on the cardiovascular system, it can decrease blood flow to the hepatic artery by a third within the first half hour of the surgery [1]. This decreases hepatocyte perfusion, which will improve during the course of the surgery, leaving however open the possibility of ischemia/reperfusion injury due to these changes. It is essential to maintain intravascular filling volume, mainly with the use of colloids and blood products, in an effort to achieve optimal perfusion, avoid or correct coagulopathy and decrease third spacing. Moreover, cirrhotic patients have a hyperdynamic systemic response with increased cardiac output, decreased systemic resistance and low arterial pressure. This makes it extremely difficult for the heart to overcome any events of hypovolemia or bleeding that may occur as part of the surgery. This further decreases the blood flow to the liver resulting in further hypoxia, thus leading to a vicious circle.

The vast majority of anesthetics reduce blood flow to the liver, which is exacerbated by the fact that liver dysfunction by itself causes significant impairment in the metabolism of a variety of anesthetics [2]. Specifically, alterations in the metabolism of cytochrome P450 enzymes and decreased biliary excretion can lead to prolonged action for several anesthetic

medications. Similarly, narcotics and benzodiazepines can have increased half-lives, thus having a detrimental effect on the hepatic encephalopathy that these patients may have. The vast majority of volatile anesthetics, such as halothane and enflurane, can cause liver damage, either by decreasing blood flow to the liver or by idiosyncratic reactions, such as the autoimmune hepatitis that halothane can cause or the postoperative liver damage that enflurane can cause [3]. Relatively newer medications, such as isoflurane and sevoflurane are not significantly metabolized by the liver, although they still can cause a diversion of the blood flow to the liver, thus augmenting any potential perioperative hepatic hypo perfusion [4-5]. The solution is the use of a combination of anesthetic agents, such as using an opioid to decrease the need for an inhalational agent and provide improved hemodynamic stability.

Categories of Non-Hepatic Surgery

This part of the Chapter will focus on the risk of cirrhosis for non-hepatic surgery. The clinical challenge presented is real and quite frequent, as 10% of patients with cirrhosis will undergo some type of non-hepatic surgery during the last two years of their lives [6]. To obtain a more accurate assessment of the risks involved, the first step is to categorize the type of surgery. There are different ways to do that, based on either the type or the severity of the surgery. Looking into the various types of surgeries for cirrhotic patients, these can be divided into the categories seen in Table 1.

Table 1. Types of non-hepatic surgery in a cirrhotic patient

Type of surgery	Comments	Examples
Elective	Can be cancelled or postponed, if risk from cirrhosis does not justify it.	Orthopedic procedures, hernia repairs, cosmetic surgery
Elective but Necessary	Need to balance the risk of the procedure with the risk of cirrhosis.	Cardiac procedures, symptomatic cholelithiasis
Cancer surgery	Although elective, this type of surgery cannot be avoided.	Surgery for colon, breast cancer
Emergency	Unavoidable due to the emergency nature. The lack of planning increases the risk further.	Trauma, bowel or hollow viscus perforation, strangulated hernias, aneurysm rupture
Required in order to become a candidate for liver transplantation	Elective, but of high necessity as it may preclude the option of transplantation.	Resection of a suspicious lung nodule, cardiac procedures

Surgical procedures in cirrhotic patients can also be divided into categories according to the severity of the surgery and the risk involved: low, moderate or high risk [7-8]. Low risk procedures include those that do not involve entry into major body cavities (abdomen or chest), and those that can generally be performed with local anesthesia or that are brief. We have to consider though that this is a relative statement, as for example hernia repairs have a

lower mortality compared to other procedures (2.5%), but still a significantly higher percentage compared to the general population [9]. Moderate risk procedures include peripheral and carotid vascular surgical procedures, gynecologic, spine and intracranial, and head and neck surgery. High-risk procedures include laparotomies, lung resections, major vascular surgeries including aneurysm repairs, orthopedic and cardiothoracic surgeries [10-11]. The latter are especially high-risk in cirrhotic patients, with the overall risk correlating with the type and time of cardiorespiratory bypass used and the need for perioperative mechanical support [12-13]. Especially surgeries involving cardiac valves, such as the tricuspid, present the additional challenge of affecting the outflow of the hepatic veins, thus creating a potential for hepatic congestion [14]. Overall, with cardiothoracic procedures the use of the bypass machine leads to added dysfunction of the coagulation system, including platelet dysfunction, fibrinolysis and hypocalcaemia. In general, in cirrhotic patients it would be wise to prefer, if possible, the less invasive options of angio- and valvuloplasty; even so, there are situations where major surgical procedures are unavoidable, and in these cases it has been necessary to combine the cardiothoracic procedure with liver transplantation [15]. Not surprisingly, in most reports, emergency procedures in cirrhotic patients have significantly higher risk than elective procedures [16-17]. This is especially true for cardiothoracic, intraabodminal surgery and trauma, with mortality in emergency orthopedic procedures reported to be as high as 50% [18].

In addition to mortality, morbidity must also be considered when considering the severity of a procedure for a given patient or group of patients. In a series of 41 cirrhotic patients undergoing colorectal procedures, there was a very high rate of postoperative complications (77%), with half of these patients developing ascites and half of those becoming infected [19]. These complications and their severity, which were more prevalent in those patients with more advanced stage of cirrhosis, serve to underscore the importance of considering morbidity in assessing the risk of a surgical procedure for a patient with cirrhosis. In another study of 135 patients with cirrhosis undergoing non-hepatic surgery, there was a 16.3% mortality rate compared to only 3.5% in the control group, a difference which was found only in patients with Child-Turcotte-Pugh (CTP) score B and C [20]. This mortality rate was comparable to other studies (range 12% to 28%), and the high CTP score, together with duration of the operation and postoperative complications were independently associated with mortality after surgery in a multivariate analysis [21-24]. The importance of the CTP score in predicting postoperative mortality also presents an opportunity for intervention, as it has been shown that preoperatively converting Child's C patients to Child's B improves survival after the surgery [25]. This can be achieved by preoperative optimization, which includes correcting coagulopathy with vitamin K and fresh frozen plasma, ascites with diuretics and paracentesis while administering albumin, hepatic encephalopathy with lactulose or other medications, malnutrition with enteral nutrition and administering antibiotic prophylaxis, all in an effort to improve the patient's extent of liver disease prior to the surgery.

We see overall that the type of surgery, as that is defined by the level of urgency and the risk involved, are major determinants in making these difficult decisions. However, the other element involved is the severity of the cirrhotic patient's liver disease.

Assessing the Severity of the Liver Disease

Over time there have been several scoring systems that have attempted to assess the severity of liver disease in a patient, in an effort to predict mortality after surgery. The main ones are the CTP score, the Model for Endstage Liver Disease (MELD) score, and, as a less specific method, the American Society of Anesthesiologists (ASA) classification.

1) CTP Score

The CTP score was the first system to be used in order to predict mortality after portocaval shunts and later for patients planned to undergo esophageal transection. Although it does utilize biochemical parameters, such as albumin, INR and bilirubin, it also includes subjective clinical symptoms such as ascites and encephalopathy. Child's A cirrhotics have a relatively low-risk of postoperative mortality (10%), compared to the significantly higher risk in Child's B (18-45%) and Child's C (75-82%) patients [26-28]. Over time it was increasingly used to evaluate the degree of liver disease and predict outcome after surgery, as well as a method of liver allocation [26-27]. It is acknowledged that the CTP score has stood the test of time concerning risk assessment, however there are significant limitations. Specifically, it has not been prospectively validated and it is also based to a certain extent on subjective parameters. Also, it only divides patients into three groups, which has become less helpful nowadays, as developments in anesthesia and intensive care have led to significant progress in the postoperative management and prognosis of these patients, thus making finer distinctions in the risk assessment necessary.

2) MELD Score

The MELD score was originally developed to predict mortality after TIPS [29]. It has since been modified and evolved into the main scoring system for liver allocation in the US and in several other countries. The score ranges from 6 to 40 based on a linear regression model with values from serum bilirubin, INR and serum creatinine. The advantages of the system are the fact that it has been prospectively validated and that it does use objective biochemical parameters. Furthermore, the fact that it is a continuous scale allows for a more specific assessment of the risk involved. As an example, for patients with a MELD score of 20, 25, and 30, the respective 3 month mortalities while on the waiting list are 50%, 75% and 85% [30].

The MELD score has been used extensively in the last decade to help determine postoperative morbidity and mortality in cirrhotic patients undergoing non-hepatic surgery. In a study of cirrhotic patients undergoing cholecystectomy, it was found that the morbidity and mortality risk was 10% if the MELD score was less than 8, whereas it was 44% with a MELD score above that number [31]. In another study of cirrhotic patients undergoing cholecystectomy, it was found that a MELD score of 14 was equivalent to a Child C category and to a mortality of 17% [32]. A retrospective study of 140 patients, half of which underwent a laparotomy, revealed a 30-day postoperative mortality of 1% for every MELD point up to 20 and 2% for every MELD point over that [33]. This last finding often serves as a quick mnemonic for making decisions for a cirrhotic patient with a non-hepatic surgical problem.

The largest published series comes from the Mayo Clinic and is a retrospective review of 772 patients with cirrhosis, where the authors compared two time periods (1980-90, and 1994-2004) and three types of surgery (cardiovascular, orthopedic and abdominal) [34]. There were two control groups used, cirrhotics that had not undergone surgery and those that had undergone only minor procedures. The main findings of this study were that a) the MELD score is an accurate predictor of 30-day, 90-day and long-term mortality in these patients for these procedures, and b) that for each point increase in the MELD system the 30-day and 90-day mortality increased by 14%, with an apparent linear relationship between mortality and increases in the MELD score above a score of 8.

3) ASA Classification

The study from the Mayo Clinic helped shed some light into the role of the ASA classification in cirrhotic patients undergoing non-hepatic surgery. This is an anesthetic assessment of the status of all patients undergoing surgery, with a range from Class 1 (healthy with no other disease, other than the one operated upon for) to Class 5 (not expected to survive 24 hours with or without an operation). Most cirrhotic patients fall into a Class 3 (severe systemic disease) or Class 4 (severe systemic disease that is a constant threat to life). In the Mayo series the ASA class was shown to be an excellent predictor of seven-day mortality, with Class 4 adding the equivalent of 5.5 MELD points to the patient's mortality [34].

Other than the CTP and MELD scores, there have been efforts to predict postoperative mortality of cirrhotic patients undergoing non-hepatic surgery using individual biochemical or clinical parameters, such as ascites, jaundice, encephalopathy, hyponatremia and the hepatorenal syndrome. However, the MELD score has been shown to be far superior to these individual factors, and as a result it is the main prognostic tool currently used [35].

The Waiting Game

Patients with cirrhosis should at some point (sooner rather than later) be evaluated as candidates for liver transplantation (LT). If the patient with cirrhosis is not on the waiting list for a LT (very low MELD score, age limitations, active substance abuse, etc), then the decision of whether to proceed with a non-hepatic surgical procedure is made simpler, as the team only has to consider and discuss with the patient and the family the postoperative morbidity and mortality risk compared to the benefit expected from the specific procedure. It should also be made very clear to the family that LT is not an option should post-operative liver decompensation occur.

The issue becomes more complicated if the patient is on the waiting list for a LT and is also suffering from a non-hepatic disease that would need surgery. The question that has to be answered is whether the treatment for the non-hepatic disease has to precede, or follow the LT, not forgetting certain special situations where the two may have to be combined. One way to group these non-hepatic surgeries is a) non-hepatic malignancies, b) non-hepatic, non-malignant diseases that do not affect the chance for a LT, and c) non-hepatic, non-malignant diseases that can affect the course and outcome of a LT. The first group includes pre-malignant (i.e. dysplastic polyps) or malignant lesions whose resection equals a cure, and as such in those cases the non-hepatic surgery should come before the LT, unless the operative

risk is prohibitive. The reason is that curing these malignancies would allow the cirrhotic patient to remain on the waiting list. The second group involves non-malignant diseases of the GI and the neuromusculoskeletal systems, among others, where because they would not affect directly the outcome of a LT, they can be performed either before or after the LT. The last group represents the toughest decisions, as it includes cardiovascular diseases, such as coronary artery disease or severe aortic stenosis, where transplantation is not possible if these are not corrected. At the same time cirrhosis poses a huge risk in proceeding with these surgeries, and as a result the decision is often made to perform the two surgeries one after the other [36].

Management Algorithms

We have seen that the factors determining the management of a patient with cirrhosis with non-hepatic disease that may require surgery include the type of surgery, as well as the severity of the liver disease, or rather the hepatic reserves of the patient, as these are expressed by the MELD score. These thoughts have led to proposed algorithms. One of these suggests that cirrhotic patients with a MELD score less than 10 with a non-hepatic disease, should only undergo the non-hepatic surgery; cirrhotic patients with a MELD score between 10 and 15 should first undergo the non-hepatic surgery and then the LT, whereas cirrhotic patients with a MELD score greater than 15 should first undergo the LT before any other procedures [37]. Although helpful in providing direction regarding the management of such patients, the application of these algorithms can frequently encounter problems, such as the lack of an adequate supply of hepatic grafts for transplantation, or the emergent nature of a non-hepatic surgery.

Nevertheless, there are some basic steps that the treating physicians can and should take in order to maximize the possibility of a successful outcome. Cirrhotic patients should be followed at a liver center by a team that would involve, among others, a hepatologist, a hepatobiliary/general surgeon, a liver anesthesiologist, and a nephrologist. These patients need to undergo a full evaluation for LT in order to decide whether they are transplant candidates or not, and more importantly what further testing is needed for them to be on the LT waiting list. This is a critical point in the decision pathway, as the waiting list can serve as a safety net for those patients with cirrhosis undergoing non-hepatic surgery. The reason is that in the unfortunate situation of hepatic decompensation following the procedure, their MELD score would potentially increase and they would have a better chance of receiving a LT. The problem is that the window of opportunity in a situation like that is very small, as the hepatic decompensation can rapidly progress and the patient may not be transplantable anymore. On the other hand, if a cirrhotic patient is not considered a transplant candidate, then this would need to be clearly discussed with the patient and the family prior to any non-hepatic procedure, as it significantly limits the options.

Some of the steps that are useful as perioperative interventions are presented in Table 2. This is by no means an exhaustive presentation, and it may be that all are not possible in every situation. However, they can serve as a guide during the perioperative period.

Table 2. Perioperative interventions for cirrhotic patients undergoing non-hepatic surgery

Issue to be addressed	Management
Ascites	-Diuresis or paracentesis with albumin replacement -TIPS
Coagulopathy	-Vit K, fresh frozen plasma -Factor VII, cryoprecipitate if severe
Renal insufficiency	-Assess for hepatorenal syndrome -Avoid nephrotoxic agents -Use of medications such as terlipressin
Portal hypertension	-TIPS -Medications such as beta blockers
Encephalopathy	-Medications such as lactulose, antibiotics -Restrict dietary protein
Infection	-Low threshold for panculturing -Low threshold for antibiotic prophylaxis, especially antifungals
Electrolytes	-Mild interventions in treating hyponatremia, such as fluid restriction
Pulmonary status	-Evaluate for hepatopulmonary syndrome -Pulmonary function tests
Cardiac status	-Assess for portopulmonary hypertension with right heart catheterization -Dobutamine stress ECHO
Nutrition	-Evaluate nutritional status -Consider tube feeds

Role of Surgery in the Treatment of Portal Hypertension (PH)

Pathophysiology of Portal Hypertension

Cirrhosis of the liver is the most common cause of PH in the Western world, accounting for almost 90% of cases, while schistosomiasis is the leading cause in many countries in the developing world. Portal hypertension is defined by a pathological increase in the portal venous pressure. When the portal pressure gradient (the difference between pressures in the portal vein and the inferior vena cava) increases above 12 mmHg (normal is below 6 mmHg), then complications of PH can occur. Predominant among these complications is the formation of varices with the risk of eventual rupture, which can often represent a catastrophic event for the patient.

As in any other vessel, the pressure within the portal vein is determined by the product of blood flow and vascular resistance as defined by Ohm's law: ΔP (portal pressure gradient) =

Q (blood flow) x R (resistance). Q is the flow within the portal venous system, and R is the vascular resistance of the portal venous system, which is the sum of the resistance of the portal vein, the portosystemic collaterals and the hepatic vascular bed. From the equation for the portal pressure gradient, it follows that the gradient can increase by either an increase in portal blood flow, an increase in vascular resistance, or a combination of both [38-39]. As seen in Figure 1, portal pressure increases initially as a consequence of an increased resistance to flow because of a) a structural problem with the architectural distortion in the liver that is the result of the fibrosis and regenerative nodules typical of cirrhosis, and b) a dynamic one, with the increased hepatic vascular tone due to endothelial dysfunction and decreased nitric oxide availability [40]. The increase in the portal pressure gradient (the difference between portal and hepatic vein pressure) leads to the formation of collaterals between the portal and systemic circulations, a process modulated by angiogenic factors [41-43]. In addition, the second part of the equation takes place with an increase in portal venous blood inflow as a result of splanchnic vasodilatation and increased cardiac output [44]. All of these lead to the emergence and increase in size of the gastroesophageal varices.

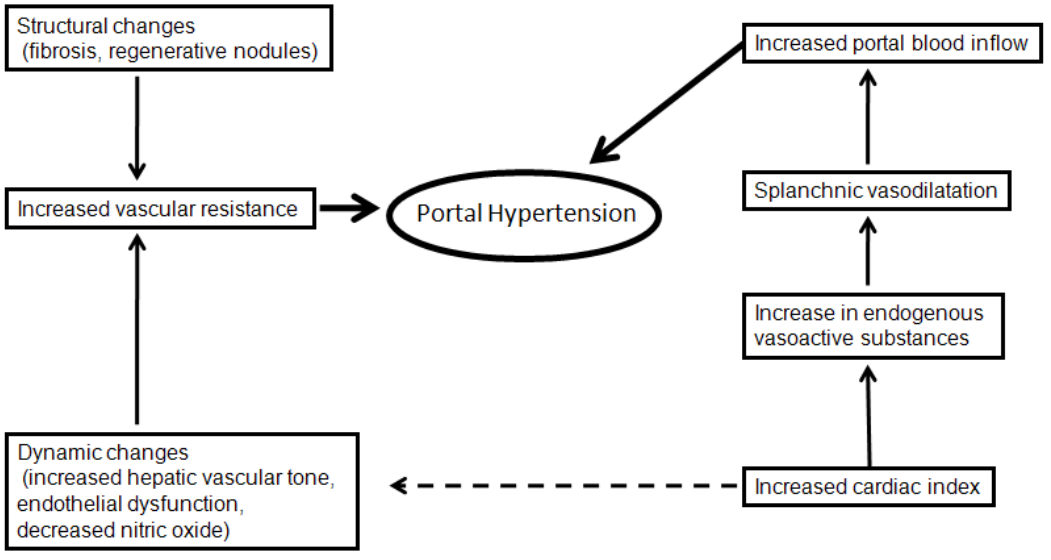

Figure 1. From the above it is clear that it is critical to be able to control PH in patients with cirrhosis, and in this next part of the chapter we will examine what, if any, is the role of surgery in that.

Surgical Shunts in the Treatment of PH: A Lost Art?

The keys to managing patients with variceal bleeding from cirrhosis and PH are controlling the acute bleeding episode and preventing a recurrent hemorrhage. Controlling the acute variceal bleeding is generally accomplished nowadays with a combination of medical and endoscopic management, whereas decompressing shunting procedures are reserved for those episodes not controlled by or refractory to these treatments [45]. Similarly, the prevention of further hemorrhagic episodes rests on medical and endoscopic therapy, with the

decompressing shunting procedures being reserved for patients with end-stage liver decompensation or refractory to the less invasive therapies. Despite urgent pharmacologic and endoscopic therapy, variceal bleeding may not be controlled or may recur in about 10% to 20% of patients [46]. Hepatic transplantation is the only curative treatment for the patient with cirrhosis and PH that experiences variceal bleeding; however, many patients for a variety of reasons (psychological, social, financial, advanced disease) may not be candidates for a LT. For all these reasons, shunting decompressing procedures still remain in the armamentarium of the team caring for the cirrhotic patient.

Shunt therapy, either as shunt surgery or transjugular intrahepatic portosystemic shunts (TIPS), has clinical efficacy as salvage therapy. Currently, TIPS is the preferred approach, as it is undertaken more than 10 times more frequently than surgical shunts to prevent or control variceal bleeding, mainly because it is a less invasive procedure [47]. TIPS is a radiologic procedure by which a tract is created between the hepatic vein and the portal vein and kept open by deployment of a coated stent, with hemostasis in 90% of cases [48-49]. TIPS-related complications include procedural ones, as well as dysfunction or stenosis, which can occur with relative frequency (50% to 80%) in the first year [50]. The latter is thought to occur less frequently with polytetrafluoroethylene (PTFE)-covered shunts. Finally, with TIPS there is the risk of worsening encephalopathy (25%), although the symptoms are mild and controlled with medications [51].

The surgical options currently used for variceal bleeding are either shunt or nonshunt procedures, with shunt procedures further divided into selective and non-selective, as can be seen in Table 3 [52]. The distal splenorenal shunt (DSRS) is the principal selective shunt, as it controls bleeding by selectively decompressing gastroesophageal varices through the short gastric veins and the splenic vein to the left renal vein, while maintaining portal flow to the cirrhotic liver [53]. This allows a lower rate of encephalopathy (<10%) compared to the other shunts [54]. Overall, it appears to be one of the more popular ones, as it has a low risk of operative morbidity, less shunt dysfunction and good long-term survival [55].

Although TIPS is used much more frequently compared to the surgical procedures, this does not necessarily mean that it is better. A large, multicenter trial of TIPS versus DSRS (the DIVERT study) found similar rates of encephalopathy, rebleeding and mortality in patients with Child's A and B cirrhosis who had failed endoscopic or pharmacologic therapy, with a higher rate of shunt dysfunction in the TIPS group [56]. Another meta-analysis in cirrhotic patients found significantly better 2-year survival and significantly less frequent shunt failure in the patients undergoing surgical shunting compared to TIPS for bleeding varices [57]. That study concluded that the meta-analysis promotes surgical shunting relative to TIPS, because of improved long-term survival and less frequent shunt failure. The study also points out that from their analysis, due to the higher rebleeding rates after TIPS and the need for frequent surveillance, the overall resource consumption to the healthcare system is greater for TIPS [58].

Table 3. Surgical shunts for PH

Type of Procedure	Advantages	Disadvantages
Shunting		
Nonselective		
Portocaval shunt (side-to-side or end-to-side)	Most effective in treating ascites and variceal hemorrhage	Completely divert portal flow from the liver predisposing to portosystemic encephalopathy and earlier onset of hepatic failure Require extensive hilar dissection
Mesocaval shunt		
Central splenorenal shunt		
Selective		
Distal splenorenal shunt	Preserve portal flow to the liver, continued delivery of hepatotrophic hormones (insulin) and continued extraction of intestinally absorbed cerebral toxins (less encephalopathy)	Not suitable for patients with significant ascites
Coronary caval shunt		
Nonshunting		
Gastroesophageal devascularization	Useful for emergency bleeding, as less technically demanding	High rebleeding rate

Those in favor of TIPS would argue that the advantage of a significantly less invasive procedure, as well as the fact that it is frequently aimed as a bridge to LT, and as such long-term patency is not key, make it an ideal choice for the cirrhotic patient. However, few patients ever undergo transplantation after TIPS with most series reporting less than 10 percent of patients undergoing liver transplant after TIPS [59-61]. Thus, the bridge to transplantation may be built, but it is rarely crossed. Overall, it should be recognized that both TIPS and surgical decompressing procedures can offer solutions to the difficult to control variceal bleed in a cirrhotic patient. As a result, what is often the determining factor in what will be used is the availability of local expertise for these highly demanding procedures.

Hepatocellular Carcinoma (HCC) in Cirrhosis

Pathophysiology of HCC in Cirrhosis

Hepatocellular carcinoma is a major health problem, being the sixth most common cancer world-wide with about 600,000 new cases a year, with the incidence increasing in Europe and the United States [62-63]. More importantly HCC is currently the leading cause of death among patients with cirrhosis, with chronic hepatitis B viral (HBV) infection the predominant risk factor in Asia and Africa, and chronic hepatitis C viral (HCV) infection in Japan and the west [64]. Hepatocellular carcinoma develops in a cirrhotic liver in 80% of cases, as cirrhosis as a preneoplastic condition is essentially the strongest predisposing risk factor [65].

Although the exact mechanism is not known, it may have to do with the uncoordinated architectural changes in the hepatic parenchyma seen in cirrhosis providing the stimulus for the malignant transformation of the hepatocytes.

The crucial factor in treating HCC in patients with cirrhosis is that any kind of treatment needs to take into consideration both diseases: the HCC and the chronic liver disease. Both of these and their severity will determine the optimal treatment. Specifically, the main surgical treatments for HCC are resection and LT, and deciding between them is a matter of the stage of the cancer and the hepatic reserve of the patient. Regarding the stage, there is no consensus, meaning that there is lack of a common language to address the problem. There are several staging systems, such as the Okuda, the CLIP (cancer of the Liver Italian Program), the AJCC/UICC (American Joint Committee on Cancer/Union Internationale Contre le Cancer), the American Liver Tumor Study Group Modified TNM Classification System, the Liver Cancer Study Group of Japan classification system, and the BCLC (Barcelona Clinic Liver Cancer) system [66]. Some of these systems depend on clinical and radiological findings before treatment, whereas others are based on histopathological findings after treatment. Perhaps the most complete approach is with the BCLC, as it has been externally validated and is advocated by several of the hepatology associations. The BCLC uses the total cancer load, the stage of the cirrhosis and the patient's level of functioning to determine the type of treatment needed, as well as the expected survival [67].

Regarding the hepatic reserve of the patient this is a matter of both quantity and quality. That is in order to consider resection as opposed to transplantation, it is important to establish beforehand that the hepatic remnant will be adequate to sustain the patient till the liver regenerates. In a cirrhotic patient this would mean a hepatic remnant of about 40%. The other issue is that of the quality of the liver, meaning the degree of liver failure, which can be determined by a variety of direct (liver biopsy) and indirect tests (EGD looking for varices, CT scan, number of platelets, INR and albumin values in serum as indicators of the synthetic ability of the liver).

After considering the issues of staging of the liver cancer in the cirrhotic patient, as well as that of the extent of the liver disease, one can proceed with the decision about the optimal surgical therapy, essentially choosing between hepatic resection and liver transplantation.

Surgery for HCC in Cirrhosis

Hepatectomy

In order to consider resection in patients with cirrhosis and HCC it is imperative that strict criteria are used, to avoid complications, such as the occurrence of postoperative liver failure. The best candidates for resection are cirrhotic patients with a single HCC lesion, and with well-preserved hepatic function; these patients can achieve five-year survival of 70% [68]. Defining well-preserved hepatic function is a matter of debate; some studies have defined it as the absence of clinically significant portal hypertension (pressure difference with the hepatic veins less than 10 mmHg, no varices or splenomegaly, and platelets over 100,000/mm3) and normal bilirubin values [69]. Based on these same studies, survival can decrease significantly (five-year survival of 50%) if there is portal hypertension, and even more if there is an elevation of the bilirubin (five-year survival down to 25%). Other studies have suggested the use of the MELD score as a way to predict the possibility of hepatic decompensation after hepatectomy, with a value of 9 as the cutoff for a safe hepatectomy without a significant risk of postoperative liver failure [70]. The biggest hurdle in the long-

term survival of these patients, even in those fulfilling the strict criteria, is the high rate of recurrence (over 70% in five years), with major determining factors being the presence of microvascular invasion, satellite lesions, and low-grade differentiation [71].

In an effort to treat patients with large HCCs that are outside the criteria for liver transplantation, there are certain preoperative maneuvers, such as the portal vein embolization, that can help optimize the result. Specifically, by embolizing the portal vein on the side of the HCC, one can expect a 10-30% increase in size of the rest of the hepatic parenchyma, thus making a liver resection feasible [72]. This carries a risk in patients with cirrhosis, as the liver regeneration that will ensue is not an orderly one. Even so, it is possible to achieve survival in cases that present a significant challenge. In a study of 166 patients with HCC over 10cm in diameter and half of them also having cirrhosis the five-year survival was 29% [73]. This may not seem like a high number, but if we consider the extent of the liver disease, the size of the HCC and the lack of any good options in these patients, these numbers have a certain value.

No discussion about liver resection would be complete without mentioning the possibility of laparoscopic resection. There was initial reluctance in the surgical community, as there was concern that the long-term results would not be the same and whether an oncologically sound resection would be possible. Recent studies show that with the proper patient selection (Child A cirrhosis and HCC present in segments 2, 3, 4b, 5 and 6) it is possible to perform a laparoscopic hepatectomy with safety and 3-year survival over 60%, that is equivalent to the open hepatectomy [74]. In addition in none of these studies were there any recurrences at the resection margins or at the sites of laparoscopic instrument entry into the abdomen [75].

Overall, we can see that it is possible with liver resection to achieve acceptable therapeutic results for HCC in patients with cirrhosis, even in challenging cases. It should be noted, that when considering the different surgical treatments for HCC in cirrhotic patients, hepatectomy is preferable to LT given the limited organ supply. However, they should not be viewed as competitive options, but rather as complimentary. That is the cirrhotic patient with HCC considered for a hepatectomy should also undergo an evaluation for LT, as the possibility of hepatic decompensation after liver resection and the need to proceed rapidly to LT should not be underestimated. These are discussions that should take place with the patient and the family before undertaking these treatments.

Liver Transplantation

Liver transplantation is the main treatment for patients with cirrhosis and HCC in whom resection is not possible, because of either the location and anatomy of the tumor or the hepatic reserve of the patient after resection. Due to the limited organ supply, there are commonly accepted criteria based on which patients can enter the waiting list. The most frequently used are the Milan criteria (one lesion less or equal to 5cm in size or no more than three lesions, none of which are over 3 cm in size), which when used can lead to a five-year survival of 70% [76]. Other studies have shown that it is possible to expand the Milan criteria, in terms of the size and/or number of lesions and still achieve comparable survival. One example are the San Francisco criteria (single lesions up to 6.5cm in size or multilobular HCC with up to 3 lesions, none of whom are larger than 4.5cm in size and with a total diameter of all lesions less than 8cm) that can lead to comparable five-year survival of 70-80% [77].

An important problem for the HCC patients was that usually their liver disease was not advanced enough to place them in the top part of the waiting list, where the main criterion in the US and in several other countries is the MELD score. As a result it was observed that after one year on the transplant waiting list, approximately 40% of HCC patients were no more candidates for LT [78]. To overcome this problem the decision was made to provide patients with HCC that were within Milan criteria additional points as their waiting time on the list increased, in an effort to get them to LT before the HCC was too advanced. That system needed to be readjusted over time in order to maintain, to the extent possible, the principle of equity with the other etiologies for liver disease, so that HCC patients would not be favored.

An additional strategy by several of the transplant centers is the use of locoregional treatment of HCC, while the patient is on the waiting list. Different types of locoregional treatment have been used, such as Transcatheter Arterial Chemoembolization (TACE), radiofrequency ablation (RFA), or microwave coagulation therapy (MCT). The goal of these therapeutic modalities is to bridge the patient to the LT, by not allowing the HCC to advance beyond the established criteria, or even to downstage the tumor, so that the patient becomes eligible for a LT. In a study of 48 patients outside Milan criteria, the use of some of these locoregional therapies led to 43 patients ending within Milan criteria and undergoing LT with results equivalent to patients originally inside Milan criteria [79]. There is the belief that this strategy also helps better select HCC patients for LT, as those patients that after TACE or RFA have stable disease can achieve five-year survival of 90%, as opposed to those in whom the tumor continues to grow after the locoregional treatments and who have a five-year survival of only 35% [80].

A possible solution to the long waiting time for LT is that of the Living Donor Liver Transplantation (LDLT). Currently in the US, LDLTs comprise about 5% of the total number of transplants, with the experience for HCC being somewhat limited, but positive. In a study from Japan, where LDLT is much more prevalent, of 316 patients with HCC that underwent LDLT, the one- and three-year survivals were 78% and 69% respectively, whereas the one- and three-year recurrence-free survivals were 73% and 65% respectively [81]. Other studies have shown improved survival of HCC patients that underwent LDLT compared to those that underwent LT with a graft from a deceased donor (86% vs. 71% one-year survival) [82]. Despite these positive results, there are certain concerns. Primarily, one has to consider that this is an operation that involves significant risk for a healthy living donor, as it is unique in surgery as a procedure that patients undergo without any health benefit for themselves. Additionally, although it does offer the possibility of a more aggressive approach, in that patients that are outside criteria can be transplanted since they have their own living donor, it does raise the question of what should happen if the living donor graft fails and whether that recipient would now have immediate access to the deceased donor waiting list, something that would not have been possible before. Finally, as seen in certain multicenter trials, such as the Adult-to-Adult Living Donor Liver Transplantation Cohort study in the US, there is a higher risk of HCC recurrence in the LDLT recipients compared to those form a deceased donor (29% vs. 0%), despite the very short time on the waiting list (160 vs. 469 days) [83].

A point worth reiterating is that hepatectomy and LT represent two important points in the spectrum of surgical management of HCC in patients with cirrhosis, and that we need to consider the role of the locoregional treatments, such as TACE, RFA, MCT and Percutaneous alcohol injection (PEI) [84-87]. Each of these therapies have their advantages and disadvantages, but their common denominator is that by themselves they do not usually

constitute complete therapy for HCC (with the possible exception of very small HCC lesions less than 2cm), but rather temporizing or complimentary measures. Their optimal use is as bridging therapies to LT or as complimentary therapies to resection. For example in cases with multifocal HCC disease it may be possible to resect one lobe and use RFA for the lesions in the other one, so as to preserve as much of the hepatic parenchyma as possible [88]. Also, in cases of recurrence after hepatectomy in cirrhotic patients or after LT, it may be possible to treat them, if they are detected promptly, with these locoregional methods [89].

Conclusion

We have examined in this chapter the role of surgery in treating patients with cirrhosis. First, we analyzed the approach to the cirrhotic patient that is in need of non-hepatic surgery, and how to assess the risks involved regarding the type of surgery and the level of hepatic function. Then, we approached the topic of portal hypertension, a common problem for all cirrhotic patients, and how surgery still has a role in treating the problem of variceal bleeding, despite the significant progress made with interventional methods, such as TIPS. Finally, we addressed the issue of HCC in cirrhosis, which is one of the more common causes of death for these patients, and we identified the whole spectrum of surgical treatments, ranging from hepatectomy to liver transplantation, with the complimentary role of locoregional treatments.

The most important point in this chapter is that patients with cirrhosis represent a special category of patients in that their hepatic and overall reserves are significantly depleted. In a situation like that, any type of stress factor, and any surgery would qualify as such, can easily undermine the fragile balance that exists. For this reason the best way to approach patients with cirrhosis is by a multidisciplinary team at a center that can offer the whole spectrum of therapies for cirrhosis.

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Chapter 8

Primary Biliary Cirrhosis – An Infection Triggered Disease?

Jochen Mattner
Cincinnati Children's Hospital Medical Center and University of Erlangen

Abstract

Primary Biliary Cirrhosis (PBC) is a chronic cholestatic liver disease that usually progresses to liver failure and death unless liver transplantation is performed. Its pathophysiological hallmarks include the destruction of small bile ducts by T cells and the production of autoantibodies to mitochondrial antigens that bind the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). Although a combination of environmental factors and genetic predisposition likely triggers PBC in susceptible individuals as suggested similarly for the etiology of a majority of autoimmune diseases, this devastating liver disorder is considered to be rather immune - mediated than autoimmune due to its (relative) unresponsiveness to immunosuppressive agents.

One frequent cause of immune activation under pathologic conditions is represented by overt or covert bacterial or viral infections, but revealing microbial involvement and understanding the cellular and molecular mechanisms leading to immune - mediated tissue damage generally remain a challenge. Recent clinical studies strongly suggest that infection with the ubiquitous alphaproteobacterium *Novosphingobium aromaticivorans* is specifically associated with susceptibility to PBC. Notably, there is compelling evidence that patients with Primary Biliary Cirrhosis (PBC) express antibodies against various lipoylated enzymes of *Novosphingobium* including the conserved bacterial homolog of the mitochondrial PDC-E2 enzyme, the major PBC antigen. This seropositivity for *Novosphingobium* is highly specific of PBC and not found in healthy subjects and other diseases.

Infection alone is likely not sufficient to confer disease, genetic susceptibility plays an important role. Naturally occurring allelic polymorphisms determine the susceptibility to autoimmunity. Underlying single nucleotide polymorphisms (SNPs) likely evolved due to microbial pressure and reveal a consequence of natural selection for altered susceptibility to certain pathogens. Notably, we have not only developed a mouse model that recapitulates key features of the human disease upon infection with

Novosphingobium, but also identified genetic susceptibility loci that drive susceptibility to PBC. Given the emerging role of genetic factors, defining their control and regulation will improve the understanding of pathogenic mechanisms in PBC. The translation of findings from the mouse model into human disease will help to define these genes as novel disease risk factors and should subsequently help guide the development of diagnostic tests and/or effective therapies next to ursodeoxychloic acid. These are needed as current immunosuppressive drugs are often not target specific, ineffective and elicit severe side effects.

1. Introduction

Primary Biliary Cirrhosis (PBC) is an incurable chronic cholestatic liver disease that usually progresses slowly to liver failure and death unless liver transplantation is performed. The diagnostic hallmarks of PBC are the immune-mediated destruction of the intrahepatic small- and medium-size bile ducts by T cells and auto-antibodies to mitochondrial antigens (AMAs) that bind the inner lipoyl domain of the E2 subunit of the pyruvate dehydrogenase complex (PDC- E2) [1] [2] [3]. These PDC-E2 subunits form the dominant autoreactive epitopes for B cells and for liver-infiltrating CD4+ and CD8+ T cells [4] [5] [6] [7] [8]. The non-suppurative inflammatory process in PBC is accompanied by increased IFN-γ and IL-17 levels while IL-10 copy numbers are reduced [9-11].

PBC has distinct geographic and demographic features. Highest prevalence occurs in Northern Europe and women between the 5^{th} and 7^{th} decade of life constitute 90% of PBC patients. The prevalence rate in the US and Europe ranges between 30 and 60 per 100,000 with an incidence rate of 3 per 100,000 [1] [12]. Although there have been no associations with particular HLA antigens described to date, the familiar accumulation of PBC and the concordance rate for PBC in identical twins being among the highest reported in immune-mediated disorders suggests a strong genetic component.

2. Clinical Symptoms

The majority of PBC patients is asymptomatic at the time of diagnosis. Overt clinical symptoms include fatigue and pruritus and develop in most patients two to four years later. Survival rates are variable and correlate with the number and severity of symptoms present in each individual patient; negative prognostic factors include jaundice, fatigue, progressive bile duct loss, liver cirrhosis and accompanying co-morbidities. In general, liver failure occurs in most patients six to 12 years after the presentation of clinical symptoms; however, large inter-individual differences have been described and patients can even remain asymptomatic after diagnosis for decades [13].

PBC is often diagnosed by accident during routinely screening tests by the detection of elevated serum levels of alkaline phosphatase and/or gamma-GT and confirmed by the presence of highly specific anti-mitochondrial antibodies (AMAs). These antibodies consisting of the IgG and IgA subclass bind to the E2 subunit of the pyruvate dehydrogenase enzyme complex (PDC-E2) present on the inner mitochondrial membrane and are detected in 90-95% of PBC patients [1]. Next to the pathogenic role of antibodies of the IgG class,

especially IgG2a, anti-PDC-E2 IgA have been implicated in the pathogenesis of PBC. It has been hypothesized in this context that PBC is a mucosal disease with generalized epithelitis. In fact, immunoglobulin IgA AMA are readily detected in the bile, saliva, and urine from patients with PBC [14] [15]. Anti-PDC-E2 IgA may be also involved in the destruction of bile duct epithelial cells in PBC either by binding PDC-E2 and forming immune complexes or by trafficking to mitochondria during the normal course of transcytosis [16]. Next to these signature anti-PDC-E2 IgG and IgA antibodies, other circulating auto-antibodies such as anti-SMA, anti-gp210, ANA or rheumatoid factor are frequently detected; while those are unspecific, hypergammaglobulinemia with selective IgM elevation is a common serum feature of PBC [1]. Hyperlipidemia, hypothyroidism and osteopenia are also frequently observed in PBC patients. As PBC is often accompanied by other autoimmune disorders such as Sjorgen's disease, keratoconjunctivitis sicca and scleroderma [1], other autoantibodies may be found as well. SLE can also occur together with PBC [17] and autoantibodies directed at nuclear antigens are found in approximately 50 percent of patients with Primary Biliary Cirrhosis, and often in patients who have anti-mitochondrial antibodies [1]. At later stages of disease, patients suffer from malabsorption, deficiencies of fat-soluble vitamins and steatorrhea. Although patients develop portal hypertension during end-stage disease, ascites, hepatic encephalopathy and hemorrhage from esophageal varices are only rarely detected [13].

3. Histopathological Features

Florid PBC bile duct lesions are characterized by the T cell-mediated injury to bile duct epithelium and the disruption of the bile duct basement membrane within the inflammatory process [1]. Expression of MHC II is observed on cholangiocytes in liver biopsies of patients with PBC [18]. Pro-inflammatory cytokines like IFN-γ, TNF-α and/or IL-1 have been also shown to increase the expression of intercellular adhesion molecule-1, HLA class I, and HLA class II on human intrahepatic biliary epithelial cells *in vitro*; these observations are consistent with the hypothesis that locally acting factors like cytokines and/or chemokines may play a part in the pathogenesis of immune mediated disorders such as primary biliary cirrhosis in which immune mediated bile duct damage occurs.

Only small and medium-size intrahepatic bile ducts form the target for autoreactive immune cells and are progressively destroyed during disease progression, while large intra- und extrahepatic bile ducts remain unaffected. The inflammatory infiltrate is composed of CD4+ and CD8+ T cells and B lymphocytes, macrophages and varying numbers of plasma cells, but also DCs, NKT and NK cells are found within the mixed lympho-/monocytic periportal infiltrate [1]. The presence of scattered eosinophils in the periportal infiltrate is one histopathologic hallmark of PBC and helps to distinguish PBC from autoimmune hepatitis (AIH) [19] [20]. During the destructive process, the proliferation of bile duct epithelial cells is accompanied by the development of fibrosis. The progressive loss of bile ducts contributes to the chronic cholestasis and subsequently causes liver cirrhosis and failure. It is not understood to date, why these mitochondrial antigens are specifically targeted and why the immune reaction is restricted to the small bile ducts of the liver, although the autoantigen is ubiquitously expressed in all tissues of the body.

4. Medications

Although PBC constituting one of the first disease conditions in which specific autoantibodies were recognized and therefore regarded as a model of autoimmune disease, patients with PBC respond very poorly to immune suppression [21]. Based on the ineffectiveness of corticoids and other immune suppressants, PBC is considered nowadays as immune-mediated rather than autoimmune disease. However, immune suppressive medications are often administered to PBC patients as their accompanying autoimmune co-morbidities are frequently treated with them.

The only FDA-approved medical treatment for PBC is ursodeoxycholic acid (UDCA). When administered at adequate doses of 13-15 mg/kg/day, up to 60% of patients experience an extended life expectancy [22] [23]; application of UDCA may slow down the progression to liver cirrhosis, prolong the time period for patients without the need for liver transplantation and improve the overall quality of life [24] [25] [26] due to the reduction of clinical symptoms like pruritus. Application of UDCA reduces serum biochemical markers such as bilirubin, AP, gamma-GT, cholesterol, and IgM levels [27] [28]. Its application is safe and it elicits only rarely few side effects [29].

Although a substantial number of patients profit from UDCA, the mechanism(s) of its action have remained unclear. Key mechanisms may involve the stimulation of impaired bile duct cellular secretion, anti-apoptotic effects, inhibition of fibrosis, immunomodulatory properties and the detoxification of bile [1] [12]. Patients that do not properly respond to UDCA therapy need liver transplantation, which is the only effective therapy for late-stage disease. Although histologic disease recurrence has been reported in up to 30% of patients, clinically significant and progressive recurrent disease is uncommon [30].

5. Pathogenesis

Although the first report describing chronic biliary cholestasis in the absence of mechanic obstruction of the extrahepatic bile ducts dates back to 1851 [115] and the term “primary biliary cirrhosis” was coined in 1949 [31], the pathogenic mechanism(s) of biliary injury in PBC are still poorly defined. This may be in part due to the only very recent development of small-animal models that mimic human disease [32] [33] [34] [35] [36] [37].

The stimuli that trigger autoreactivity are unknown but include both genetic and environmental factors [38]. PBC illustrates the importance of genetic factors well because of its high degree of concordance in identical twins [39]. Both genetic and environmental influences are implicated by its clinical uniformity and the specific presence of a highly specific serologic marker, autoantibodies (AMA) directed against the E2 subunits of 2-oxo-acid dehydrogenase complexes (PDC-E2) in the inner mitochondrial membrane [1] [40].

Molecular mimicry has been proposed as mechanism for the development of autoimmunity in PBC [41]. Environmental factors implicated in the pathogenesis of PBC include tobacco, reproductive hormones, exposure to nail polish or toxic waste, xenobiotics and repeated urinary tract infections [42] [43]. Some bacteria and viruses have also been suggested as causative agents [1]. One particular bacterium, however, *Novosphingobium aromaticivorans*, stands out because it has a 100-1,000-fold greater homology with the

immunodominant region of human PDC-E2 than any microorganism thus far studied [44] [45] [46].

6. Novosphingobium SPP

Novosphingobium aromaticivorans are gram-negative, LPS-negative, oxidase positive, non-fermentative rods of the *Sphingomonodaceae* family found ubiquitously in the environment [47] [48] [49] [50] and at mucous surfaces and in the feces of humans [44]. Although the genera *Novosphingobium*, *Sphingobium*, *Sphingomonas*, *Sphingophyxis* form the genus *Sphingomonas* [51] [52], these terms are often synonymously used. In order to simplify the complex taxonomy, we will refer to all species under the genus *Sphingomonas* as *Novosphingobium spp*. from now on.

Several *Novosphingobium* strains degrade a wide variety of environmentally hazardous compounds, including polycyclic aromatics [53], dioxine compounds [54] and chlorinated phenols [55]. These unique capacities of *Novosphingobium spp.* are used for the detoxification of industrial polluted soils [56] [57] and explain the survival of *Novosphingobium spp.* at polluted locations where they can metabolize these hazardous compounds as sole carbon source. The presence of *Novosphingobium spp.* in these locations may co-incide with the increased incidence of PBC in areas of toxic waste. Several *Novosphingobium* strains are also xenobiotics [57] [58] [59], a feature that may impact on steroid metabolism and autoimmunity in humans.

Although the number of clinical case reports describing nosocomial infections and septic shock associated with *Novosphingobium spp.* is limited [60] [61] [62], one has to keep in mind that *Novosphingobium spp*. had to be renamed because of their unique cell wall composition [63] (see below). Preferentially *Novosphingobium paucimobilis* (formerly *Pseudomonas paucimobilis*) has been implicated in a variety of community-acquired and nosocomial infections, including bacteremia, catheter-related sepsis, meningitis, peritonitis, pneumonia, cutaneous infections, visceral abscesses, urinary and biliary tract infections, adenitis, and diarrheal disease [64] [65] [66] [67] [68] [69] [70] [71]. Administration of antibiotics resulted in the resolution of infection in all patient groups. As nosocomial *Novosphingobium* infections can be as resistant as *Pseudomonas aeruginosa*, a combination therapy of a third generation cephalosporin with an aminoglycoside is recommended [116].

The lack of a typical lipopolysaccharide constituent of the cellular membrane of *Novosphingobium spp.*, with the accompanying deficiency of endotoxin activity, may explain the lack of deaths attributed to this organism [72] [73]. Instead, Glycosphingolipids (GSLs) build the cell wall of *Novosphingobium* [72] [73]; their discovery led not only to the renaming of these bacteria, but also uncovered a novel pathway of innate immune recognition through natural killer T (NKT) cells.

7. Recognition of *Novosphingobium* GSLs by Natural Killer T (NKT) Cells

NKT cells that are most abundantly found in the liver constitute a population of innate (-like) T lymphocytes that predominantly use a conserved semi-invariant mouse Vα14-Jα18/ Vβ2, Vβ7, Vβ8 or human Vα24-Jα18/Vβ11 TCR with specificity for CD1d combined with GlycoSphingoLipid (GSL) ligands [74] [75]. CD1d presents GSL antigens on the surface of B cells, macrophages and dendritic cells (DCs) to NKT cells after sampling the late endosomes and lysosomes of these cell populations and loading GSLs in these compartments. The CD1d binding groove is composed of connected hydrophobic channels where the fatty acid portion of the GSL is buried, whereas the polar head is exposed for recognition by the TCR.

NKT cells participate in a prompt and widespread activation cascade *in vivo*. The sequence initially involves cognate interaction with, and crossactivation of CD1d expressing DCs through CD40L/CD40 interactions, explosive release of cytokines and chemokines and powerful activation of NK cells within 30-60 minutes [76] [77]. Likewise, B cells, which also express CD1d, are activated to upregulate their costimulatory properties [78]. GSL ligands have been shown to constitute one of the most efficient adjuvants available [79] [80], particularly for the production of antibodies and vaccines for cytotoxic lymphocytes (CTLs), and are widely used in clinical trials [81] [82] [83] [84].

In mice and in humans, natural killer T (NKT) cells recognize GlycoSphingoLipid (GSL) antigens of *Novosphingobium* that replace LPS in its cell wall [85] [86] [87]. In the absence of *tlr-4* signaling, the NKT cell population may therefore represent the major innate immune recognition pathway for this class of bacteria. Striking redistribution of NKT cells from the blood to the livers in patients with PBC [88] [89] and aberrant expression of CD1d on bile duct epithelium [90] may reflect the recognition of *Novosphingobium* GSLs by NKT cells during a (latent) infection of PBC with this bacterium.

8. Specific Recognition of PDC-E2 Homologues From *Novosphingobium Aromaticivorans* by Sera of PBC Patients

As outlined before, the E2 subunits of the pyruvate dehydrogenase complex form the target antigens for autoreactive T and B lymphocytes in PBC patients. Notably, there is compelling evidence that patients with Primary Biliary Cirrhosis (PBC) express antibodies against various lipoylated enzymes of *Novosphingobium aromaticivorans* including the bacterial homolog of the mitochondrial enzyme PDC-E2, the major PBC antigen [44] [45] [46]. In fact, seropositivity for *Novosphingobium* is found in seronegative PBC patients and is therefore more closely associated with disease than the presence of anti-PDC-E2 antibodies. Seropositivity for the alphaproteobacterium *Novosphingobium* is highly specific of PBC and not found in healthy subjects and other diseases. Analysis of mitochondrial encoded genes and their genomic organization/ distribution imply that mitochondrial genomes are derived

from an alphaproteo-bacterium (-like) ancestor that invaded an Archea-type host more than 1.5 billion years ago [91]. This evolutionary association of alphaproteobacterial and mammalian mitochondrial antigens might be critical for the development of PBC (molecular mimicry) and the target specificity of the immune attack to mitochondrial enzymes.

9. Mouse Model of Infection-Induced Primary Biliary Cirrhosis

Notably, we have replicated these clinical reports in mice and established a model where infection of mice with *Novosphingobium aromaticivorans* induces anti-PDC-E2 IgG responses and liver lesions resembling PBC in humans [37]. Although NKT cells accelerate the clearance of *Novosphingobium aromaticivorans* like other *Novosphingobium spp.*, their activation upon microbial encounter may be deleterious for the host, providing innate signals that contribute to the breakdown of tolerance and unleash autoimmune effector cells. As CD1d-deficient mice that lack NKT cells exhibited significantly less severe liver disease and developed significantly reduced anti-PDC-E2 IgG titers than wild type mice and as NKT cells, that are abundantly found in the liver, dominate the innate immune response to *Novosphingobium*, that preferentially persist in the liver upon infection, the activation of NKT cells by *Novosphingobium*-GSLs may explain the liver specific pathology. B6.Vα14 transgenic mice, which overexpress NKT cells, exhibited also more severe histological lesions and higher anti-PDC E2 IgG titers than wild-type B6 mice suggesting that NKT cells provide help for anti-PDC-E2 IgG producing autoreactive B cells. While polyclonal IgM responses including anti-nuclear antibodies have been described in infected mice [92] [93] [94] [95], the high titer of the IgG response months after infection and its detection in mouse strains including B6 that are not prone to autoimmunity has not been reported previously. Generation of these persistent IgG autoantibody responses requires innate signals that are associated with *Novosphingobium*- infection, which recruits NKT cells help for B cells (cytokines, CD40L, cognate). Direct cognate interactions between NKT and B cells are required to produce in particular anti-PDC-E2 IgG2a responses, the subclass of auto-antibodies that is in particular considered pathogenic. In contrast, TLR signaling alone, in the case of other bacteria, fails to provide similar helper signals as suggested by the drastically reduced anti-PDC-E2 IgG titers in CD1d-deficient compared to wild-type mice. Once disease is established in the mouse model, liver lesions can be adoptively transferred by conventional CD4+ and CD8+ T cells from wild type, but not NKT-deficient mice after bacterial clearance into irradiated congenic recipient mice. This illustrates the importance of early microbial activation of NKT cells in initiating autonomous, organ-specific autoimmunity and suggests a critical role of NKT cells in the propagation and/or expansion of autoreactive T and B cells.

This model does not only unleash an innate immune mechanism for the organ specific development of (auto-) immune responses to ubiquitously expressed antigens, but also reflects the situation in humans that is characterized by the redistribution of NKT cells from the blood to the liver [88] [89]. Although the inherent autoreactivity to self GSL antigens [96] may well underlie the role of NKT cells in some forms of autoimmunity, the discovery of microbial GSL ligands of NKT cells suggests an alternative, complementary scenario whereby unrecognized infection by alphaproteobacteria may drive autoimmunity. This

hypothesis, which challenges the idea that autoreactivity is the "be all and end all" pressure for NKT cells [96] [74], is particularly relevant in the context of our infection induced PBC model that replicates the recent clinical reports associating human PBC with a striking antibody response against *Novosphingobium* and with tissue redistribution of NKT cells to the liver. Based on their potent adjuvant functions [79] [80] [81] [82] [83] [84], vaccination strategies with NKT cell ligands may provide a new tool for the prevention of infectious diseases, considering the fact that some of the pathogens re-emerge due to altered migration features of the population. However, the consequences of NKT cell activation under these circumstances and in the respective infectious models need to be elucidated in further detail. This will help also to evaluate in which situations the extremely powerful adjuvant properties of NKT cells can be utilized in vaccination strategies without inducing side effects as exemplarily shown in the *Novosphingobium* - infection model of PBC.

10. Genetic Susceptibility to PBC

Although autoimmunity often clusters together in individuals and families, indicating the potential for a broad-spectrum genetic defect in immunological tolerance mechanisms, the triggers that target specific antigens and induce tissue-/organ-specific pathology have remained often unknown [97]. The nature of environmental triggers and/or tissue-specific events that enable a defined genetic allele to be protective for one or more autoimmune diseases on the one hand but increase susceptibility to different autoimmune targets on the other require therefore definition. NOD congenic mice - generated through the introgression of genetic regions from B6 and B10 mice that exhibit great allelic variations from the NOD background - mimic this situation in humans and proved to be an useful tool for the identification of genetic regions associated with autoimmunity: for example, introgression of diabetes susceptibility loci (*Idd*) from chromosome 3 and 4 of B6 and B10 mice render these mice resistant to spontaneous type 1 diabetes (T1D), but increase their susceptibility to primary biliary cirrhosis (PBC) [33] [32]. While NOD congenic mice containing the complete chromosome 3 and 4 develop spontaneous liver disease, NOD congenic mice containing smaller introgressed regions do not develop spontaneous PBC while being (partially) protected from type 1 diabetes; some of these NOD congenic mice are particularly susceptible to *Novosphingobium* induced PBC, while some are protected. Naturally occurring genetic polymorphisms between the NOD and the B6 or the B10 background may underlie the susceptibility to autoimmune disease in these mice similarly as suggested for humans. The underlying single nucleotide polymorphisms (SNPs) most likely evolved due to microbial pressure and reveal a consequence of natural selection for enhanced resistance/susceptibility to certain pathogens. Association studies mainly focused on immune genes belonging to both the human leukocyte antigen (HLA) family and non-HLA immune modulator genes. Next to allelic variations within the MHC class II gene (DR, DQ), components of the innate (C4*Q0, C4B*2, NRAMP1/SLC11A1, MBL, VDR) and the adaptive (CTLA4, IL-1beta, TNF alpha, IL12A, IL12RB2) immune systems have been shown to be associated with susceptibility to PBC [98] [99] [100] [101] [102] [103] [104] [105] [106] [107]. The possible role of allelic variation of several components of the innate and adaptive immune system suggests some disturbances of host resistance to microbial infection in PBC and their implication in the

initiation or perpetuation of the inflammatory process. This may apply in particular for genetic variations of the IL12 pathway since several data link inherited deficiencies of IL12, IL12R, and interferon gamma to increased susceptibility and severity of infectious diseases in particular mycobacterial diseases [108].

As the candidate genes within the introgressed regions in NOD congenic mice can be identified using haplotype analyses and genetic deletion approaches and the function and/or expression of the proteins encoded in the respective candidate genes can be evaluated, translational studies allow a direct correlation of the regulation of susceptibility genes in the mouse system to candidate genes in human disease. Considering the fact that PBC is induced by *Novosphingobium* in the mouse system, genetic susceptibility in the context of autoimmunity may not only reflect the tendency of susceptible individuals to develop aberrant immune responses against self- antigens, but also the inability to prevent an infection with a particular microbe and/or the inability to clear the microbe without causing collateral tissue damage. In addition, a latent infection with *Novosphingobium* may alter the expression and/or function of the in the candidate gene encoded proteins regulated by genetic alterations in susceptible individuals which may contribute to enhanced autoantibody production and/or redirection of an antigenic specificity due to augmented NKT- and/or T-B cell interactions. By translating our findings from the mouse system into human disease, our goal is to develop a predictive test for patients at risk to develop PBC that is based on the expression and/or function of the respective candidate genes.

11. Conclusion

The observation that PBC might be triggered by an ubiquitous alphaproteobacterium that is also found in the intestines of humans [44] opens new routes and possibilities for treating and diagnosing this devastating disease. Therefore, signs of infection need to be more carefully examined as they may not be immediately apparent. In order to understand these mechanisms animal models need to be developed which complement clinical and epidemiological studies. Uncovering the etiologies for these devastating liver diseases in the context of genetic susceptibility requires therefore further attention and research efforts. NOD congenic mice provide thereby not only a unique tool for the identification of genetic susceptibility region, but allow also the translation into human disease as SNPs in orthologous candidate genes have been also frequently associated with autoimmune disease in humans [109] [110].

Given the emerging role of microbial pathogens in autoimmune diseases, defining their control and regulation by the host immune response will improve the understanding of pathogenic mechanisms in these disorders. The discovery of infectious triggers of autoimmunity and the elucidation of the mechanisms by which they induce autoimmune tissue damage will change our current concepts about the etiology of various autoimmune syndromes and may suggest new, simpler ways to diagnose and treat these debilitating diseases. As anti-PDCE2 responses can proceed the development of liver lesions (reported even for 2 decades in advance) [111] [112] [113] [114], the presence of anti-PDC-E2 antibodies in the serum might be a good indicator for the application of antibiotics to patients. However, pre-screening tests would be necessary as patients in general just present at stages

of disease where liver lesions are already present. These tests in combination with the detection of anti-PDC-E2 antibodies may help to identify the actual time window in which the application of antibiotics may prevent the onset of symptomatic disease.

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Chapter 9

(Surgical) Treatment of Portal Hypertension

Falk Rauchfuss [*] ***and Utz Settmacher***
Department of General, Visceral and Vascular Surgery, University Hospital Jena, Jena, Germany

Abstract

Portal hypertension is a major complication of liver cirrhosis and may lead to life-threatening hemorrhage of esophageal varices, ascites, hepatic encephalopathy and/or renal failure.

From the pathophysiological point of view, this hypertension is caused by an increased intrahepatic resistance due to the liver cirrhosis and is aggravated by a decreased sensitivity to endogenous vasopressors and an increased local concentration of vasodilators.

The therapeutic options range from symptomatic medical treatment to endoscopic treatment (band ligation) to radiological interventions (placement of a transjugular intrahepatic portosystemic shunt) to surgical procedures.

In this review, we present different treatment regimes for the treatment of portal hypertension focusing on the different shunt procedures for surgical treatment of portal hypertension. The operative options include side-to-side shunts as well end-to-side anastomoses between the portal vein and the inferior vena cava, distal spleno-renal shunts (Warren-shunts) and mesocaval shunt procedures (Drapanas-shunts). Furthermore, there

[*] Corresponding author:
Falk Rauchfuss, MD
Department of General, Visceral and Vascular Surgery
University Hospital Jena
Erlanger Allee 101
07747 Jena
Germany
Phone: +49-(0)3641-9322695
Fax: +49-(0)3641-9322602
Mail: Falk.Rauchfuss@med.uni-jena.de

are several rarely performed shunts (e.g. Linton-shunts) and devascularization procedures.

While liver transplantation is an option for curative treatment of liver cirrhosis, the different shunt procedures are important for the symptomatic treatment of portal hypertension, especially in CHILD-PUGH A patients. Furthermore, due to the persistent shortage of donor organs the shunt procedures are important interventions prior to transplantation to prevent potential life-threatening complications of portal hypertension, such as massive hemorrhage of esophageal varices, as a bridging therapy to liver transplantation.

Introduction

Portal hypertension (PH) is a consequence of liver cirrhosis that may develop life-threatening complications. PH is defined as a porto-caval pressure gradient of more than 5 mmHg.

Key points for the development of PH are an increased resistance to portal flow and increased portal venous blood inflow [1]. There are several mechanisms in the development of PH depending on whether this originates pre-hepatically, intra-hepatically or post-hepatically (see Table 1).

Table 1.

Location	Cause (examples)
Prehepatic	Malformation of the portal vein; extrahepatic portal vein thombosis
Intrahepatic presinusoidal	Congenital liver fibrosis; schistosomiasis
Intrahepatic postsinusoidal	Liver cirrhosis
Posthepatic postsinusoidal	Budd-Chiari syndrome

In liver cirrhosis, the increase in resistance is on the one hand structural due to a distortion of liver vascular architecture by fibrosis and regenerative nodules and on the other hand dynamic due to an increased hepatic vascular tone caused by endothelial dysfunction and increased nitric oxide bioavailability [1, 2].

Parallel with the development of porto-systemic collaterals (which develop between the portal and systemic circulation), portal venous blood inflow often increases because of splanchnic vasodilatation and increased cardiac output [1, 3] (see Figure 1).

Markers for PH are esophageal varices, platelet count decrease, spleen size increase and hypergammaglobulinemia [4]. Complications are mainly esophageal varices with subsequent bleedings, ascites and portal hypertensive gastropathy.

Treatment options for PH range from conservative treatment to endoscopic therapy to interventional therapy to surgery. Surgical treatment includes several shunt procedures as well as orthotopic liver transplantation.

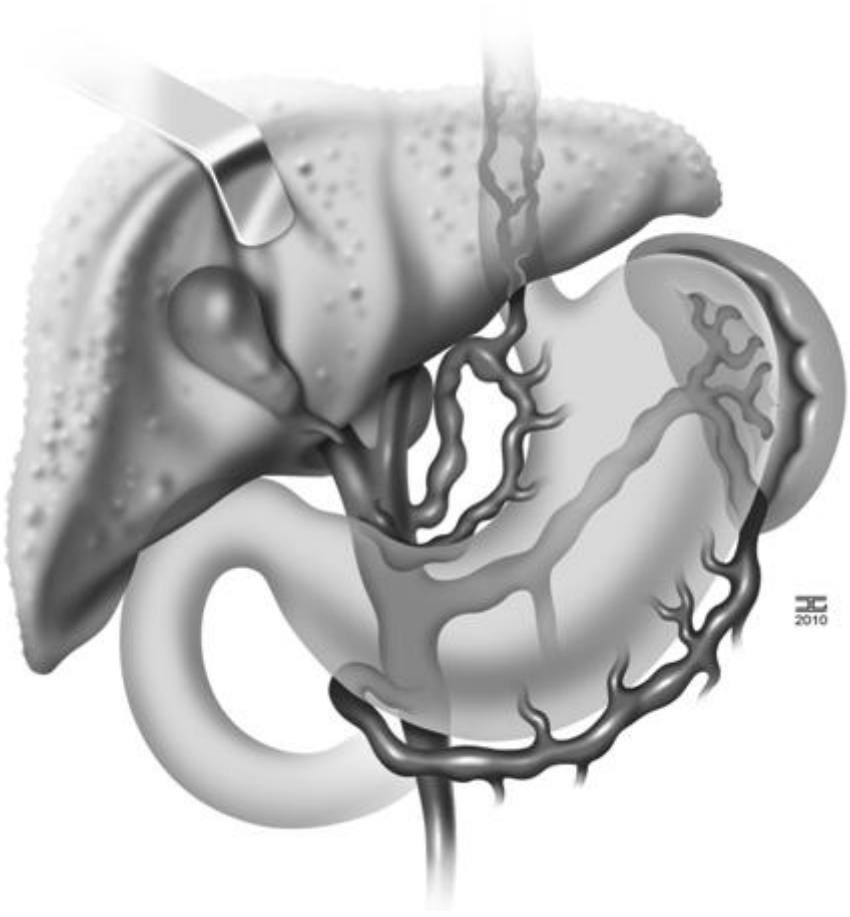

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 1. Illustration of the variceal dilation of the esophageal and the gastric venous vessels due to the portal hypertension.

Conservative Treatment

There are several approaches for the conservative treatment of PH: splanchnic vasoconstriction, reduction of cardiac output and increase of nitric oxide provision to the intrahepatic circulation [1].

Splanchnic vasoconstrictors (mainly vasopressin and somatostatin) induce arteriolar vasoconstriction in the splanchnic area with subsequently decreased portal pressure. Furthermore, they improve renal blood flow due to redistribution of effective arterial blood volume [5].

Major disadvantage of these substances is the necessity of parenteral administration. Therefore, they are primarily used in emergency settings or during in-patient treatment.

Splanchnic vasoconstriction can be also achieved by β_2-blockade inducing an arteriolar vasoconstriction. Use of non-selective β-blockers (e.g. propanolol or nadolol) may be

beneficial because the cumulative effect of the additional β_1-blockade with a subsequent reduction of cardiac output [1].

Induction of intrahepatic vasodilatation is another approach for the treatment of PH. It may be achieved with nitrates or simvastatin (via an increase of nitric oxide provision to the intrahepatic circulation) or substances, such as prazosin or clonidine which block adrenergic activity. Furthermore, angiotensin antagonists (e.g. losartan) can induce an intrahepatic vasodilation [6].

Attention should be paid to the fact that vasodilators not only induce intrahepatic vasodilatation but also systemic vasodilation which may lead to a pronounced sodium retention and renal vasoconstriction [1]. This might induce a further deterioration of renal function.

In case of the development of hepato-renal syndrome (especially type 1), a combination therapy using terlipressin and albumine should be used. This therapy reduced the 15-days-mortality in patients suffering from hepatorenal syndrome type 1 [7].

Interventional Treatment

Endoscopic Band Ligation, Endoscopic Sclerotherapy

There are many published data concerning endoscopic treatment of esophageal varices from portal hypertension. In brief, the American Association for the Study of Liver Diseases (AASLD) recommendations are as follows:

Endoscopic band ligation treatment in combination with β-blockers is associated with a significantly lower incidence of first variceal hemorrhage but with no differences in mortality compared to β-blocker treatment alone for the prevention of the first variceal hemorrhage in patients with medium or large varices. β-blocker therapy should be the first-line treatment, whereby endoscopic band ligation should be offered to patients who have contraindications or intolerance to β-blockers [4].

Comparing endoscopic band ligation and endoscopic sclerotherapy, band ligation appears to be more effective than endoscopic sclerotherapy offering a better control of hemorrhage, lower rates of re-bleeding and less adverse events without showing differences in mortality [4].

Transjugular Intrahepatic Portosystemic Shunt (TIPS)

TIPS was more or less an incidental invention while planning a transjugular cholangiography. Josep Rösch described the feasibility of transjugular portography and consecutive shunting [8, 9]. In 1982, Colapinto et al. described the performance of a TIPS in a 54-years old man suffering from treatment-refractory variceal bleedings using a so-called Grüntzig balloon catheter [10]. The disadvantage of this method was a collapse of the created channel. The stent implantation was first described by Palmaz et al. [11] in dog experiments. Hereby a shunt was created and a stent was introduced between the portal vein and the vena cava using a transjugular access, as we know the shunt procedure today [8, 11].

The key points of the TIPS procedure are:

- Puncture of the right jugular vein and placement of a catheter in the right hepatic vein
- Puncture of the portal vein
- Perforation of the hepatic vein and passage through the parenchyma up to the portal vein
- Dilation of the dissected parenchyma
- Placement of the prosthesis

The principle of a TIPS is depicted in Figure 2.

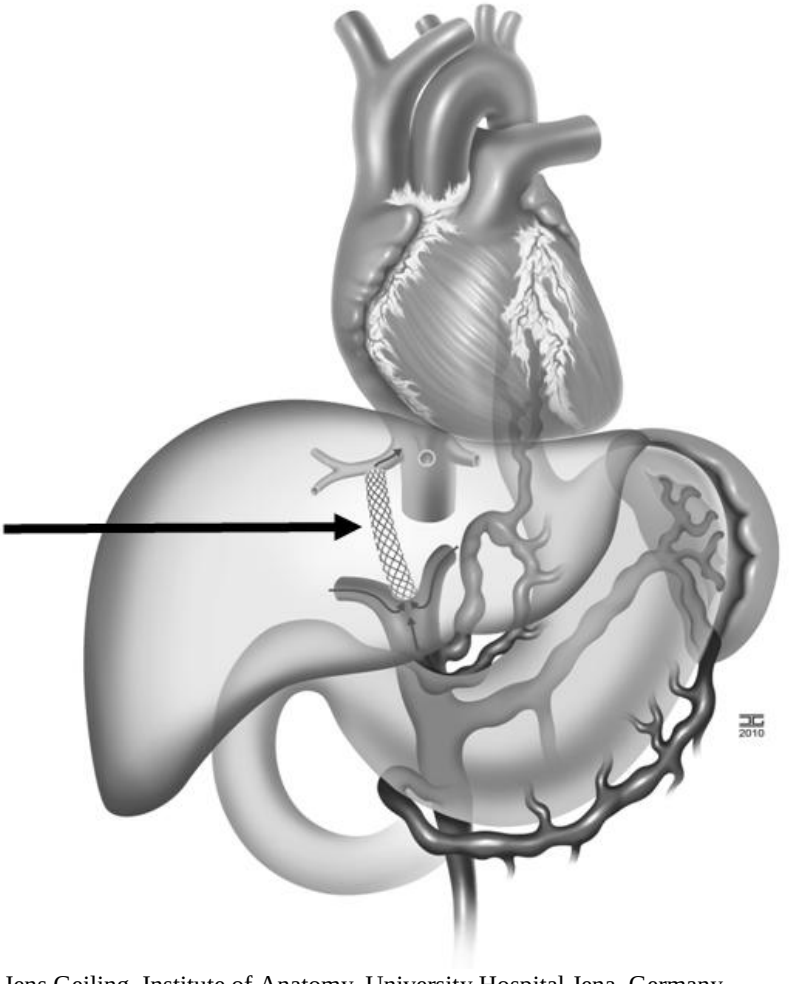

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 2. Illustration of a TIPS. The shunt (marked with the black arrow) is a connection between the portal vein and the right hepatic vein. The aim is the bridging of the cirrhotic liver parenchyma (and therefore bridging the blockage which contributes to the portal hypertension).

Major complications of the TIPS procedure are aggravation of hepatic encephalopathy, laceration of vessels, arterio-portal fistula, hepatic infarction, hemoperitoneum, sepsis, progressive hepatic failure and migration of the stent.

However, with the use of bare metal stents, the rate of TIPS dysfunction (due to stent occlusion, thrombosis or stenosis) is high and reintervention is frequently required.

Nowadays, the shunts should be covered with extended polytetrafluoroethylene (e-PTFE) and should have a diameter of approximately 8 mm [12].

There has been controversy about the proper timing for TIPS placement. However, TIPS should not be considered for primary prophylaxis for variceal bleedings after the diagnosis of esophageal varices. The AASLD recommends that TIPS should not be used after a single bleeding incident from esophageal varices. Only in case of treatment failure (using pharmacological and endoscopic ligation or sclerosis therapy) a TIPS should be performed. TIPS should be considered after a re-bleeding incident.

This recommendation is challenged by the results of the studies performed by Monescillo et al. [13] and by García-Pagán et al. [12]. In 2004, Monescillo et al. [13] compared an early TIPS-treatment with a medical treatment regime. In their work, TIPS was associated with an improved prognosis among patients with high risk for uncontrolled variceal bleeding or rebleeding. In a recently published study, García-Pagán et al. [12] evaluated an early use of TIPS in patients with acute variceal bleeding in CHILD-PUGH B or C liver cirrhosis. They compared patients treated with vasoactive substances and endoscopic therapy to treatment with an e-polytetrafluoroethylene (e-PTFE) -covered stent within 72 hours after randomization or continuation of vasoactive-drug therapy, followed after 3 to 5 days by treatment with propranolol or nadolol and long-term endoscopic band ligation. The medical treated patients showed a significantly higher rate in rebleeding or failure to control bleeding. The one year-probability of a rebleeding free interval was significantly higher in TIPS group as was the one-year-survival. The authors concluded that the early use of TIPS was associated with significant reductions in treatment failure and in mortality in patients with cirrhosis and acute variceal bleeding who were supposed to have a high risk for treatment failure. The authors emphasized that the excellent results of this study are associated with the use of e-PTFE-covered stents.

The recommendations of the AASLD [14] concerning the TIPS procedure are summarized in Table 2.

Comparison between TIPS and Surgical Shunts

A retrospective case-control study, performed by Helton in 2001 [15], compared patients with CHILD-PUGH A or B liver cirrhosis who received a TIPS or a surgical shunt for variceal bleeding. The 30-day-mortality was higher in the TIPS group while the longterm outcome showed no difference between both groups. The rate of rebleeding events, rehospitalizations, diagnostic studies of all types, shunt revisions as well as hospital, professional and total charges were significantly higher in the TIPS group. The authors concluded that surgical shunt procedures are more effective, more durable and less costly than TIPS procedures in patients with satisfactory liver function.

Table 2. Recommendation for the usage of TIPS in liver cirrhosis by the AASLD [14]

- ***Evidence level I (Controlled randomized trials)***
- ePTFE-covered stents are preferred to bare stents because of a lower incidence of shunt dysfunction
- Prophylactic use of non-absorbable disaccharides or antibiotics does not appear to lower the risk of hepatic encephalopathy after TIPS performance
- TIPS should not be performed in patients who bled once from esophageal varices. In this case, TIPS should only be performed in patients who failed pharmacological and endoscopic therapy
- Patients who failed medical therapy in the prevention of re-bleeding, either a TIPS or surgical shunts are appropriate, a good liver function provided
- TIPS should be performed in patients who do not tolerate repeated large volume paracentesis due to refractory ascites

Evidence level II-1 (Controlled trials without randomization)

- TIPS performing institutions should offer a regular TIPS surveillance (e.g. Doppler ultrasound in regular intervals)

Evidence level II-2 (Cohort or case control analytic studies)

- Target values:
- Reduction in hepatic venous pressure gradient to less than 12 mmHg when indication are bleeding esophageal varices
- Reduction in hepatic venous pressure gradient when indication is therapy refractory ascites is unclear, but hepatic venous pressure gradient less than 12 mmHg should be achieved
- In patients with a high expected 30-days-mortality, TIPS should only be performed in the absence of other options
- In case of ultrasound findings indicating TIPS dysfunction or in case of complications caused by a recurrence of portal hypertension, a shunt venography or intervention should be contemplated

Evidence level II-3 (Multiple time series, dramatic uncontrolled experiments)

- TIPS performers should be aware of potential complications (procedural complications as well as complications due to portal diversion) and should be experienced in complication management
- TIPS stenosis is common in bare stents, which is often not reflected in ultrasound (due to low sensitivity and specificity). Thus, TIPS catheterization or upper endoscopy should be considered one year after TIPS performance
- TIPS is effective in control of acute bleeding from varices that are refractory to medical therapy and should be performed rather than surgery
- TIPS is effective in preventing re-bleeding from gastric or ectopic varices and should be preferred for the prevention of re-bleeding in these patients
- In patients with portal hypertensive gastropathy, a TIPS should only be performed in case of recurrent bleeding despite β-blocker therapy
- TIPS is ineffective in bleeding control from gastric antral vascular ectasia and is therefore not recommended
- TIPS is effective in the therapy of hepatic hydrothorax but should be only used in case of therapy failure of diuretics and sodium restriction
- The use of TIPS for the therapy of hepato-renal syndrome type I is of investigatory use

Table 2. Continued

- Use of TIPS in the treatment of hepato-pulmonary syndrome is not recommended

Evidence level III (Opinions of respected authorities, descriptive epidemiology)

- Performance of TIPS only by experienced radiologists (or specially trained physicians); monitoring of success and complication rates, in case of failure review of the program
- TIPS indication should be reached in a team decision of gastroenterologist/hepatologist, interventional radiologist and, if possible, transplantation surgeons
- Before TIPS, tests of liver and kidney function as well as imaging of the liver should be performed (exclusion of liver masses; patency of portal system)
- In high-risk patients, need for liver transplantation should be evaluated before TIPS performance
- TIPS for prevention of bleedings from esophageal varices that never bled is contraindicated because of an increased risk of morbidity and mortality
- In patients with poor liver function who failed medical therapy in the prevention of re-bleeding, a TIPS should be preferred the surgical shunts

Henderson et al. [16] performed a randomized controlled trial comparing patients with CHILD-PUGH A or B liver cirrhosis and recurrent variceal bleeding. Patients either received TIPS or distal splenorenal shunt. Interestingly, there was no difference in the rebleeding rate, encephalopathy or 2- and 5-year-survival between both groups. The TIPS group showed a significantly higher rate of shunt thrombosis or stenosis with a need of reintervention. As both procedures had equivalent outcomes the authors concluded that the choice of intervention should be based on the center's expertise experience, the ability of adequate post-interventional surveillance and the experience in re-intervention.

In patients with poor liver function (CHILD-PUGH C) TIPS should be performed.

From the pathophysiological point of view, TIPS acts as a side-to-side porto-caval shunt [17].

Surgical Treatment of PH

The number of surgical performed porto-systemic shunts for PH in liver cirrhosis has decreased in the past years. Improved endoscopic management of esophageal varices, the more frequent performance of TIPS and the increasing performance of liver transplantation as a definitive treatment of liver cirrhosis and, therefore, PH might be reasons for this observation.

However, due to the shortage of donor organs surgical shunt procedures are still indicated for bridging until a donor organ becomes available or as a palliative procedure in patients who are not suitable for liver transplantation (e.g. due to regular alcohol or drug consumption).

There are different techniques performing surgical procedures for PH:

- portosystemic shunt procedures, which are subdivided in
  - total portosystemic shunts and

- selective portosystemic shunts
- (gastric) devascularization procedures.

At our center, we divide the shunts in central and peripheral shunts.

The choice of the procedure depends on the individual surgical experience and preferences, previously performed abdominal operations which might make a certain shunt procedure difficult and the option of future liver transplantation.

The following section gives an overview about the commonly used surgical shunt procedures.

Selective Shunt Procedures

In selective shunt procedures, the portal blood flow is at least partially maintained. Thus, the rate of liver failure and hepatic encephalopathy after decompression of the esophageal varices is reduced. However, the rate of shunt thrombosis is higher in selective shunts compared to total porto-systemic shunts.

Distal Splenorenal Shunt (WARREN-Shunt)

The distal spleno-renal shunt is a connection between the splenic vein and the left renal vein (see Figure 3). It was first described by Warren and Zeppa in 1967 [18].

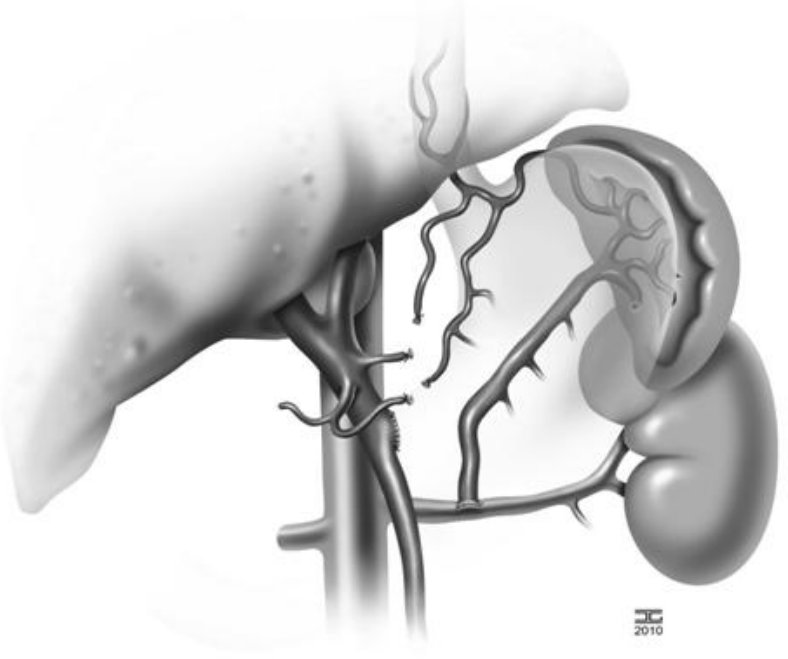

Figure 3: Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 3. Illustration of a distal spleno-renal shunt (Warren-shunt). The principle is an end-to-side anastomosis between the splenic vein and the left renal vein.

It provides a selective decompression of the esophageal and gastric varices via the fundus of the stomach and the spleen to the left renal vein. In approximately 70 % of patients, the orthogonal portal blood flow is maintained after distal splenorenal shunt procedure.

The occurrence of hepatic encephalopathy is low. Recurrent bleedings from the varices are also rare (< 15 %).

Warren et al. restricted the indication for this shunt procedure. They considered ascites as being a contraindication for the shunt because a distal splenorenal shunt does not significantly lower the pressure in the intestinal venous system and in the hepatic sinusoids. Thus, a distal splenorenal shunt procedure might fail if treatment refractory ascites is the indication for the shunt [19].

Several factors should be taken in account, which may make a distal splenorenal shunt procedure difficult or impossible:

- chronic pancreatitis
- previously performed splenectomy
- anatomical factors (abnormalities in the splenic or left renal vein)
- thrombosis of the splenic vein [19, 20].

Livingstone et al. published the largest series with long-term outcome in 2006 [21]. The authors published their experience with 507 distal splenorenal shunts.

The best survival was observed in patients with portal vein thrombosis and biliary cirrhosis whereas patients with alcoholic liver cirrhosis showed the worst outcome.

Postoperative complications included recurrent bleeding, ascites, and encephalopathy. The encephalopathy was low with 13,9 %. The authors concluded that distal splenorenal shunts provide long-term survival and control of bleeding in most patients with portal hypertension and liver cirrhosis [21].

Distal splenorenal are also commonly performed in children with portal hypertension [22, 23].

Splenorenal End-to-Side Shunt with Splenectomy (LINTON-Shunt)

A Linton-shunt is a splenorenal shunt in combination with a splenectomy (see Figure 4).

It was first decribed by Robert Linton in 1947 [24]. Linton started the operation with a left thoracoabdominal incision. Prior to the removal of the spleen, the splenic vein was dissected at the splenic hilus until a maximum length of the vein was available. Then the spleen was removed. The left renal vein was isolated. The segment of the splenic vein lying on the posterior aspect of the pancreas was then dissected. The small pancreatic branches draining into the splenic vein were isolated and ligated individually. Once reaching an optimal length of the splenic vein, an end-to-side splenorenal anastomosis was performed [24, 25].

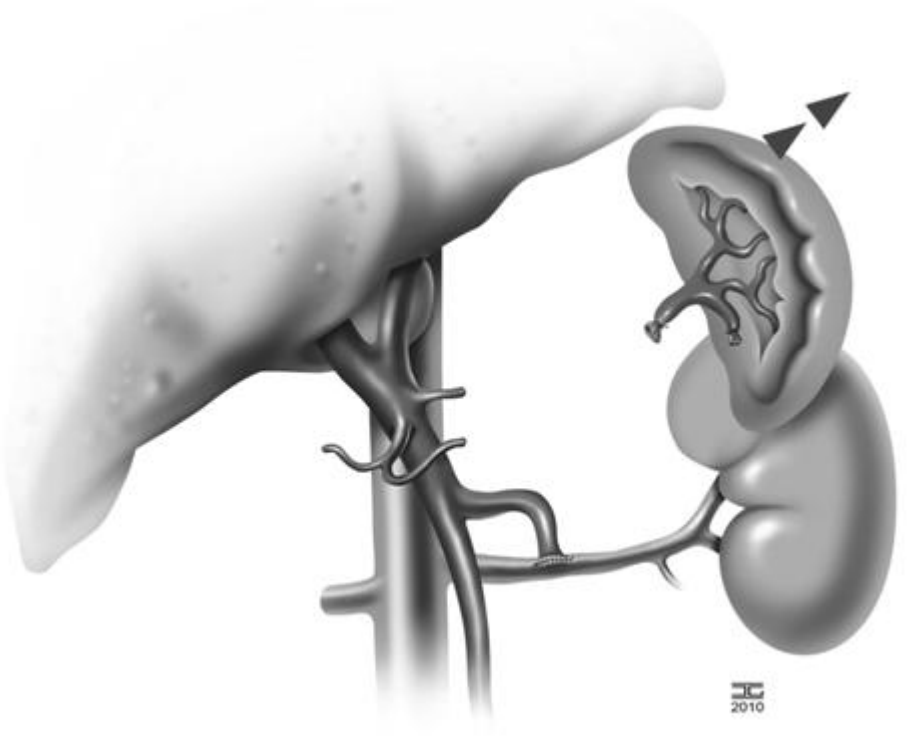

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 4. Illustration of a splenorenal end-to-side shunt with splenectomy (Linton-shunt). The principle is an end-to-side anastomosis between the splenic vein and the left renal vein after a splenectomy.

In 1961, Linton published a comparative analysis between splenorenal shunts with splenectomy and portocaval (end-to-side) shunts. Hereby, 169 patients were reviewed. The authors concluded that the operative procedure of choice for patients with portal cirrhosis and bleeding esophageal varices with or without ascites should be a splenectomy and an end-to-side splenorenal anastomosis. The authors justified their results argueing that „more people so treated live longer and are happier than with other types of portacaval shunts“ [26].

In 1982, Oettinger et al. published the results of an analysis after having performed 140 Linton-shunts. Patients with a CHILD-PUGH A liver cirrhosis had the most favourable outcome. Liver failure was the most common cause of death. In 17 patients a distal pancreatectomy had to be performed (for the improvement of the shunt position (11); due to devascularization of the pancreas (4); unknown reasons (2)) [27].

In 2005, a simplified technique of the Linton-shunt was introduced by Shah et al. [25]. The principal element of so called „Omar's technique“ is the incision through a portion of the fusion fascia of Toldt (along the body and tail of the pancreas) to gain access to the splenic vein. The authors described a significantly reduced operation time as well as a significantly reduced intraoperative blood loss.

However, the combination of a splenorenal shunt with a splenectomy is not recommended anymore except for special indications such as severe hypersplenism nowadays [20].

Splenorenal Side-to-Side Anastomosis (COOLEY-Shunt)

In 1963, Cooley introduced a technique performing a side-to-side anastomosis between the splenic and the left renal vein (see Figure 5) which provides a wide anastomosis, good decompression and reduces the likelihood of shunt thrombosis [28].

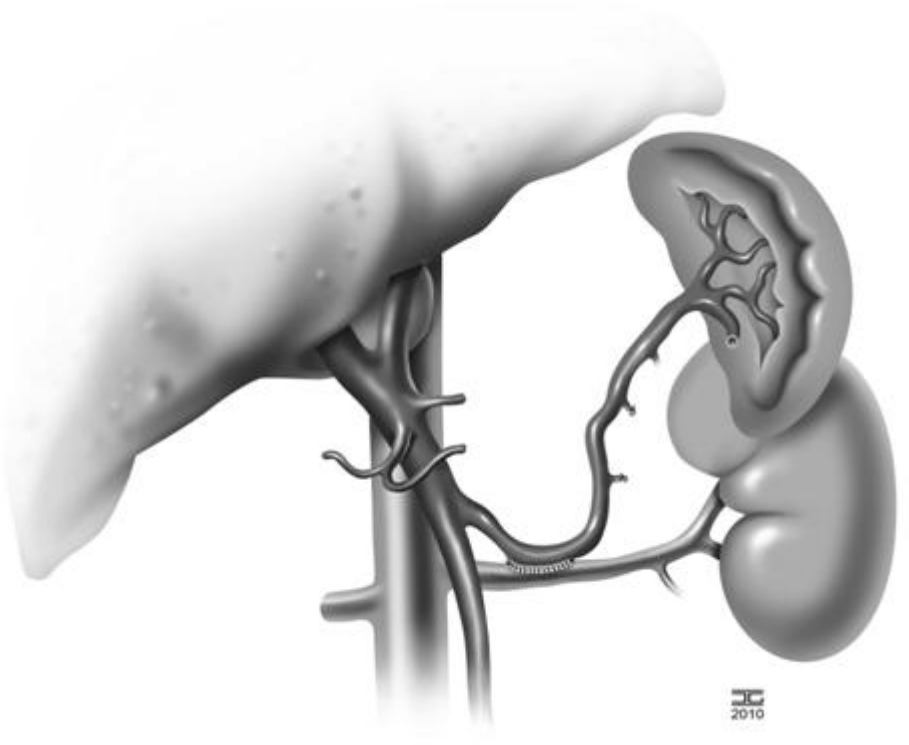

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 5. Illustration of a spleno-renal side-to-side shunt (Cooley-shunt). Principle is a side-to-side anastomosis between the splenic vein and the left renal vein.

In the current literature, this technique is rarely mentioned in publications. Mostly, Cooley-shunts are performed in children because the shunt diameter is larger compared to end-to-side splenorenal shunts. This is important in pediatric surgery because the shunts do not grow with the development of the children [20].

Mesocaval H-Shunt (DRAPANAS-Shunt)

The Drapanas-Shunt, introduced by Drapanas in 1972, is a mesocaval shunt [29]. Thereby, a synthetic graft (e.g. Dacron, e-PTFE) is positioned between the superior mesenteric vein and the vena cava (see Figure 6).

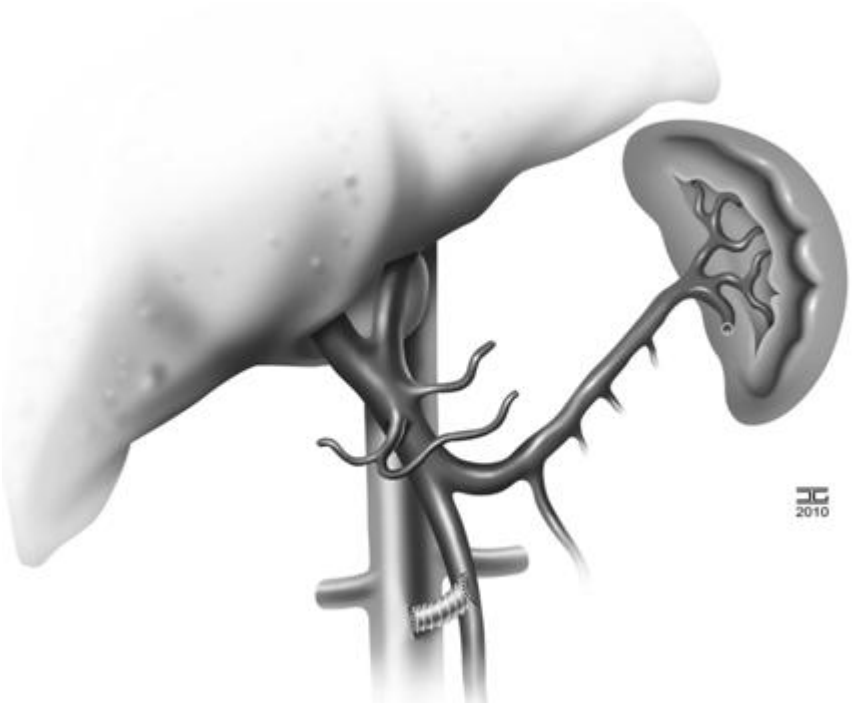

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 6. Illustration of a meso-caval H-shunt (Drapanas-shunt). Principle is the interposition of a graft between the superior mesenteric vein and the inferior vena cava.

Drapanas recommended the graft especially for patients with complications of PH in CHILD-A cirrhosis.

In 1987, Kaufman et al. described a modification of the Drapanas-shunt using the greater saphenous vein. The technique of performing shunt interposition with a spiral vein was described to be superior in children because the venous graft was supposed to compensate for discrepancies in alignment once the exposed underside of the mesentery is replaced. Furthermore, Kaufmann et al. anticipate the venous graft to be less prone to failure [30].

A large study published by Stipa et al. compared a side-to-side porto-caval shunt with a meso-caval interposition shunt (whereby a jugular vein was used as graft). There were no significant differences between the shunt groups regarding operative mortality, rebleeding rates, encephalopathy rates or survival. Especially in liver transplantation candidates, the authors favour the meso-caval interposition shunt because it offers excellent protection against rebleeding and carries the same risk of encephalopathy as other shunt procedures. Furthermore, it has the advantage of leaving the hepatic hilum undissected,and it can easily be interrupted by ligation at the time of transplantation [31].

Total Porto-Caval Shunts

Porto-Caval Side-to-Side Shunts

Porto-caval side-to-side shunts are performed in patients suffering from treatment refractory ascites that might be a consequence of diuretic-resistant or diuretic-intractable ascites. Thus, the clinical presentation of most patients who receive porto-caval shunts is worse compared to those who receive a peripheral shunt procedure, at least in the elective shunt procedures. Most patients in the published literature are in CHILD-PUGH class B or C [32, 33]. In clinical study by Orloff [33], side-to-side porto-caval shunt were performed in 34 patients with treatment refractory ascites (CHILD-PUGH A: n = 0; CHILD-PUGH B: n = 23; CHILD-PUGH C: n = 11). After the shunt interposition, mean portal-caval pressure gradient was significantly reduced. Ascites was relieved in all patients without further requirement of diuretic treatment. Mortality rates were low (30-day-mortality: 6 %; 15-year-survival: 73 %). The authors reported a significantly improved patient quality of life after the shunt procedure. The shunts were patent in all patients over the whole follow-up period. Orloff et al. concluded that side-to-side porto-caval shunts are very effective in the treatment of treatment-refractory ascites in liver cirrhosis patients.

In a comment to the aforementioned paper, Rodés [17] remarked critically that „the number of patients in whom portacaval shunts was indicated was so small (1 patient per year) that this therapy should be considered as anecdotal and only useful in very selective patients".

Capussotti et al. [34] performed a prospective randomized clinical trial comparing an interposition porto-caval H-graft with a small-diameter side-to-side porto-caval shunt in patients with variceal hemorrhage. The rate of encephalopathy was higer in patients who received the side-to-side shunt. Futhermore, postoperative liver function was deteriorated in these patients. These results are similar to those of Spina et al. [35] who described a better quality of life in patients after distal splenorenal shunt compared to patients who received a side-to-side porto-caval anastomosis.

Recently, the use of ENDO-GIA staplers for the perfomance of venous side-to-side anastomosis was introduced [35].

In conclusion, side-to-side porto-caval shunts are effective in the treatment of otherwise therapy-refractory ascites. In the treatment of variceal bleedings, other shunts should be preferred due to the better quality of life after the shunt procedure.

TIPS are functional side-to-side shunts, so the performance of operative porto-caval side-to-side shunts has become less frequent in the last years.

Portocaval End-to-Side Shunts

As early as 1877, Eck described his first experience with the performance of a porto-caval end-to-side shunt in dogs. Thus, these shunts are also known as „Eck's fistula" [36] (see also Figure 7).

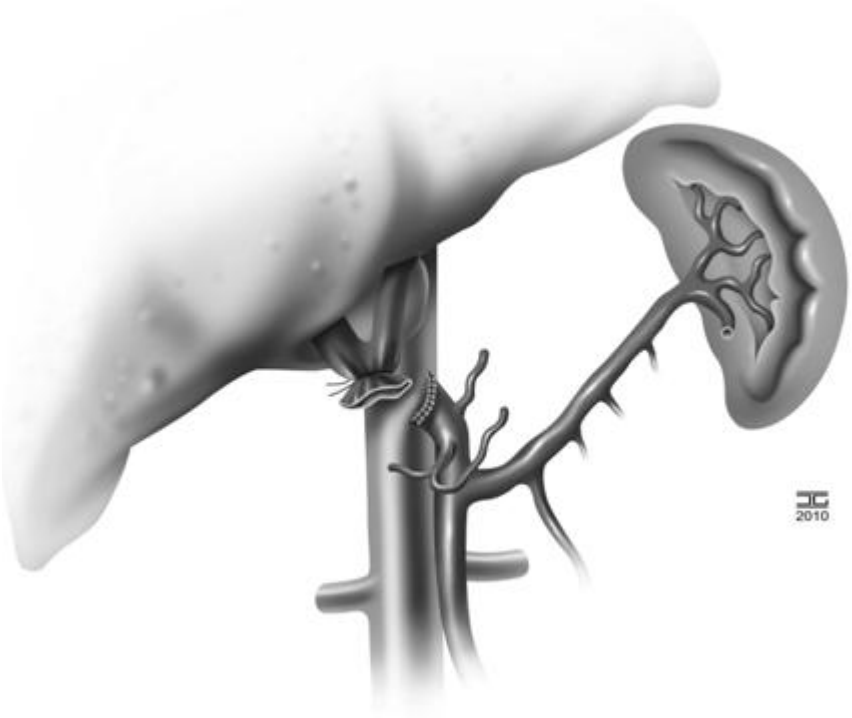

Illustration by Jens Geiling, Institute of Anatomy, University Hospital Jena, Germany.

Figure 7. Illustration of a porto-caval end-to-side shunt (Eck's fistula). Principle is an end-to-side anastomosis between the portal vein and the inferior vena cava.

Performance of a porto-caval end-to-side shunts provides a excellent relief of the portal hypertension even in emergency cases. However, due to the complete diversion of the portalvenous blood to the circulation without a hepatic passage, the rate of neuro-nutritional disturbances is high [26]. The encephalopathy rate is reported as being high as 40 % [20].

In 1986, Harley et al. [37] showed no differences between the encephalopathy rates in patients after the performance of a end-to-side porto-caval shunt compared to patients receiving a distal splenorenal shunt.

Especially in a emergency situation, a porto-caval end-to-side shunt is advisable due to the fast and relatively easy implementation [20].

Devascularization Procedures

In case of a treatment-refractory variceal bleeding or in case of veins not suitable for shunt procedures, so called devascularization procedures should be considered [20]. The aim of the devascularization procedures is the achievement of a porto-azygos disconnection through the interruption of the intramural as well as the extramural vessels feeding gastroesophageal varices while maintaining hepatic portal perfusion.

Mainly, there are two devascularization procedures: on the one hand the devascularization introduced by Sugiura and Futagawa [38-40] and on the other hand the devascularization described by Hassab and Paquet [41].

The Sugiura-Futagawa devascularization procedure was introduced in 1973 [38]. It includes extensive paraesophagogastric devascularization, esophageal transsection, splenectomy, vagotomy, and pyloroplastic. It was performed via a thoracic and a seperate abdominal approach, in some cases in two stages. The results published by Sugiura and Futagawa demonstrated very low re-bleeding rates and a 10-year-survival of more than 70%. Furthermore, no encephalopathy was observed postoperatively.

The devascularization procedure described by Hassab-Paquet includes devascularization of the diaphragm, the distal esophagus, the proximal two-third of the stomach, a selective vagotomy, a splenectomy and/or a fundoplication with a pyloroplasty [41].

In 2006, Johnson et al. [42] published their results of a modified Sigiura procedure. Hereby, only a laparotomy had to be performed. The extramural disconnection was achieved by the external devascularization procedure and the intramural disconnection was achieved by either stapler or manual esophageal transsection or endotherapy. The authors compared a group of patients, who received a devascularization procedure with esophageal stapler transsection to patients undergoing only devascularization without esophageal stapler transsection. There were no differences between both groups with respect to variceal rebleeding, residual varices and recurrence. The morbidity was significantly higher in patients who received devascularization treatment with esophageal stapler transsection. The authors concluded that the devascularization without esophageal stapler transsection is a safe and effective procedure with an adequate (emergency and long-term) control of variceal bleeding.

Furthermore, splenectomy is not mandatory in the devascularization procedures and should only be performed in patients suffering from symptoms related to hypersplenism [20].

Helmy et al. [43] described the feasibility of a laparoscopic performance of a devascularization procedure even in emergency cases.

Conclusion

Portal hypertension can lead to life-threatening complications such as acute variceal bleeding. The primary prophylaxis should be performed conservative (medical/endoscopic).

In case of recurrent, treatment-refractory bleedings, a shunt procedure should be considered. In the past years, the performance of a TIPS was performed more frequently. In our opinion, TIPS should be performed in patients with liver cirrhosis CHILD-PUGH B and C, where surgical shunt procedures can only be performed with a high risk.

However, due to the high stenosis rates of a TIPS, surgical shunt procedures seem to be more favourable, at least in patients with liver cirrhosis CHILD-PUGH A. There are several shunt procedures, whereby the distal spleno-renal shunt appears to be the most frequently performed one.

We recommend performing surgical shunts in patients with CHILD-PUGH A liver cirrhosis in case of treatment-refractory complications of portal hypertension, even as a bridging treatment until a liver transplantation can be performed.

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Chapter 10

Cell-based Therapy of Liver Cirrhosis

Dong-Sheng Li[1], Ya Liu[2], Sheng-Ming Zhang[2], Yong-Tang Feng[2] and Long-Jun Dai[*1, 3,]

[1] Hubei Key Laboratory of Embryonic Cell Research, Yunyang Medical College, Shiyan, China

[2] Department of Surgery, Weifang Medical College, Weifang, Shandong, China

[3] Department of Surgery, University of British Columbia, Vancouver, Canada

Abstract

Hepatic cirrhosis is the end-stage of chronic liver diseases. The majority of patients with hepatic cirrhosis die from life-threatening complications at early age. Liver transplantation has been the most effective treatment for patients with hepatic cirrhosis. Since liver transplantation is critically limited by the shortage of available donor livers, searching for an effective alternative therapy has attracted great interest in preclinical studies. The encouraging advances in stem cell research have paved the way towards the treatment of the end-stage of chronic liver diseases. In view of the pathogenic fundamentals of hepatic cirrhosis, cell-based treatment should be aimed to complement or replace damaged liver cells and to correct the imbalanced extracellular matrix regeneration/degradation. Understanding the transition of hepatocyte regeneration to hepatic fibrogenesis during chronic liver injury could guide the appropriate utilization of cell-based therapy. This chapter is intended to describe the characteristics and therapeutic potential of various stem cells, including hepatocytes, liver progenitor cells, hematopoietic stem cells, mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells. Since autologous adult stem cells have the least obstacles for clinical application, their potential interventions on cirrhosis are especially illustrated in terms of the cellular and molecular mechanisms of hepatic fibrogenesis.

[*]Corresponding author. Department of Surgery, University of British Columbia, 400-828 West 10th Avenue, Vancouver, BC, Canada V5Z 1L8. Tel.: +1 604 875 4111x62501; Fax: +1 604 875 4376. E-mail addresses: ljdai@interchange.ubc.ca, longjundai@gmail.com (L.-J. Dai).

Introduction

Cirrhosis represents the common final pathological outcome for the majority of chronic liver diseases. Most patients with cirrhosis die from one or more clinical complications including ascites, hepatic encephalopathy, and variceal hemorrhage [1]. The majority of chronic liver diseases are initiated by the infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) [2, 3]. Liver transplantation has been the most effective therapy for the patients with advanced liver disease. Unfortunately, most patients are unable to obtain transplantation due to the limited availability of donor livers. Over 10% of patients die while waiting for liver transplantation. Among the fortunate patients who received liver transplants, the survival rate is 94% at 3 months, 88% at 1 year and 79% at 3 years [4]. Thus, it is of great clinical interest to search for an effective alternate to treat this type of life-threatening disease. Recently, stem cell-based cytotherapy has shown promising benefits on animal models and some clinical trials. Since chronic liver failure is caused by both inadequate hepatocytes and fibrosis-induced cirrhosis, the therapeutic strategy should be based on hepatocyte regeneration or replacement and the correction of imbalanced fibrogenesis. This chapter will describe the development of cirrhosis and the stem cells which have the potential for treating hepatic cirrhosis, starting with the transition of hepatocellular regeneration to hepatic fibrogenesis. Considering recent progress in clinical applications, special emphasis will be placed on adult stem cells.

Hepatocellular Regeneration versus Hepatic Fibrogenesis

The liver is one of the most regenerative organs in the body. Rapid hepatic regeneration is very well documented with acute liver injury models such as partial hepatectomy. However, in the case of chronic liver injury, which correlates with most clinical liver diseases, the pathogenic factors induce hepatocellular senescence and/or apoptosis rather than hepatocellular regeneration. The uncontrolled cellular growth and imbalanced fibrogenesis lead to the destruction of the liver architecture, and ultimately to hepatic cirrhosis. It is of great interest to shed light on the possible pathomechanisms by which different outcomes arise under different pathological conditions.

Hepatocellular Regeneration

Liver regeneration is a fundamental liver response to hepatic injury or the loss of hepatic tissue. The normal liver bears a relatively low level of cell turnover, but when an abnormal hepatocyte loss occurs, a regenerative response is rapidly elicited to restore the organ to its pristine state [5]. The ancient Greeks recognized liver regeneration in the myth of Prometheus. Prometheus is credited as a benefactor of mankind, having provided humans with knowledge of fire, medicine and mathematics. As a result of his action, Prometheus was punished by being chained to a mountain, when, every day, a great eagle would come to eat

his liver. At nightfall, the eagle would leave, and Prometheus' liver would regenerate, only for the process to be repeated the next day (Fig. 1) [6]. In modern times, it is widely recognized that mature adult hepatocytes are not terminally differentiated cells and they have the most significant regenerative potential during acute liver injury. In the healthy adult liver, hepatocytes are quiescent cells and renewed slowly, thus there is only minimal "wear-and-tear" renewal [5]. They have a life expectancy of over a year, as estimated by telomere loss which is 50-120bp per year in healthy individuals [7, 8]. However, liver damage or loss of liver mass can extensively stimulate the regenerative capacity until the tissue mass has been restored by the proliferation of mature parenchymal liver cells.

Figure 1. The Greek Myth of Prometheus symbolizes the unyielding strength that resists oppression. The detailed description is indicated in the text. Reproduced with permission from Trends in Biotechnology (Elsevier).

Modern studies have validated the ancient myth of Prometheus by providing experimental evidence that liver has an almost unlimited capacity to regenerate. Up to 75% of surgically removed liver mass can be regenerated within 1 week in rodents [9]. Using partial hepatectomy to its limit, it was shown that rat liver regenerated each time after 12 sequential hepatectomies [10]. Liver generation after partial hepatectomy is carried out by the proliferation of all existing mature cellular populations composing the intact organ. These include hepatocytes, biliary epithelial cells, fenestrated endothelial cells, Kupffer cells, and stellate cells. More recently, Zappa et al [11] analyzed the human liver regeneration after partial hepatectomy on 27 consecutive patients without chronic liver disease or liver

dysfunction. They revealed a 64% increase in volume of the liver remnant from the future liver remnant at day 7 after hepatectomy of right liver. However, partial hepatectomy is not a typical liver injury. From a human disease standpoint, chronic or long-standing iterative liver injury (e.g., chronic viral hepatitis) is often associated with hepatocyte replicative senescence [12, 13].

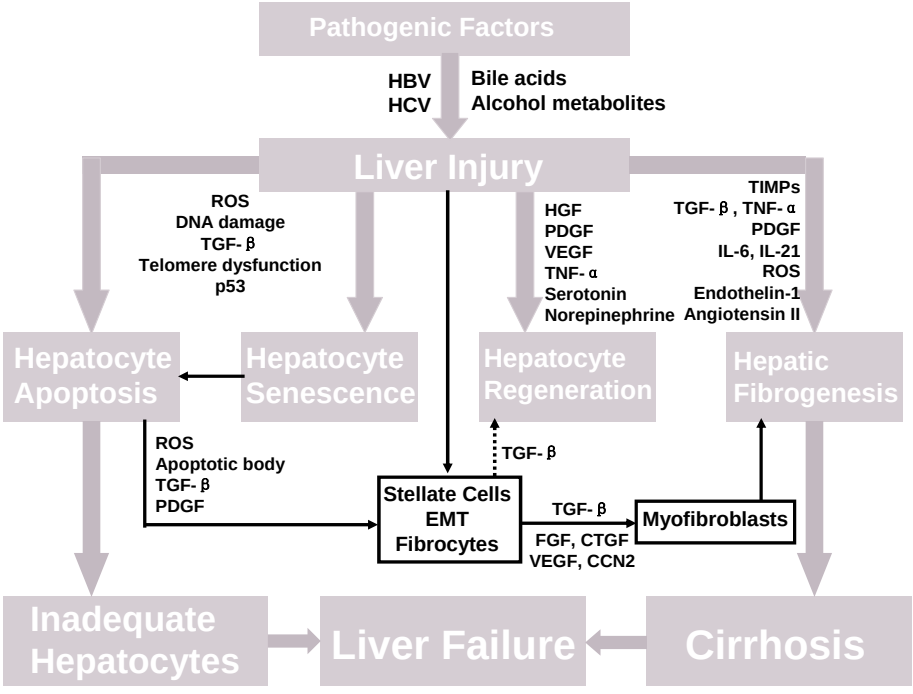

Figure 2. A flowchart of pathogenesis of liver cirrhosis. Liver injury is initiated by various liver-targeted pathogenic factors. Hepatocyte regeneration dominates the acute responses during early phase of liver injury. Under the circumstances of chronic liver injury, the major pathogenic responses are hepatocyte senescence, hepatocyte apoptosis and hepatic fibrogenesis. Both inadequate hepatocytes and fibrosis-induced cirrhosis are the direct causes of liver failure. The activation of liver stellate cells plays a pivotal role in hepatic fibrogenesis. The related pathogenic factors and mediators are indicated in the appreciate steps. Solid lines represent stimulation and dotted line indicates inhibition.

Hepatocellular Senescence and Apoptosis

Pathogenesis of Hepatic Fibrosis and Cirrhosis

Persistent insult-induced liver injury is infallibly accompanied by hepatic fibrogenesis. This is determined by the nature of the tissue repair process, which commences from the initial tissue damage [33]. Hepatic fibrosis refers to the excessive accumulation of extracellular matrix (ECM) with the formation of scar tissue encapsulating the area of injury. Progressive fibrosis is the hallmark of chronic injury; it can eventually result in cirrhosis, liver failure, or HCC [20]. Cirrhosis, the end-stage of progressive fibrosis, is characterized by

septum formation and rings of scar that surround the nodules of hepatocytes. Accumulation of ECM results from both increased synthesis and decreased degradation of ECM. Recognizing the mechanisms underlying hepatic fibrogenesis will provide the guidelines for an effective remedy of liver cirrhosis.

Cellular Sources of ECM in Cirrhotic Liver

Liver stellate cells (LSCs) are the main ECM-producing cells in the injured liver. LSCs reside in the space of Disse and are the major storage sites of vitamin A. When a liver injury occurs (e.g., viral hepatitis), LSCs proliferate and undergo a dramatic phenotypical alteration, which is characterized by the acquisition of a proliferative, contractile, migratory, fibrogenic and inflammatory phenotype. Activated LSCs secrete a large amount of ECM proteins, including collagen (I, III, and IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans [1]. The accumulating interstitial ECM constituents that collectively form the hepatic scar replace the low-density type IV collagen with the normal subendothelial space of Disse. These interstitial fibril-forming collagens (especially types I and III collagens) become distributed primarily in the connective septa surrounding the regenerative hepatic nodules. A cirrhotic liver may contain up to six times more collagen and proteoglycan than a healthy organ [34]. In addition to the resident LSCs, periportal fibroblasts, bone marrow-derived fibrogenic cells, epithelial-mesenchymal transition, and possibly circulating fibrocytes can contribute to the fibrogenesis in the liver [34]. The relative importance of each cell type in liver fibrogenesis depends on the origin of liver injury. While LSCs are the main fibrogenic cell type in pericentral areas, periportal fibroblasts predominate when liver injury occurs around portal tracts. Since the majority of patients with chronic liver diseases are induced by HBV and HCV infection [2, 3], LSC-mediated pericentral fibrosis plays an important role in the development of hepatic cirrhosis. Therefore, prevention of LSC activation has been the most promising therapeutic strategy for this disease.

Some other cell types also contribute to the progression of liver fibrosis. These include liver resident cell types (for example, hepatocytes, Kupffer cells, sinusoidal endothelial cells, and bile duct epithelial cells, etc.) and non-resident or circulating cells (for example, T and B lymphocytes). They may not secrete ECM proteins directly, but upon being damaged they activate LSCs through a variety of inflammatory mediators, apoptotic bodies, free radicals, and fibrogenic cytokines [35-38].

Molecular Regulations of ECM Accumulation

Numerous factors are involved in liver ECM synthesis and/or degradation, including growth factors, cytokines, and chemokines. They play direct or indirect roles in fibrogenesis or anti-fibrogenesis depending on their effects and targets. For example, matrix metalloproteinases (MMPs) are directly responsible for matrix breakdown, and an increase of tissue inhibitors of metalloproteinases (TIMPs) indirectly favors the accumulation of ECM. The general molecular basis of fibrosis has been summarized in recent reviews [24, 33].

The expression of inflammatory mediators determines the fibrogenic response to liver injury. Hepatocytes are the targets of most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and bile acids [39]. Damaged hepatocytes and involved inflammatory

cells release a variety of inflammatory mediators. A number of fibrogenic molecules are also inflammatory mediators, such as free radicals, IL-1b, IL-6, IL-10, IL13, IFN-γ, SOCS-1, and osteopontin [1, 40]. Inflammatory molecules activate LSCs, and the resulting activated LSCs secrete inflammatory chemokines, express cell adhesion molecules, and modulate the activation of inflammatory cells [34, 41]. Therefore, a vicious circle exist between inflammatory and fibrogenic cells. Any approach able to break this circle can presumably lead to the development of an anti-fibrotic treatment.

Reversibility of Hepatic Cirrhosis

Hepatic cirrhosis is traditionally thought to be irreversible. However, recent evidence from animal studies and human clinical observations indicate that even advanced fibrosis is still reversible [42, 43]. The most effective intervention in the treatment of liver fibrosis is to remove the causative agents, such as the utilization of anti-viral therapy and the obstruction of alcohol intake. It may take years for significant recovery to be achieved; the time varies depending on the underlying cause of the liver injury and its severity. It is unlikely to reach a complete return to normal histology. So, the term of "regression" is more relevant to the real situation rather than "reversal" [44]. As described above, liver fibrosis results from the imbalance between ECM production and ECM degradation. Theoretically, any approach which decreases ECM synthesis and/or increases ECM degradation could accelerate the regression of hepatic fibrosis/cirrhosis. For example, the inhibition of activated LSCs by modulating their activation and/or proliferation or the promotion of their apoptosis would be a useful strategy.

Cell-Based Therapy of Liver Cirrhosis

Cell-based therapy can be defined as "the use of living cells to restore, maintain or enhance tissue and organ function" [45]. Cells from a variety of tissue sources can be classified into three groups. Autologous (self), which offers the advantage of manipulation with minimal risk of adverse host response and disease transmission; allogeneic (non-self, same species), which offers the advantage of banking prior to need, but is more likely to be complicated by the presence of disease-transmitting viruses; and xenogeneic (animal, other species). Both allogeneic and xenogeneic cells would be more likely to generate an adverse response from the host. Since the pluripotent plasticity of stem cells was first discovered by Potten and Loeffler [46], their therapeutic potential have attracted numerous and broad investigations. It is believed that cell-based therapy will eventually revolutionize conventional medicine and make many incurable diseases manageable. Stem cells are defined as cells that are clonogenic, self-renewing, and capable of differentiating into multiple cell lineages. Stem cells may be divided into four different groups according to their potential for differentiation: totipotent, pluripotent, multipotent, or unipotent. The earliest embryonic stem cells are totipotent, from which the trophoblast and all three germ layers (endoderm, mesoderm, and ectoderm) necessary for future development of an organism are derived. Pluripotent cells contribute to all three germ layers. Stem cells that give rise to different lineages within a

single germ layer are considered multipotent and constitute the adult stem cell population in later life. These cells are tissue-specific cells, and are capable of maintaining, generating, and replacing aging or damaged cells within the organ [47]. In terms of cell-based therapy for liver disorders, the potentially related stem cells can be grouped as intrahepatic stem cells, extrahepatic stem cells, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). The cellular content, cell origin, differentiation capacity, and major advantages and disadvantages are summarized in Table 1. As the liver contains many different cell types, which are organized in three-dimensional structures, growth and regeneration processes are very complicated.

Table 1. Cell types and potential applications of hepatic therapy-related stem cells

Cell type	Differentiation Capacity	Origin	Advangtages	Disadvantages
Intrahepatic				
Hepatocytes	Unipotent	Liver	-Direct cell replacement	-Limited by donor -Allo-Tx in nature
LPCs	Bipotent	Liver	-Function demonstrated in animal models	-Rare availability -Tumorigenesis
Extrahepatic				
HSCs	Multipotent	Bone marrow Blood Cord Blood	-Handy availability -Function demonstrated in animal models and some clinical trials -Possibility for auto-Tx	-Potential for fusion and nuclear disruption -Tumorigenesis
MSCs	Multipotent	Bone marrow Adipose Cord blood	-Handy availability -Ex vive expansion -Less immunogenic -Anti-inflammatory -Immunoregulation -Possibility for auto-Tx	-Limited differentiation -Fibrogenic potential
ESCs	Pluripotent	Embryo	-Unlimited source -Function demonstrated in animal models	-Tumorigenesis -Allo-Tx in nature -Ethical issues
iPSCs	Pluripotent	Somatic cells	-Unlimited source -Function demonstrated in animal models -Possibility for auto-Tx	-Tumorigenesis

ESCs: embryonic stem cells; HSCs: hematopoietic stem cells; iPSCs: induced pluripotent stem cells; LPCs: liver progenitor cells; MSCs: mesenchymal stem cells; Tx: transplantation.

Intrahepatic Stem Cells

The liver contains at least two endogenous populations of stem cells: hepatocytes and hepatic oval cells. Hepatocytes fulfill many of the definitions of a stem cell in that they are able to self-renew almost limitlessly, and often play the principal role in liver regeneration [48, 49]. Nevertheless, this replenishment would appear to be compromised in chronic liver disease [18, 50]. Hepatic oval cells are also known as liver progenitor cells (LPCs) in humans. They have been shown to differentiate into hepatocytes and cholangiocytes, leading to the restoration of hepatic volume in different experimental models [51, 52]. Intrahepatic stem cells are the first line of cells to be utilized during hepatocellular regeneration.

Hepatocytes

As described earlier, mature adult hepatocytes have the most significant regenerative potential in acute liver injury. They are able to repopulate the injured liver prodigiously within a short period of time [53, 54]. Based on their regenerative capacity, hepatocytes have attracted extensive interest in exploring an ideal substitution of liver transplantation. About 30 years ago, the first animal experiments showed isolated hepatocytes to be suitable for the treatment of liver diseases [55]. For example, the Gunn rat is a model for UDP-glucuronosyl transferase (UGT1A1) deficiency (Crigler-Najjar syndrome type 1) resulting in high serum bilirubin levels. After transplantation of primary adult hepatocytes into these animals, the bilirubin level is markedly reduced to near normal values [56]. Many other animal models for human liver diseases have also been studied for the therapeutic potential of hepatocyte transplantation, including metabolic disorders such as Wilson's disease (Long-Evans cinnamon rats) [57], tyrosinemia (FAH^- mice) [48], hypercholesterolemia (Watanabe rabbit) [58], intrahepatic cholestasis $mdr3^{-/-}$ mice [59], and ornithine transcarbamylase deficiency (spf-ash mice) [60].

Clinical hepatocyte transplantation was first attempted by Mito et al in a small number of patients with chronic liver disease in 1992 [61]. They isolated hepatocytes from PTH specimens and transplanted them into patient's own spleen. These cells could be detected with radionucleotide scanning up to 1 year after autotransplantation; however, their presence was not correlated with any clinical improvement. A considerable number of patients treated for metabolic disorders have been reported. In 1998, a 10-year old girl suffering from Crigler-Najjar syndrome type I was treated with an infusion of 7.5×10^9 hepatocytes through the portal vein for partial correction of the metabolic defect. By 11 months after transplantation, the need for daily phototherapy had decreased from 10–12 h to 6–7 h and lowered the serum bilirubin from 450 μmol/l to 240 μmol/l [62]. A conventional liver transplantation was successfully performed 2.5 years after the cell transplantation. Some other cases have been described in the last few years [63-65]. Over eighty patients have now been transplanted word-wide and the safety of the procedure together with medium-term success has been established [66]. Few studies on liver cell therapy for the treatment of acute liver failure in humans have the intention to bridge the gap until patients receive orthotopic liver transplantations [67-69]. The main challenges for this approach are the quantity of transplanted cells, the availability of freshly prepared cells or the quality of cryoconserved cells and the need for immunosuppression to prevent the rejection of the transplanted cells [55].

In contrast to orthotopic liver transplantation, which is a well-established, life-saving procedure, hepatocyte transplantation is still experimental. It is ethically difficult to assign cadaveric livers to experimental approaches while many patients still pass away on the waiting list. Although one could establish banks of cryopreserved hepatocytes, with which some clinical success has been reported [68, 70], this may not solve the problems of cell shortage because human hepatocytes are easily damaged during the freezing–thawing procedure. Therefore, there is a need for alternative source of cells, such as stem cells or progenitor cells that can differentiate into hepatocytes.

Liver Progenitor Cells

Liver progenitor cells (LPCs) are a population of bipotent liver cells with the ability to give rise to either hepatocytes or choangiocytes. Liver-intrinsic LPCs would be the most abundant cell source for liver repopulation. LPCs are also known as facultative liver stem cells, small hepatocytes or specifically, oval cells in rodents because of their oval-shaped nuclei. They are located in the smallest distal branches of the biliary tree known as the canals of Hering. LPCs could be activated by overwhelming liver injury, chronic liver injury or large-scale hepatocyte senesence [5]. In both humans [71] and mice [72], the extent of this reaction is dependent on the severity of the damage. While the mechanisms controlling progenitor cell activation are partially understood in rodent models, very little is known about the mechanisms in humans. This so-called 'oval cell' or 'ductular reaction' amplifies a cholangiocyte-derived population before these cells differentiate into either hepatocytes or cholangiocytes [73, 74].

Oval cells/LPCs have attracted little attention in terms of therapeutic potential, perhaps because isolated human cells only differentiate in vitro into biliary cells [54, 75]. However, the hepatocyte differentiation of oval cells from rodents has been clearly demonstrated in animal models [76]. Upon transplantation oval cells make a modest contribution to the hepatocyte population in the CCl_4-damaged mouse liver. After 1 and 6 months of transplantation, the transplanted LPCs comprise 2 and 0.4% respectively [77]. In the adult human liver, LPCs are difficult to track and isolate because of the lack of a known definitive marker. Only recently LPCs have been isolated and showed to have bipotential in vitro. LPCs can be isolated based on their expression of epithelial cell adhesion molecule and neural cell adhesion molecule [78] and using the oval cell surface marker Thy-1 in combination with magnetic sorting [79]. These LPCs have the ability to repopulate the liver in animal models. Meanwhile, results of such experiments in humans are anxiouly anticipated [80].

Extrahepatic Adult Stem Cells

The first demonstration of the existence of precursor liver cells in the bone marrow was reported by Petersen et al. in 1999 [81]. They showed that bone marrow cells transplanted into lethally irradiated mice engrafted in the recipient's liver and differentiated into liver stem cells (oval cells) or mature hepatocytes. These in vivo results were confirmed in animal models and in patients who received bone marrow transplantation for hematological disorders [82, 83]. Hematopoietic stem cells (HSCs) consist of the majority of stem cell population in the bone marrow, while nonhematopoietic, i.e. mesenchymal stem cells (MSCs) are only a

very small fraction of the population, representing 0.001- 0.01% of the nucleated cells in adult human bone marrow [84]. Although these two cell types are reported to differentiate into cell lineages of all three germ layers, i.e. ectoderm, mesoderm and endoderm, MSCs show different characteristics compared to other components in differentiating into hepatocytes [85]. Schwartz and colleagues provided direct evidence of in vitro hepatogenic differentiation of MSCs [86]. A subpopulation of MSCs isolated from bone marrow of human, mouse and rat, cultured on Matrigel with FGF-4 and HGF, differentiated into hepatocyte-like cells. These cells express hepatic markers, such as HNF-3, GATA4, CK19, transthyretin, fetoprotein, albumin and CK18. They also possess functional characteristics of hepatocytes, such as secreting urea and albumin, having phenobarbital-induced cytochrome p450, taking up LDL and storing glycogen. In vitro liver-specific differentiation of MSCs can also be induced by co-culture with liver cells [87] and pellet culture [88]. MSCs can be isolated from several other tissues, including adipose tissue, placenta, amniotic fluid and umbilical cord blood.

MSCs appear to be the most promising cells for cell-based therapies [5]. The feature of self-origin, readily availability, ex vivo expansion, and no requirement of immunosuppression render MSC transplantation a practical approach. MSCs represent an advantageous cell type for allogeneic transplantation as well. They are immuno-privilegd with low major histocompatibility complex I (MHC I) and no MHC II expression, therefore have a reduced risk of allogeneic transplant rejection. However, it is worth noting that the expression of MHC I and MHC II in undifferentiated MSCs could be proportionally increased with the differentiation extent of MSCs. The clinical application will be described in a later section.

Embryonic Stem Cells

Embryonic stem cells (ESCs) are highly proliferative and pluripotent cells which have recently received much more attention in the field of cell-based therapy. Derived from the inner cell mass of the early developing embryo, ESCs are capable of undergoing multilineage differentiation into highly specialized cells representing all three germinal layers [89]. Owing to their properties of self-renewal and pluripotency, ESCs hold great potential to be an unlimited source for targeted therapies and regenerative medicine. Despite their undoubted therapeutic promise, their effective clinical application has remained elusive to date.

ESCs differentiate spontaneously towards the hepatic lineage simply by the removal of factors that prevent their differentiation, or can be guided towards a hepatocyte-like fate with the appropriate cytokines [90]. Mouse ESCs, which have been differentiated to hepatocyte-like cells, were transplanted into models with liver injury ad demonstrated evidence of improved survival without malignancy [91, 92]. Ishii et al [93] reported the use of ESCs that were differentiated in vitro into an AFP-producing hepatocyte-like phenotype. These cells were transplanted into mice with liver injury induced by diphtheria toxin. By day 7 and day 35 after cell transplantation, the donor cells represented 3.4 and 32.8% of the liver mass, respectively. These engrafted donor cells decreased 18.3% at day 40 and 7.9% at day 50, and few donor cells were observed by days 60-90. The survival rate of the AFP-producing cell-transplanted group was significantly higher than that of the control group. Two months after transplantation, splenic teratomas were found in a large number of animals.

Human ESC lines have been effectively differentiated to hepatocyte lineage with evidence of repopulation of immunosuppressed mouse model of liver injury [94, 95]. However, undifferentiated human ESCs transplanted into immunodeficient mice have also resulted in teratoma formation [91, 96]. Apparently ESCs are allogeneic in nature, and transplantation of hepatocyte-like cells generated from ESCs will require immunosuppression. The major obstacles against ESC-based cellular therapy are its tumorigenic potential and ethical issues associated with using ESCs.

Induced Pluripotent Stem Cells

Recent efforts on somatic cell reprogramming opened a novel avenue towards more practical regenerative medicine or cell-based therapy. Since the cloning of Dolly demonstrated that nuclei from mammalian differentiated cells can be reprogrammed to an undifferentiated state by trans-acting factors present in the oocyte [97], many groups have been searching for factors that could mediate similar reprogramming without somatic cell nuclear transfer. In addition to dedifferentiation being triggered by placing the nucleus of a differentiated cell in the cytoplasmic milieu of an egg cell, a small number of transcription factors can reprogram cultured adult cells to pluripotent stem cells termed induced pluripotent stem cells (iPSCs). iPSCs were successfully developed from animal and human somatic cells at different research institutes [98-100]. The related studies point to the possibility of regenerating mammalian tissues by first reverting skin or other adult cells to iPSCs and then redifferentiating these cells into various cell types. For the first time, Song et al [101] demonstrated that human iPSCs can be directly induced to differentiate into hepatocyte-like cells using a stepwise differentiation protocol. The hepatic differentiation efficiency of human iPSCs is comparable to that of human ESCs.

Direct reprogramming of somatic cells to iPSCs provides an invaluable resource for regenerative medicine, enabling the generation of patient-specific cells of any lineage without the use of embryonic material. The key steps involved in this process are the choice of factors, their delivery method, the choice of target cell type, and the parameters of factor expression [102]. Patient-specific cell transplantation could avoid ethical issues and the problem of tissue rejection. However, several hurdles must be cleared before the reprogramming approach can be applied to clinical patients, such as the potential risks involving genetic manipulation during in vivo treatment, viral carriers associated with insertional mutagenesis and hence tumor initiation. Recent studies conducted by Yu et al. [103] and Zhu et al. [104] demonstrated that reprogramming human somatic cells does not require genomic integration or the continued presence of exogenous reprogramming factors and removes one obstacle to the clinical application of human iPSCs.

Furthermore, differentiated adult cells of one type can be directly and efficiently converted into functional cells of another type within an organism and without the activation of dedifferentiation, which is described as transdifferentiation. By using a strategy of re-expressing key developmental regulators in vivo, Zhou et al [105] identified a specific combination of three transcription factors (*Ngn3, Pdx1 and Mafa*) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble ß-cells. The induced ß-cells express genes that are essential for ß-cell function and can ameliorate hyperglycemia by remodeling local vasculature and secreting insulin. Hopefully, further

precisely designed studies could bring about a practicable approach by which fibrogenic cells can be reprogrammed into hepatic parenchymal cells in the cirrhotic liver.

Mechanisms of Cell-based Intervention

As described earlier, hepatic cirrhosis is caused by both hepatocellular damage or cell loss and imbalanced fibrogenesis, therefore, the ideal cell-based therapeutic strategy should be designed to replace the damaged cells and diminish the fibrogenesis in the cirrhotic liver. Recognizing the special structure of the liver could be helpful for strategic decision. The liver contains many different cell types, including hepatocytes, sinusoidal endothelial cells, stellate cells, hepatic macrophages (Kupffer cells) and biliary epithelium. These hepatic cells are rigorously organized in three-dimensional structures, which are termed as hepatic lobules. The hepatic lobule is the smallest functional unit in the liver. There are about half a million lobules in the adult human liver, which comprise 96% of total volume of the liver. The main component of the hepatic lobule is hepatocyte (75%), which is the major attack target of various insults. In order for transplanted cells to functionally replace the damaged hepatic cells, the transplanted cells need to be located in the right location and differentiated into the right cell types. Considering the bone marrow-derived HSCs and MSCs are the only cell types that are employed in clinical trials up to date, these extrahepatic adult stem cells especially MSCs are exemplified in this section.

Homing and Functional Integration

The potential therapeutic benefit of MSCs can only be realized through their homing efficiency to the required site. The ultimate success or failure of cell therapy will rest on its ability to show clinical efficacy rather than the underlying mechanisms. However, a variety of evidence from clinical and animal studies have indicated that MSCs' direct differentiation and indirect effect through its secretion play important roles in promoting tissue recovery. An animal study by Aurich et al. [106] showed the functional integration of MSC-differentiated hepatocytes in the liver. Human MSCs were pre-differentiated and directly transplanted into immunodeficient mouse liver. Engraftment of transplanted pre-differentiated human MSCs in the mouse liver was observed three weeks after transplantation. Functional hepatic integration was also revealed using un-differentiated human MSCs xenografted directly onto rat liver [84].

The following questions have been frequently encountered while pursuing cell-based therapeutic investigations. What is the best method of delivery of cells? And how do the cells get to the sites of injury and by what mechanisms are they targeted? As previously discussed, the methods of cell administration can be classified into three categories: directional or site-specific delivery, semi-directional delivery, and systemic delivery [107]. Among mentioned examples, Sato et al. [85] administered MSCs to the injured liver by intrahepatic injection, which is considered directional delivery; intrasplenic injection by Aurich et al. [106] and intravenous infusion by Fang et al. [108] are examples of semi-directional and systemic deliveries. Under certain circumstances, the combination of more than one method and repeated administration may also be considered. The possible routes of cell delivery are illustrated in Figure 3. The most likely widely accepted in clinical practice is intraportal delivery. The transplanted cells move across the sinusoids and ideally engraft in the liver. The

number of cells transplanted into the portal vein at a time may be limited by portal pressure, thus intrasplenic transplantation has been suggested as an alternative [109]. The spleen has good capacity as a site of transplantation and may be particularly useful in chronic liver disease where the deranged liver architecture prevents transplanted cell engraftment. The exposure of transplanted cells to the immunological environment of the spleen is a disadvantage, and injection into the splenic artery has also been associated with splenic necrosis. Peripheral intravenous infusion is the most convenient and practical systemic delivery of stem cells. This deliver route is widely utilized in both experimental and clinical studies. The pulmonary passage is a major obstacle for intravenous stem cell delivery. This is the so called pulmonary first-pass effect [110].

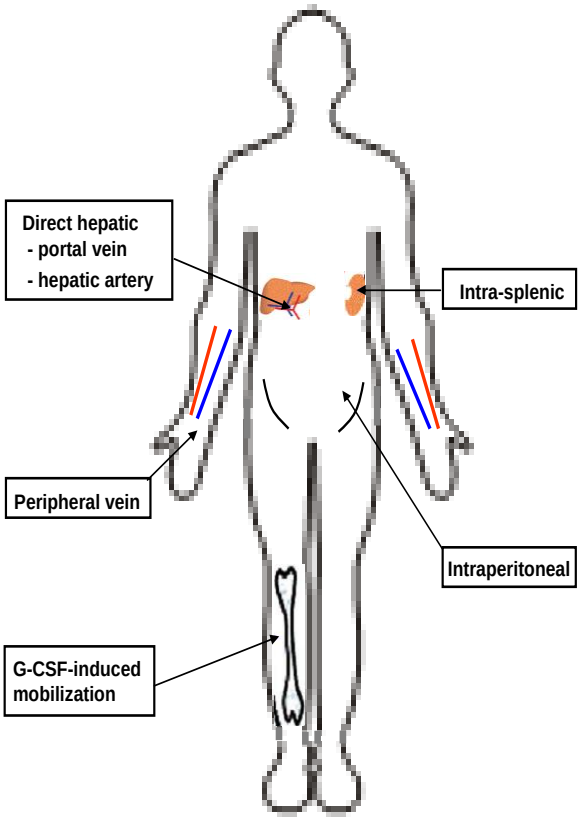

Figure 3. The possible routes of cell administration. G-CSF: granulocyte colony-stimulating factor.

The migration of MSC from the circulation into damaged or pathological tissues is the most crucial step in bringing MSC into play. Biological signals released from the injured area and corresponding receptors expressed on the transplanted cell surface are critical determinants in this step. Xiang et al. [111] reported that, in rats with CCl_4-induced liver injury, the timing and numbers of MSC homing to the liver are closely related to the presence of liver injury but not to the route of MSC infusion, e.g., through the tail vein or the portal vein. The process of leucocyte homing to specific inflammatory sites in response to inflammatory stimuli is a well characterized sequential process, which involves selectins,

chemokines, integrins and other adhesion molecules. As MSCs are known to be selectively recruited to injured tissue it can be reasonably assumed that they utilize comparable mechanisms of recruitment, such as transendothelial migration directed by chemokine gradient [112]. This assumption has been evidenced in the studies of myocardial infarction [113] and ischemic brain injury [114]. The detailed process of transendothelial migration of MSC was elaborated in a recent review [112]. It is worth noting that MSC chemokine receptor expression has been shown to diminish with in vitro culture and decreased receptor expression leads to a corresponding decrease in chemotactic responsiveness of the cells [115].

Cell engraftment is the predisposition for the function of transplanted cells. The quality of cells may affect the success of engraftment after transplantation. The size of hepatocyte or candidate stem cell is roughly 20-40 um in diameter; they become entrapped in sinusoids which have 6-9 um fenestrations. This causes portal hypertension and transient ischemia-reperfusion injury, thereby stimulating Kuppfer cells. Activated Kuppfer cells release TNF-α thus inducing vascular permeability. Transplanted cells stick to the activated endothelium and translocate through the sinusoidal fenestrations where they integrate into the liver parenchyma forming gap junctions and bile canaliculi between transplanted cells and hepatocytes [66]. Hepatic remodeling occurs in rodents in 3 to 7 days when the transplanted cells become histologically indistinguishable from host cells. Cell-to-cell interactions between transplanted cells, Kuppfer cells and stellate cells modulate the engraftment, and the extracellular matrix also play an important role in this process [116, 117]. From a clinical perspective, several problems need to be addressed for optimal cell engraftment, including cell quality, cell quantity, the requirement of immunosuppression, and the route of cell administration.

Replacement of Hepatocytes in the Injured Liver

Hepatic insufficiency or malfunction can be restored by the replacement of damaged hepatocytes, which is fundamental for the treatment of chronic liver diseases. Hepatocytes and hepatocyte-like cells differentiated from stem cells are the main sources for the hepatocyte replacement. Sufficient cell mass (approximately 10-15% of liver cell mass) is needed to provide enough function to sustain metabolic function [66, 118]. In chronic liver diseases, or acute chronic dysfunction, the aim of hepatocyte or hepatocyte-like cell transplantation is to allow the cells to engraft, to repopulate the liver and to restore liver function. As the liver architecture is disrupted in chronically damaged liver, there are difficulties with engraftment. The fate of the transplanted cells is dependent on their interaction with the local microenvironment, the extracellular matrix, soluble mediators such as cytokines and growth factors, and the immunological response of the host [119].

Although hepatocyte-induction protocols work well in cultured MSCs, an organ-specific microenvironment is the most suitable place for them to differentiate into required cell types. Sato et al. [85] demonstrated the first in vivo hepatic differentiation of MSC. In that study, human bone marrow-derived MSCs were directly xenografted to allylalcohol-treated rat liver, and the most human MSC-differentiated hepatocyte-like cells were observed at day 28, as revealed by positive immunostaining for human specific AFP, albumin, CK19, CK18 and asialoglycoprotein receptor. A recent study by Chamberlain et al. [120] provided further evidence of in vivo hepatic differentiation of MSC. Clonal human MSCs were xenotransplanted to fetal sheep liver by intrahepatic injection in their study. A widespread distribution of human MSC-originated hepatocytes throughout the liver parenchyma was

exhibited at days 56 to 70. In addition to the bone marrow-derived MSC, hepatogenic differentiation of MSCs from other sources, such as adipose tissue [121], umbilical cord blood [122] or commercially available MSCs [123] have also been achieved. It is worth noting that a significant challenge for stem cell research is the interpretation of in vitro data concerning the content of hepatic differentiation, which is often problematic and controversial [124]. Useful stem cell-derived hepatocytes will need to not only express the genes found in mature liver cells, but the level of the expression need to be at or near those found in the normal liver.

The gene signaling pathways in hepatic differentiation are essential for MSC-based therapies for the treatment of cirrhotic liver disease. Hepatic-differentiated cells are characterized by the expression of hepatocyte-specific genes. This specific gene expression is ingeniously regulated by numerous transcription factors and is also influenced by microenvironmental conditions. Costa et al. [125] elaborated the transcription factors in liver development, differentiation and regeneration. Recently, Yamamoto et al. [126] demonstrated the hepatic differentiation of human adipose tissue-derived MSCs. They utilized microarray analysis to identify the genes responsible for hepatic differentiation and found evidence of transdifferetiation through mesenchymal-epithelial transition in the process of hepatic differentiation. Further studies identifying the complex network of interaction between gene signals during hepatic differentiation of MSCs may facilitate the development of novel methods of therapeutic intervention in human liver cirrhosis.

The functional recovery of the injured liver is proportionally relevant to the amount of hepatocyte regeneration. At least 2.5 to 5% of a human liver needs to be replaced by healthy cells to reverse a pathological condition [127]. However, most transplantation studies have indicated that MSC-derived hepatocytes did not comprise more than 1% of the total liver mass. One plausible explanation for this low efficiency is the heterogeneity of MSCs employed in the studies. On other hand, due to the xenogeneic nature in some xenotranspantation studies, a considerable number of inoculated MSCs might have been rejected even in an immunosuppressive state. This viewpoint is consistent with Chamberlain's recent finding in a preimmune fetal animal model [120]. Human MSC-derived hepatocytes comprised over 12% of the total liver mass after xenotransplanted into fetal sheep liver. Apparently, homing efficiency of MSC could be greatly improved by autologous transplantation.

In addition to the direct hepatic differentiation of implanted stem cells in the injured area, MSC paracrine-mediated hepatic regeneration from endogenous liver stem cells may also contribute to the hepatocyte replication and recovery of hepatic function. Parekkadan et al. [128] reported the first experimental evidence of therapeutic use of MSC paracrine. A significant survival benefit was observed by MSC-conditioned medium perfusion in Gal-N-induced fulminant hepatic failure rat model. More recently, van Poll et al. [129] provided further evidence that MSC-derived molecules directly inhibit hepatocellular death, enhance liver regeneration and ultimately improve survival in rats undergoing D-galactosamine-induced fulminant hepatic failure. These investigations validate the therapeutic benefits of MSC-derived molecules on liver disease and may create potential new avenues for the treatment of advanced liver disorders.

Attenuation of Hepatic Fibrogenesis Progression

As described earlier, hepatic fibrosis or cirrhosis results from the imbalance of ECM production and degradation. Any approach that resets the balance could lead to the resolution of fibrogenic liver disorders. The fact that MSCs have antifibrosis effects in injured liver has been clearly demonstrated in animal models of liver fibrosis [108, 130, 131]. MSCs have a significant impact on hepatic fibrogenesis through their ability of inhibiting activated LSCs and re-regulating the fibrogenic process.

Induction of LSC apoptosis LSCs are the major source of fibrillar collagens and other ECM proteins that characterize liver fibrosis. Following liver injury, LSCs undergo a phenotypic switch from quiescent, vitamin A-storing cells into proliferative, α-smooth muscle actin positive, myofibroblast-like cells, a process termed activation [34]. Activation of LSCs is central to liver fibrosis and induction of LSC apoptosis is a potential antifibrotic treatment. This was directly evidenced in a study of Parekkadan et al. [128]. Indirect co-culture of activated LSCs and MSCs led to a significant decrease in collagen deposition and cell proliferation, while inducing apoptosis of activated LSCs. The underlying mechanisms in the modulation of LSC activity by MSCs were attributed to paracrine mediators, IL-10, TNF-α and HGF. Blockade of MSC-derived IL-10 and TNF-α abolished the inhibitory effects of MSCs on LSC proliferation and collagen synthesis. MSC-derived HGF was responsible for the marked induction of LSC apoptosis as determined by antibody neutralization studies. IL-6 secretion from activated LSCs induced IL-10 secretion from MSC, suggesting a dynamic response of MSCs to LSCs in the microenvironment. LSC apoptosis can also be triggered by MSC-secreted nerve growth factor (NGF) stimulation. MSC-originated NGF was directly identified from human MSC culture supernatant by quantitative ELISA measurement [132]. Trim et al. [133] found that LSCs express p75, a low affinity NGF receptor, and respond to NGF stimulation by undergoing apoptosis. They also identified the presence of activated LSC in the fibrotic bands of cirrhotic human liver biopsies and claimed p75 as a novel marker of activated LSC. The interactions between NGF and activated LSCs have been further demonstrated in mouse models [134, 135]. Zhao et al. [131] have provided additional evidence that in vitro co-culture of MSCs and LSCs increases the number of LSCs in the G_0 phase and reduces the number of LSCs in the S phase. Thus, MSCs play an inhibitory role in the process of LSC transition from the quiescent state to the activated state. However, it is worth noting the existence of some discrepancies in this field. Russo et al. [136] reported that bone marrow-derived cells significantly contributed LSC and myofibroblast populations in the cirrhotic mouse liver. These bone marrow-derived cells were found to be active for collagen type I transcription. Also with a murine model, Higashiyama et al. [137] reported that there were few, if any, bone marrow-derived cells expressing α-smooth muscle actin (α-SMA, a marker of activated LSCs) in the fibrotic liver. Presumably, the altered microenvironment in the cirrhotic liver favors to transplanted MSCs' fibrogenic differentiation rather than hepatocellular differentiation. Further studies are required to clarify the inconsistencies.

Re-regulation of fibrogenesis Collagen turnover and ECM remodeling is regulated by various MMPs and their inhibitors, i.e. the tissue inhibitors of metalloproteinases (TIMPs).

MMPs and TIMPs are crucial for matrix remodeling processes during hepatic fibrogenesis. The balance of ECM synthesis/ECM degradation is mainly determined by the balance of MMPs/TIMPs. During spontaneous recovery from liver fibrosis, there is a decrease in TIMP expression, an increase in collagenase activity, and an increase in apoptosis of LSC. The close correlation between the reduction of TIMP expression and apoptosis of LSC observed in vivo highlights a potential role for TIMP in regulating LSC survival. An in vitro study of Murphy et al. [138] indicated that the inhibition of apoptosis of HSC by TIMP-1 is mediated via effects on MMP inhibition. MSC-mediated TIMP-1 reduction was demonstrated in an in vivo MSC transplantation study in myocardial infarction rat model [139]. Comparing to the control, the expression of TIMP-1 was significantly decreased in the infarcted myocardium, along with declined expressions of collagen I, collagen II, TGF-ß1, and the protection of cardiac function. Similar molecular mechanisms may apply to liver injury-healing process, though direct evidence is absent from liver studies. As described in the recent review, most MSC-secreted and fibrogenesis-related molecules are anti-fibrogenic and this might make MSC favorable to ECM degradation rather than accumulation during ECM remodeling [24]. More recently, the secretion of two major anti-fibrogenic molecules (MMP-2 and MMP-9) was revealed in cultured mouse bone marrow-derived MSCs, which provides the direct evidence of MSCs' anti-fibrogenic effect [140]. However, the delicate mechanism is very complicated and far from clear, since some MSC-derived molecules are fibrogenic, or favorable to ECM accumulation. Di Bonzo et al. [141] identified a significant number of myofibroblast-like cells of human origin after transplanting human MSCs to the mice with liver injury. It is presumable that the effect of MSC varies with the nature of liver injury, time-frame of MSC application and different experimental models. In a rat model of severe chronic liver injury, MSC failed to reduce fibrosis and improve liver function [142]. Further investigations are required to identify the factors which affect the fate of MSC in the injured area.

Clinical Application and Perspectives

The translation of preclinical research on MSC to clinical use on cirrhotic patients has generated great interest, due to the growing population of patients with advanced liver diseases and the critical shortage of available donor livers. The choice of stem cell in human clinical trials reflects, in part, the investigator's belief on the mechanism of action. These mechanisms include transdifferentiation, stimulation of endogenous hepatocyte proliferation, and antifibrotic and immunomodulatory effects. If investigator believes plasticity is the major mechanism, then there is literature supporting both HSCs and MSCs, although it is more compelling for MSCs. Many of the initial studies suggested that HSCs were the predominant cell type involved [143], but more recently in vitro and in vivo experiments have supported transdifferentiation of MSCs into hepatocytes [85, 144, 145]. If stimulation of endogenous hepatocyte proliferation is desired, there is evidence to support the use of HSCs [146, 147]. The effects of MSCs are not known in this setting. As for antifibrotic effects, the literature is less clear in that unsorted bone marrow cells have been used. There is a growing body of evidence supporting an immunomodulatory effect of MSCs [148, 149].

To date, there are 11 published human clinical studies investigating the effects of bone marrow stem cell therapy in patients with liver disease [47]. All reported clinical trials are

small-scale, uncontrolled safety and feasibility studies [150]. Terai et al. [151] implemented a clinical trial on nine patients with decompensated liver cirrhosis. These patients were infused with $5.2\pm0.63\times10^9$ autologous bone marrow cells from the peripheral vein. At 24 weeks after transplantation, significant improvements were observed. These improvements included total protein, serum albumin, Child-Pugh scores, and ?Fetoprotein and proliferating cell nuclear antigen expression in liver biopsy tissues. Recently, Mohamadnejad et al. performed two small scaled clinical studies. In their first trial, four patients with decompensated liver cirrhosis were infused 3.17×10^7 (mean) MSCs through a peripheral vein [152]. At the end of follow-up (after 12 months), the model for end-stage liver disease scores (MELD) of two patients improved by four and three points. The mean physical and mental component scaled more than doubled by the end of follow-up. Computed tomography (CT) showed the increase of liver volumes of three patients by the sixth month. However, the results of their second trial [153] were not satisfactory. Four patients received 5.25×10^6 (mean) autologous bone marrow hematopoietic stem cells infused through hepatic artery. Only marginal improvements were observed in some patients. The results of their MSC transplantation were more promising than the study of hematopoietic stem cell transplantation. They also indicated that hepatic artery delivery of stem cells was not a safe procedure. Because of the lack of reliable means of identifying transplanted stem cells in the human body [154], caution is advised during the evaluation of the clinical outcomes.

Autologous $CD133^+$ bone marrow stem cells (BMCs) were used successfully to boost regeneration in human liver [155]. Three patients with large liver tumors were subjected to portal vein embolization of the tumor-bearing lobe to induce atrophy. Portal vein embolization could cause contralateral lobe hypertrophy and increase the size of the future remnant liver volume before an extensive partial hepatoectomy. Based on CT criteria, the contralateral lobes injected with BMCs enlarged by more than 2.5-fold compared with the contralateral lobes of non-BMC-injected patients. A number of phase I clinical trials involving the injection of autologous BMCs or BMC mobilization in patients with cirrhosis have demonstrated modest improvements in clinical scores [47]. For example, the application of G-CSF to patients with end stage liver disease resulted in a reduction in MELD scores from 17.5 to 14.5 [156]; the injections of autologous BMCs to patients with hepatic cirrhosis were attributed to the elevation of serum albumin and reduction of serum bilirubin levels [151, 157]. Similarly, nine patients with alcohol-induced cirrhosis showed modest improvements in Child-Pugh scores after infusion of autologous $CD34^+$ cells via the hepatic artery [158]. A case report described the use of autologous unsorted bone marrow stem cells as a rescue treatment for hepatic failure in a 67-year-old man ineligible for liver transplantation [159]. Apparent rapid improvement in hepatic synthetic function was obtained after the portal venous infusion of the cells. A liver biopsy performed 20 days after cell transplant was reported as showing increased hepatocyte replication around necrotic foci, although transplanted cells were not identifiable as they were not labeled with markers before transplantation.

Based on the knowledge from preclinical studies and previous clinical trials, the following considerations should be addressed during clinical trials for cirrhotic patients:

1. Utilization of purified MSC population. As discussed in the earlier section, MSC possess the abilities of hepatic engraftment and hepatic differentiation, and in addition, their easy accessibility and quick in vitro expansion make MSC an ideal resource for clinical use. Because bone marrow-originated fibrogenic cells play a role in the progression of liver fibrogenesis, the use of purified MSC population could avoid this risk. Peng et al. [160] successfully isolated and in vitro expanded MSC from advanced hepatitis B patients. MSCs isolated from patients share the same surface markers and similar biological characteristics to those isolated from healthy humans. Their study reveals the capability of autologous MSC transplantation in patients with advanced liver disorders. About one billion or at least one million/kg body weight of MSCs are required for transplantation at a time [161].

2. MSC passage. Longer in vitro culture and unnecessary manipulation may introduce more unexpected effects to ex vivo expanded MSCs. Our experience indicates that 3 to 5-passage cultures are safe and practical. During this culture period MSCs retain their cytogenetic stability, and enough number of cells can be obtained for transplantation starting from 10 to 15 ml of bone marrow aspirate [107, 162].

3. MSC delivery route. Intravenous infusion is the first choice under most circumstances. Although portal vein or hepatic artery delivery may enhance MSC homing efficacy, precautions must be taken when catheterization is applied to cirrhotic patients.

4. Evaluation standard. In order to objectively assess the therapeutic effect of MSC transplantation, a standardized criterion must be established prior to MSC administration. The following aspects should be covered in a practical criterion: (1) patient enrollment requirement, e.g., age, gender and type of hepatic disease; (2) hepatic function assessment; (3) liver parenchyma assessment, e.g., ultrasonography, CT and MRI; and (4) hepatic fibrosis assessment. Liver biopsy is considered to be the gold-standard method for the assessment of liver fibrosis, but the invasive nature and sampling error restrict its use as a routine laboratory assay. Recently, Fontana et al. [163] established a three-variable model to evaluate cirrhosis. Serum hyaluronic acid, TIMP-1 and platelet count are closely correlated with Ishak fibrosis scores based on liver biopsy samples from 513 subjects. This model can be used as a surrogate marker of liver fibrosis. Some parameters governing the success of using MSCs and characteristics of various delivery approaches were described in recent reviews [164, 165].

Figure 4 outlines the possible interventions of stem cells in the treatment of hepatic cirrhosis. It is likely that some types of stem cell play a part in differentiating to hepatocytes, stimulating the regeneration of endogenous parenchymal cells, and enhancing fibrous matrix degradation. Exploring the therapeutic potential of stem cells on hepatic cirrhosis will benefit millions of people who suffer from end-stage of chronic liver diseases. An obvious advantage of using mesenchymal stem cells is their autotransplantable nature, so as to bypass the ethical hurdles and avoid the use of expensive immunosuppression drugs. However, iPSCs have the same feature and hold great therapeutic potential. It remains unclear about the long-term fate of the engraftment, and some unexpected effects may be encountered during their application. Large-scale controlled and double-blinded clinical trials are required before stem cell transplantation becomes a regular therapy for patients on the end-stage of chronic liver diseases.

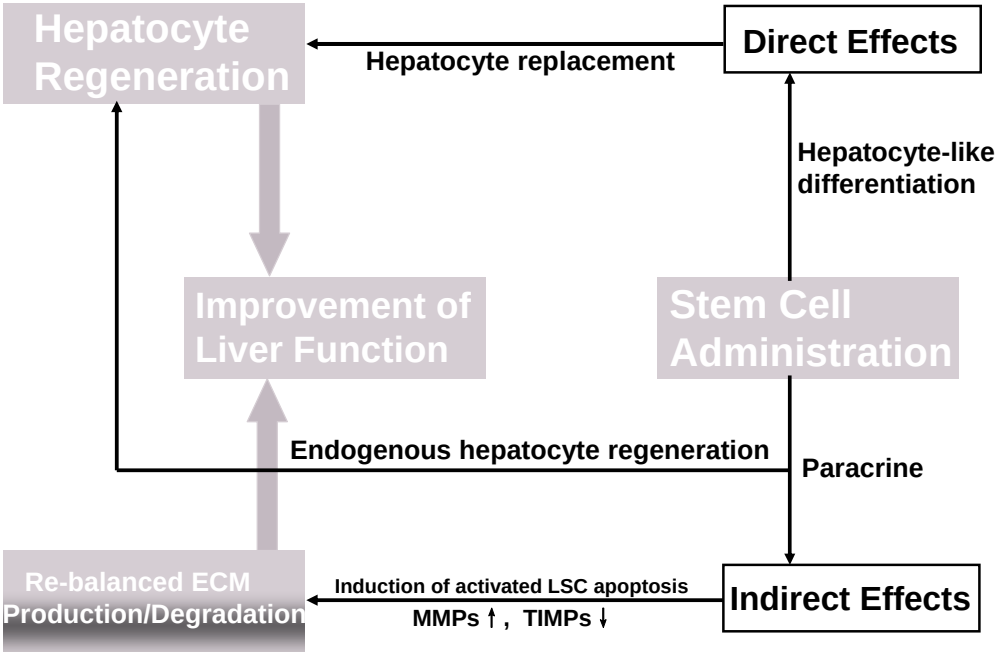

Figure 4. Summary of therapeutic potential of stem cells on hepatic cirrhosis. Administrated stem cells could improve the liver function directly through hepatocyte replacement and/or indirectly through their paracrine effects. ECM: extracellular matrix; LSC: liver stellate cell; MMPs: matrix metalloproteinases; TIMPs: tissue inhibitors of metaloproteinases.

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Chapter 11

Zinc, Copper, Manganese and Magnesium in Liver Cirrhosis

Dario Rahelic[1], Milan Kujundzic[2] and Velimir Bozikov[1]

[1]Division of Endocrinology, Diabetology and Metabolic Disorders,
[2]Division of Gastoenterology,
Dubrava University Hospital, Zagreb, Croatia.

Abstract

The role of trace elements in the pathogenesis of liver cirrhosis and its complications is still not clearly understood.

Zinc, copper, manganese and magnesium are essential trace elements whose role in liver cirrhosis and its complications is still a matter of research.

Zinc is associated with more than 300 enzymatic systems. Zinc is structured part of Cu-Zn superoxide dismutase, important antioxidative enzyme. Zinc acts as an antioxidant, a membrane and cytosceletal stabilizator, an anti-apoptotic agent, an important co-factor in DNA synthesis, an anti-inflammatory agent, etc. Copper is an essential trace element which participates in many enzymatic reactions. Its most important role is in redox processes. Reactive copper can participate in liver damage directly or indirectly, through Kupffer cell's stimulation. Scientists agree that copper's toxic effects are related to oxidative stress. Manganese is a structural part of arginase, which is an important enzyme in the urea metabolism. Manganese acts as an activator of numerous enzymes in Krebs cycle, particularly in the decarboxilation process.

Magnesium is important for the protein synthesis, enzyme activation, oxidative phosphorilation, renal potassium and hydrogen exchange etc.

Since zinc, copper, manganese and magnesium have a possible role in the pathogenesis of liver cirrhosis and cirrhotic complications**,** the aim of our study was to investigate the serum concentrations of mentioned trace elements in patients with liver cirrhosis and compare them with concentrations in controls.

Serum concentrations of zinc, copper, manganese and magnesium were determined in 105 patients with alcoholic liver cirrhosis and 50 healthy subjects by means of plasma sequential spectrophotometer. Serum concentrations of zinc were significantly lower (median 0.82 vs. 11.22 μmol/L, $p<0.001$) in patients with liver cirrhosis in comparison to controls. Serum concentrations of copper were significantly higher in patients with liver cirrhosis (median 21.56 vs. 13.09 μmol/L, $p<0.001$) as well as manganese (2.50 vs. 0.02 μmol/L, $p<0.001$). The concentration of magnesium was not significantly different between patients with liver cirrhosis and controls (0.94 vs. 0.88 mmol/L, $p=0.132$). There were no differences in the concentrations of zinc, copper, manganese and magnesium between male and female patients with liver cirrhosis. Only manganese concentration was significantly different between Child-Pugh groups ($p=0.036$). Zinc concentration was significantly lower in patients with hepatic encephalopathy in comparison to cirrhotic patients without encephalopathy (0.54 vs. 0.96 μmol/L, $p=0.002$). The correction of trace elements concentrations might have a beneficial effect on complications and maybe progression of liver cirrhosis. It would be recommendable to provide analysis of trace elements in patients with liver cirrhosis as a routine.

Keywords: zinc, copper, manganese, magnesium, liver cirrhosis, trace elements.

1. Liver Cirrhosis

1.1. Epidemiology

According to data from the World Health Organization (WHO) chronic liver disease causes more than 1.4 million death outcomes per year. Liver cirrhosis mortality rates vary across the countries. A cirrhosis mortality rate in Great Britain is now among the highest in Europe (28.9 per 100.000 in man and 12.8 in women). Between 1987-1991 and 1997-2001, cirrhosis mortality in man in Scotland doubled more than once (112% increase) and in women increase almost by two-thirds (67%) (Leon et al., 2006 a,b). On the contrary, in Sweden decrease in liver cirrhosis mortality has been observed. In the last fifteen years an age standardized mortality rate of liver cirrhosis was about 4 per 100.000 deaths per year (Stokkeland et al., 2006).

The regional differences in cirrhosis mortality rates follow a north-south gradient, with the lowest rates in Northern and the highest in Southern Europe. In 1995 cirrhosis mortality rates in Northern Europe for men were about 12.2 per 100.000 and for women 4.8. On the contrary, in Southern Europe cirrhosis mortality rates were about 33.7 for men and 12.1 for women. Some exceptions were noticed in order to obtain general pattern. France has substantially higher death rates than other southern European countries like Greece, Spain and Italy (Ramstedt, 2002).

Taking into account that liver cirrhosis has important impact on overall mortality, even small progress in explaining the liver cirrhosis pathogenesis could have positive influence on disease progression.

1.2. Definition

Liver cirrhosis is, according to the WHO, a diffuse process characterized by fibrosis and conversion of normal liver architecture to structural abnormal nodules without normal lobular organization.

1.3. Etiology

There are numerous causes of liver cirrhosis. Alcohol is the most common cause in the Western countries but viral infection is the most common cause world-wide. Other causes, like hereditary hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, galactosemia, autoimune disease, Budd-Chiari syndrome, secondary biliary cirrhosis, hepatic venous congestion, thrombosis of portal vein, infection, sarcoidosis, drugs, etc. are less common.

1.4. Alcoholic Liver Disease

Ethanol is metabolized in the liver through three pathways, by alcohol dehydrogenase / aldehyde dehydrogenase (ADH), microsomal ethanol oxidative system (MEOS) and by catalase.

More than 80% of ethanol is metabolized through first pathway. Consequently, an increase in the NADH/NAD ratio occurs, what inhibits many NAD+ dependent enzymes.

Liver changes caused by alcohol consumption include fatty liver, alcoholic hepatitis and alcoholic (Laenec's) liver cirrhosis. Fatty liver is initial, reversible phase of alcoholic liver disease characterized by the development of fatty vacuole in hepatocytes due to the impaired fatty acid metabolism.

Alcoholic hepatitis is characterized by hepatocyte necrosis, infiltration of polymorphonuclear leucocytes mainly in zone 3, then by dense cytoplasmic inclusions called Mallory bodies and by hiatal sclerosis (Jensen et al, 1994 a,b). Mallory bodies are not specific for alcoholic liver disease, because they can be found also in Wilson's disease and primary biliary cirrhosis.

Liver cirrhosis is pathohystological term characterized by necrosis of liver parenchyma, fibrosis and development of regeneratory lobules. According to the lobular size, liver cirrhosis can be divided on micronodular, macronodular and mixed cirrhosis. Micrinodular cirrhosis is characterized by lobules smaller than 3 mm in diameter and macronodular by lobules above 3 mm in diameter.

Alcoholic liver cirrhosis is usually micronodular, but during the time can transform to macronodular cirrhosis.

1.5. Pathogenesis of Alcoholic Liver Disease

Alcoholic liver cirrhosis is caused by toxic effects of alcohol on the liver, lipid peroxidation and immunologic alterations. After absorption, alcohol is transformed to

acetaldehyde. Accumulation of acetaldehyde alters hepatocyte function. Also, the product of alcohol metabolism is reduced nicotinamide-adenine dinucleotide (NADH) which accumulates in the cytosol and mitochondria of hepatocytes. That results in inhibition of many NAD+ dependent enzymes like the enzymes of β-oxidation. All that cause decreased oxidation of fatty acids with increased export of very low density lipoprotein (VLDL) and hypertriglyceridemia which are commonly associated with alcohol use (Crabb & Estonius, 1998). Consequently, fat deposition occurs within the liver what cause so called *fatty liver*. Acetaldechide can also modify liver proteins and DNA forming neoantigens which can be recognized by the immune system and cause immunological reaction. Acetaldechyde stimulates collagen synthesis by stellate cells. Acetaldechyde reduces mitochondrial glutathione concentration. Alcohol by itself causes increased oxygen consumption with consequent centrilobular hypoxia.

In the condition of low blood and tissue alcohol concentration, ADH has a key role in the metabolism of alcohol. When tissue concentration increases above 10 mmol/L MEOS starts to metabolize alcohol. MEOS is situated in microsomes of smooth endoplasmic reticulum. Chronic alcohol consumption causes an increase in MEOS activity 5 to 10 times. Main role in MEOS has isoenzyme CYP2E1 from cytochrom P450 family. CYP2E1 metabolizes ethanol but also acetaldechyde and many other drugs what explains the increased toxicity of therapeutic doses of some drugs in alcoholics (Vucelic et al., 2002).

Chronic alcohol consumption leads to the induction of CYP2E1 with generation of oxygen radicals and lipid peroxidation. Also, alcohol sensitizes Kupffer cells and stellate cells and probably inhibits regeneration of injured hepatocytes (Crabb & Estonius, 1998). Reactive oxygen species cause hepatocyte injury by lipid peroxidation.

In pathogenesis of liver cirrhosis important occurrences are necrosis of hepatocytes, hepatocellular regeneration, fibrosis and destruction of lobular structure with forming pseudolobules.

In fibrogenesis, necrosis of hepatocytes occurs first. In the early phase, as mentioned above, products of cell inflammation, like proteinases and free oxygen radicals, cause necrosis of hepatocytes with consequent release of numerous cytokines. Changes of extracellular matrix occur in further phase. Those changes include non-proportional increase in a concentration of some extracelular matrix molecules, changes on molecules and their transposition within the matrix.

There is a multiple increase in concentration of extracellular matrix molecules like collagen, glycoproteins and proteoglycans. Chronic alcohol intake also stimulates production and releasing of numerous cytokines by stimulated Kupffer cells. One of the most important cytokine in fibrogenesis is certainly transforming growth factor β (TGF-β). Released cytokines stimulate stellate cells on proliferation and collagen synthesis.

On the other hand, degradation of protein matrix has also important role in fibrogenesis (Sherlock et al., 2002). Matrix degradation is regulated by metalloproteinase (Arthur & Iredale, 1994; Arthur, 2000). The most important metalloproteinases are collagenases (MMP-1 and MMP-13, which disolve type I, II and III collagen), gelatinase (MMP-2 and MMP-9, which disolve type IV collagen) and stromelisins (MMP-3 and MMP-10, which disolve proteoglycans, laminin, fibronectin, etc.) (Takahara et al., 1995). All these enzymes are synthesized by Kupffer cells and activated stellate cells. Metalloproteinases are inhibited by tissue metalloproteinase inhibitors (TIMP). Activated stellate cells synthesize TIMP-1.

Therefore, stellate cells have important role in degradation and synthesis of connective tissue (Iredale et al., 1992).

Fibrosis alters the liver structure and obstructs biliary and vascular pathways with clinical consequences.

1.6. Clinical Manifestations of Liver Cirrhosis

Fatty infiltration causes nonspecific symptoms or abnormal laboratory results of liver function. The liver is usually enlarged. In advanced alcoholic liver disease common symptoms are loss of appetite, weight loss, nausea, jaundice, abdominal pain and edema. Liver cirrhosis is characterized by an enlarged liver in the beginning and small, fibrous liver in advanced stages, enlarged spleen, portal hypertension with esophageal varices, hepatic encephalopathy, anemia, etc.

1.7. Treatment of Patients with Liver Cirrhosis

Treatment of liver cirrhosis is mostly symptomatically. Usually, the main problem in patients with liver cirrhosis is the treatment of complications like hepatic encephalopathy, hepatorenal syndrome and bleeding from esophageal varices.

Numerous studies have tried to focus on prevention of alcoholic liver cirrhosis and progression of alcoholic liver disease to liver cirrhosis. There were also numerous studies which have tried to slow down progression of liver disease and prevent complications. As the oxidative stress has important role in pathogenesis of liver cirrhosis, trace elements have become important substrate for investigations of pathogenesis of liver cirrhosis and its complications.

Trace elements certainly have important role in pathogenesis of liver cirrhosis and its complications.

2. Trace Elements

2.1. Definition

Trace elements are present in human body in very small concentration *(ppm - pars per million*).

Development of new laboratory methods has allowed better quality and quantity determination of trace elements, as well as better understanding of their role in numerous metabolic processes.

There are essential and nonessential trace elements. Essential trace elements are those necessary for life and normal body function. According to Cotzias (Cotzias, 1967), essential trace elements are iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co), iodine (J), molybdenum (Mo), selenium (Se) and chromium (Cr). There is an additional division of trace elements on incontestable essential trace elements and »probably essential trace elements for

humans«. Incontestably essential trace elements are iron (Fe), zinc (Zn), copper (Cu), chromium (Cr), iodine (I), cobalt (Co), molybdenum (Mo) and selenium (Se). Probably essential trace elements are manganese (Mn), tin (Sn), nickel (Ni), fluor (F), silicon (Si), vanadium (Va), calcium (Ca) and magnesium (Mg).

Trace elements entry includes ingestion by food and water. Normal nutrition satisfies requirements of human body for trace elements.

Concentration of trace elements is different in different drinking waters. Food processing, especially industrial, influences (mostly decrease) concentration of trace elements. There is also a question of trace element supplementation in patients with long-term parenteral nutrition.

All above mentioned suggests that an intake of trace elements in human body by food or by drink water varies.

Trace elements form moderately stable complexes with enzymes, nucleic acids and other ligands, changing and controlling their function, while other form compact static complexes and become integral functional components of enzymes (Speech et al., 2001). Therefore, it is obvious that some trace elements participate in numerous biochemical reactions which are necessary for life, like oxygen transport and release, redox processes, etc.

Optimal concentration of trace elements is within narrow range between their deficiency and toxicity.

Deficiency of trace elements is usually caused by the inadequate intake or other factors, like an increased loss caused by diarrhea, malabsorption after surgical resection of small intestine, by forming metal complexes with food ingredients which do not allow absorption, increase urinary losses, increase losses caused by pancreatic juices or other exocrine secretions, etc. The deficiency can as well be caused by the antagonistic actions of some trace elements on absorption or transportation of other trace elements, for example, in the case of intake of zinc and copper or copper and molibden. The intake of one trace element can also cause the deficiency of the other trace elements.

Toxic effects of trace elements depend on chemical shape, way of entry in the body, biological ligands, tissue distribution, concentration and velocity of elimination.

Toxicity mechanisms include enzyme inhibition by binding to essential amino-acid residuum, changing both function and structure of nucleic acids, inhibition of synthesis, influence on membrane permeability, inhibition of phosphorilation, etc (Kasper et al., 2005).

As it would be difficult to present role of all trace elements in liver cirrhosis we choose to present only zinc, copper, manganese and magnesium according to data from our research.

2.2. Zinc, Copper, Manganese and Magnesium in Liver Cirrhosis

The role of trace elements in pathogenesis of liver cirrhosis and its complications is still not clearly understood. In fibrogenesis the initial occurrence is hepato-cellular necrosis. In the early phase, inflammation cell products, proteinases and reactive oxygen radicals, may initiate hepato-cellular necrosis with consecutive releasing of numerous cytokines. Following hepatic injury, there is an increase in extracellular matrix, the activation of stellate cells, the increase in rough endoplasmatic reticulum and expression of smooth muscle specific α-actin (Friedman, 1993).

Activated stellate cells are influenced by numerous cytokines. Some of them have proliferative effect on stellate cells while others stimulate fibrogenesis (Pinzani, 1995).

Zinc, copper, manganese and magnesium are essential trace elements whose role in liver cirrhosis and its complications is still a matter of research. There are contrary reports about their serum concentrations in patients with liver cirrhosis.

2.2.1. Zinc

Zinc is incontestably essential trace element in humans. In nature zinc is especially present in sea food, cereals, vegetable, milk, walnuts etc. Average daily intake is approximately 12-15 mg. From oral intake, only 20-30% will be absorbed. In the enteric cell zinc induces synthesis of metalothionein, low molecular weight protein and when this process ends further absorption of zinc decreases. About two thirds of absorbed zinc is bounded on albumin and other is bounded on ß-2 microglobulin. Normal plasma concentration of zinc is 0.85-1.10 μg/mL.

In adult people renal excretion is approximately 300-600 μg per day. Renal tubular absorption decreases with tiazid diuretics administration (Prasad et al., 1996). Increased urinary losses are common in nephrotic syndrome, liver cirrhosis and other hypoalbuminic states, during penicillamine administration, catabolic states after burning, trauma, surgery, hemolytic anemia, etc. Decreased serum concentration of zinc is common in patient with acute myocardial infarction, infection, hepatitis, etc. (Kasper et al., 2005).

Lower serum concentration of zinc is common in patients with liver cirrhosis due to decreased intake, decreased absorption, decreased bioavailability and increased losses due to malabsorption, diarrhea or increased urinary losses.

There is a reduced liver protein synthesis in patients with liver cirrhosis with consequently decreased zinc bioavailability.

Zinc participates in more than 300 enzymatic systems (Christianson, 1991). Zinc is involved in synthesis of nucleic acid, protein synthesis, testosterone secretion, cerebral function etc. Zinc presents natural defense from reactive oxygen radicals through antioxidative enzyme, Cu-Zn superokside dysmutase (Speich et al., 2001). Its role in storage and release of hormones, neurotransmision, visual processes and cognitive processes were also described (Truong-Tran et al., 2000; Vallee et al., 1993).

Zinc acts as an antioxidant, membrane and cytoskeletal stabilizator, anti-apoptotic agent, important cofactor in DNA synthesis, anti-inflamatory agent etc. (Truong-Tran et al., 2001).

In the last decade role of zinc in apoptosis was considered intensively (Truong-Tran et al., 2000; Zalewski et al., 1993; Sunderman, 1995; Wyllie, 1997). Apoptosis is important in early embryonic development, what has been found in investigations with zinc deficient rats (Record et al., 1985).

Ethanol consumption induces apoptosis in liver and lymphoid tissue as well as many other. According to the published data it seems that zinc has influence on apoptosis of blood mononuclear cells by inhibiting the mitochondrial pathway of cell death. It was suggested that mitochondrial pathway of ethanol-related immune cell death may be inhibited by zinc supplementation (Szuster-Ciesielska et al., 2005).

On the other hand, it seems that zinc at pharmacologic concentrations stimulates cytokine expression and induces apoptosis of peripheral blood mononuclear cells (Chang et al., 2006).

The role of zinc in Alzheimer disease has also been investigated (Anderson et al., 1996).

Zinc has also role in glucose metabolism. Decreased secretion of insulin and impaired glucose tolerance were found in zinc deficient patients (Marchesini et al., 1998). Zinc is also integral part of insulin molecule and crucial for the synthesis, storage and secretion of insulin in pancreatic islet cells (Grungreiff et al., 2005; Chausmer, 1998; Blostein-Fuji et al., 1997). There has been hypothesed that zinc deficiency could be a link between liver cirrhosis and "liver" diabetes mellitus (Grungreiff et al., 2005). Zinc supplementation increases glucose disposal due to the increased non-insulin-mediated glucose uptake, without any systematic effect on insulin secretion and sensitivity (Marchesini et al., 1998).

Zinc has an important role in fibrogenesis. Some of zinc metalloenzymes, like DNA and RNA polymerases, have a great impact on regeneration of liver parenchyma. In fibrogenesis, zinc acts antagonistically to copper (Arakawa et al., 2003).

Zinc inhibits the cross-linking of covalent bonds in collagen through lysyl oxidase (Sato et al., 2005).

Zinc is a structure part of collagenasis. On the other hand, zinc is the inhibitor of prolyl hydroxilase, which is important enzyme in collagen synthesis (Camps et al., 1992).

In some studies, in the early phase a positive correlation between liver regeneration and zinc tissue concentration was found (Milin et al., 2005). In regenerated liver translocation of metallothionein to the nuclei is noticed where zinc participate in cell cycle processes (Tsujikawa et al., 1994).

Zinc is an activator of ornithin transcarbomoilase which participates in ammonia metabolism.

Zinc also participates in amino-acid metabolism, therefore its role in portal encephalopathy was investigated. Studies have showed that long-term oral zinc supplementation in patients with liver cirrhosis improve urea synthesis from ammonia and amino-acids with consequently decrease in concentration of ammonia and improvement clinical features of liver cirrhosis (Marchesini et al., 1996).

According to the results from clinical trials, zinc has a positive effect on the oxydative stress. Zinc supplementation in protein deficient rats resulted in increased activity of catalase, glutathion peroxidase, glutathion reductase and glutathion-S-transferase (Sidhu et al., 2005).

Zinc supplementation in those patients leads to significant increase in reduced glutathion concentration (GSH) and increased superoxide dismutase (SOD) activity in comparison to control group. By zinc supplementation in mentioned research serum concentration of copper, iron and selenium were normalized.

The results suggested possible influence of zinc on antioxidative enzymes activity and its possible effect on concentration of other trace elements.

The research on animal models showed that zinc supplementation has a protective effect on ethanol induced liver damage.

Zinc supplementation decreases ethanol induced zinc depletion and decrease in cytochrom P450 2E1 (CYP2E1) activity. Also, zinc supplementation increases activity of alcohol dehydrogenase in liver. That partially can explain zinc influence on the oxidative stress.

Zinc has also the important role in preserving of intestinal integrity as well as in prevention of endotoxemia with consequent inhibition of TNF-α synthesis induced by endotoxine (Kang et al., 2005).

Mentioned effects of zinc are independent from metallothioneine. Zinc supplementation has protective effect on ethanol induced decreasing of glutathione concentration, decreasing

glutathione peroxidase activity and increasing glutathione reductase activity in liver (Zhou et al., 2005).

Zinc inhibits free oxygen radicals generation and increases antioxidative pathways activity.

According to Camps (Camps et al., 1992) zinc supplementation leads to decreased lipid peroxidation, collagen deposition, inhibition of prolyl-hydroxilase and increased collagenase activity.

Zinc induces synthesis of metallothionein. Metallothionein is effective cytoprotective agent against ethanol induced liver damage. Its protective effect can be explained by its influence on oxidative stress (Zhou et al., 2002).

Also, ZNF 267 (*zinc finger protein* 267) mRNA expression is increased in stellate cells of patients with liver cirrhosis. ZNF 267 is binding for MMP-10 and presents negative regulator of transcription MMP-10 and indirectly enhances fibrogenesis in the liver (Schnabl et al., 2005). The role of MMP-10 was mentioned above. All mentioned suggests the important role of zinc in fibrogenesis and pathogenesis of liver cirrhosis and its complications.

2.2.2. Copper

Copper is an essential trace element which participates in many enzymatic reactions. Copper has the most important role in redox processes, where presents donator of electron on mitochondrial level.

Copper absorbed in duodenum, binds for ceruloplasmin, albumin and transcuprein. More than 90% of copper in plasma binds for ceruloplasmin, and the rest binds for albumin and transcuprein (Luza et al., 1996).

In hepatocite copper is incorporated into ceruloplasmin and metallothionein, cistein rich protein, which binds also other heavy metals, like cadmium and mercury. Metallothionein acts as a factor of detoxicaton in gastrointestinal mucosa, but also prevents copper induced cytotoxicity (Luza et al., 1996; Sato et al., 2005). Copper enters into the cell by two copper transporting enzymes, ATP-ase ATP7A i ATP7B, products of genes for Menkel and Wilson's disease. Copper elimination is mainly through hepatobiliary tract, and around 4% by urinary tract.

Copper participates in gene expression. Copper is a cofactor of many enzymes, like superoxide dismutase, important antioxidative enzime, furthermore enzyme tirosinase, which is necessary for melanin synthesis in human body, as well as many other enzymes. Copper influences on metabolism of iron, its absorption, incorporation in hemoglobin, etc.

Toxic effect of copper was focus of interest of many scientists. One of proposed model was that metallothionein saturated with copper entry to lysosomes, where is incompletely demolished and polymerized, forming insoluble material containing reactive copper which, together with iron, run lisosomal lipid peroxidation. That consequently causes hepatocyte necrosis.

Reactive copper participates in liver damage directly or indirectly, through stimulation of Kupffer and other cells (Klein et al., 1998). Toxic effect of copper is explained through its role in production of oxygen radicals (Bremner, 1998). Oxygen radicals can cause destruction of cell lipids, nucleic acids, proteins and carbohydrates, what results in the impairment of cell function and cell integrity.

Copper is also very important in fibrogenesis. Copper is the cofactor of lysil oxidase, which is involved in the formation of molecular bridges in collagen. An excessive

accumulation of copper in the liver and an increase in the copper concentration promote hepatic fibrosis (Arakawa and Suzuki, 1993; Sato et al., 2005).

2.2.3. Manganese

Manganese is an essential trace element discovered in 1774. Manganese entries the human body by ingestion. Only 3-4% of ingested manganese is absorbed. Proportion of absorbed manganese can increase in specific states, like hypochromic anemia. In plasma manganese is transported by transmanganin (Kasper et al., 2005). In the body manganese is accumulated in mitochondria. The highest concentration of manganese is in enteric system, liver, pancreas, kidney, lungs and muscles. It passes hemato-encephalic barrier and can be accumulated in the brain in the state of prolonged exposition. Mainly, manganese is excreted through hepatobiliary system. Partly is reabsorbed in small intestine and the rest is excreted by feces.

Ingestion of manganese substances can cause destruction of gastrointestinal mucosa with bleeding. Chronic intoxication with manganese leads to degenerative changes in basal ganglia, especially in globus pallidus and corpus striatum (Spahr et al., 1996; Rose et al., 1999). Decreased synthesis of dopamine and decreased conversion causes decreased concentration of dopamine in corpus striatum. Possible explanation could be decreased activity of tyrosine kinase and other oxidative enzymes situated in mitochondria, where manganese is especially accumulated. Gradually, in three phases, symptoms like those in Parkinson disease develop in patients intoxicated with manganese.

Manganese is a structural part of arginase, which is an important enzyme in the urea metabolism. Manganese acts as an activator of numerous enzymes in Krebs cycle, particularly in the decarboxilation process.

Glutamine synthesis is also manganese metalloenzyme what confirms the role of manganese in antioxidative system. Manganese influences on skeletal growth, synthesis of nucleic acids, proteins, hemoglobin, lipid and carbohydrates metabolism, etc.

According to the results of the studies, there is a common increased serum concentration of manganese in patients with hepatic encephalopathy. There are opinions that toxicity of manganese contributes to occurrence of hepatic encephalopathy. Prevention of manganese accumulation and decrease in serum manganese concentration could have beneficial effect on mental status of patients with liver cirrhosis (Hauser et al., 1996).

Several studies have showed accumulation of manganese in basal ganglia in patients with liver cirrhosis. Extrapyramidal symptoms could be explained as a result of copper toxicity on dopaminergic function of basal ganglia (Spahr et al., 1996; Rose et al., 1999).

2.2.4. Magnesium

Magnesium is the fourth frequent cation in human body. It occurs in soft tissues and bones. Only 1-5% of magnesium is situated extracellularly. The main part of magnesium originates from ingested food. About 1/3 of ingested magnesium will be absorbed. Mostly, magnesium will be excreted through urinary system. Around 30% is bounded to serum proteins, 15% is in complexes and around 50% is available in ionized form. Magnesium bounded to serum proteins is mainly (75%) bounded to albumins, α-1 and α-2 globulins. Parathyroid hormone is important regulator of magnesium concentration acting through regulation of renal tubular reabsorption.

Magnesium is important in protein synthesis, activation of enzymes, oxidative phosphorilation, etc.

Magnesium also has an important role on the level of neuromuscular connection where slows down neuromuscular impulse inhibiting acetylcholine. That is the main reason why disturbance in magnesium equilibrium causes neuromuscular symptoms.

Magnesium deficiency is usually caused by kidney disease, chronic alcoholism, excessive diuresis, malabsorption, sever diarrhea, etc. Magnesium deficiency causes increased muscular excitability due to the increased acetylcholine activity, with consequent muscular tremor. Mental disorders include confusion and hallucinations. Magnesium influences on heart conductive system and magnesium deficiency can cause arrhythmia. Magnesium intoxication usually caused by acute or chronic renal insufficiency, cause hyporeflexity, cardiac arrhythmia, respiratory depression and coma.

According to the results of published studies, there is a decreased serum concentration of magnesium in patients with liver cirrhosis (Rocchi et al., 1994). Study of Stergiou has showed that spironolactone decreases magnesium excretion by decreasing furosemide-induced renal excretion of potassium and magnesium (Stergiou et al., 1993).

Since zinc, copper, manganese and magnesium have the possible role in the pathogenesis of liver cirrhosis and cirrhotic complications**,** the aim of our study was to investigate the serum concentrations of mentioned trace elements in patients with liver cirrhosis and compared them with concentrations in controls.

3. Material and Methods

3.1. Subjects

The study included 105 patients with diagnosed liver cirrhosis of ethylic etiology who were hospitalized from 2000 to 2005 in the Division of Gastroenterology at Dubrava University Hospital, with median age 55 years. Seventy eight (74%) of them were male and twenty seven (26%) were female. According to the Child-Pugh classification patients with liver cirrhosis were divided in Child-Pugh A, B and C group. There were 35 subjects in every Child-Pugh group.

Inclusion criteria were alcoholic liver cirrhosis (diagnosed by anamnestic data of alcohol consumption, laboratory and pathohistological findings, negative markers of viral hepatitis and normal values of ceruloplasmine), ability to sign the Informed consent and age 18 to 70.

Exclusion criteria were vegetarianism, Wilson's disease, malign disease, acute liver failure, impaired renal function (creatinine clearance <60 ml/min), multiorganic failure and inability to sign the Informed consent.

The control group consisted of 50 healthy subjects (median age 52 years) who were performed laboratory analysis as part of systematic medical examinations. There were 35 (70%) males and 15 (30%) females.

The Informed consent was obtained from all study subjects. The study protocol was approved by the Ethics Committee of Dubrava University Hospital. The protocol was carried out in accordance with the ethics guidelines of the Helsinki Declaration.

3.2. Methods

Blood samples were collected without anticoagulans and serum was stored in a freezer on -20°C until processing. In processing 1 ml of serum was taken, 1.5 ml of concentrated nitric acid and 0.5 ml 30% H_2O_2 were added on account of the digestion. After the digestion the sample was cooling for 20 minutes. The solution was transferred into a 10 ml container and was supplemented with ultra clean water. The concentrations of trace elements were determined by means of plasma sequential spectrophotometer TraceScan (Thermo Jarrell Ash, USA). Data were presented with median and 5-95 percentile range and compared using Wilcoxon and Kruskal-Wallis non-parametric tests. Statistics was done using MedCalc software (MedCalc Software, Mariakerke, Belgium). Only $p<0.05$ was considered significant.

4. Results

The serum concentrations of zinc, copper, manganese and magnesium in patients with liver cirrhosis and controls are presented in Table 1. The serum concentration of zinc was significantly lower in patients with liver cirrhosis in comparison to the controls (0.82 μmol/L vs. 11.22 μmol/L, $p<0.001$). The serum concentration of copper was significantly higher in patients with liver cirrhosis in comparison to the controls (21.56 μmol/L vs. 13.09 μmol/L, $p<0.001$) as well as manganese concentration (2.50 μmol/L vs. 0.02 μmol/L, $p<0.001$). The concentration of magnesium was not significantly different between patients with liver cirrhosis and controls (Table 1, $p=0.132$). There were no differences in the concentrations of zinc, copper, manganese and magnesium between male and female patients with liver cirrhosis (Table 2).

Table 1. Serum concentrations of zinc, copper, manganese and magnesium in patients with liver cirrhosis and controls

Trace elements	Subjects (N=105) median and 5 - 95 percentiles	Controls (N=50) median and 5 - 95 percentiles	Statistics	
			z	p
Zinc (μmol/L)	0.82 (0.24–1.74)	11.22 (9.23–15.10)	10.05	<0.001
Copper (μmol/L)	21.56 (11.17–30.60)	13.09 (11.17–19.95)	-7,66	<0.001
Manganese (μmol/L)	2.50 (0.01–29.65)	0.02 (0.01–0.40)	-8,21	.001
Magnesium (mmol/L)	0.94 (0.63–1.36)	0.88 (0.56–1.12)	-1.51	.32

Table 2. Serum concentrations of zinc, copper, manganese and magnesium in male and female patients with liver cirrhosis

Trace elements	Male (N=78) median and 5 - 95 percentiles	Female (N=27) median and 5 - 95 percentiles	Statistics	
			z	p
Zinc (μmol/L)	0.84 (0.25–1.70)	0.74 (0.20–1.99)	-0.32	0.750
Copper (μmol/L)	21.18 (9.86–30.07)	23.56 (15.49–32.12)	1.58	0.113
Manganese (μmol/L)	2.10 (0.01–31.20)	3.70 (0.08–29.55)	1.45	0.146
Magnesium (mmol/L)	0.96 (0.58–1.40)	0.88 (0.71–1.36)	-1.05	0.293

Table 3. Serum concentrations of trace elements in Child-Pugh groups

Trace elements	Child-Pugh A (N=35) median and 5 - 95 percentiles	Child-Pugh B (N=35) median and 5 - 95 percentiles	Child-Pugh C (N=35) median and 5 - 95 percentiles	Statistics H	Statistics p
Zinc(μmol/L)	1.06 (0.38–1.49)	0.78 (0.26–1.94)	0.54 (0.14–1.45)	19.24	0.053
Copper (μmol/L)	19.98 (13.75–29.84)	22.30 (10.51–31.65)	23.20 (9.75–29.76)	1.00	0.608
Manganese (μmol/L)	2.00 (0.12–9.42)	2.10 (0.01–27.62)	6.30 (0.01–35.75)	9.21	0.036
Magnesium (mmol/L)	0.93 (0.65–1.18)	0.96 (0.65–1.38)	0.88 (0.40–1.53)	5.34	0.084

The data in Table 3 show that the serum levels of manganese were significantly different between Child-Pugh groups (H=9.21, p=0.036). An additional analysis showed that the serum levels of manganese were significantly higher in patients with Child-Pugh C liver cirrhosis (6.30 μmol/L) in comparison to patients with Child-Pugh A (2.00 μmol/L , z=-3.09, p=0.002) and B liver cirrhosis (2.10 μmol/L, z=-2.06, p=0.039). The concentrations of zinc, copper, and magnesium did not differ significantly between Child-Pugh groups (Table 3).

The serum concentrations of zinc, copper, manganese and magnesium in cirrhotic patients with and without hepatic encephalopathy are represented in Table 4. The concentration of zinc was significantly lower in patients with hepatic encephalopathy in comparison to cirrhotic patients without encephalopathy (0.54 μmol/L vs. 0.96 μmol/L, p=0.002). There were no differences in serum concentrations of other trace elements between patients with or without encephalopathy. The serum concentrations of zinc, copper, manganese and magnesium in cirrhotic patients with and without ascites are represented in Table 5. Only manganese concentration was significantly different between patients with and without ascites. Namely, serum manganese concentration was higher in cirrhotic patients with ascites in comparison to the cirrhotic patients without ascites (4.10 μmol/L vs. 1.80 μmol/L, p<0.001).

Table 4. Serum concentrations of trace elements in cirrhotic patients with and without hepatic encephalopathy

Trace elements	Without encephalopathy (N=83) median and 5 - 95 percentiles	With encephalopathy (N=22) median and 5 - 95 percentiles	Statistics z	Statistics p
Zinc (μmol/L)	0.96 (0.25–1.77)	0.54 (0.19–1.11)	-3.07	0.002
Copper (μmol/L)	21.56 (13.07–31.43)	21.31 (9.85–29.31)	-1.21	0.227
Manganese (μmol/L)	2.20 (0.01–31.38)	4.90 (0.01–26.94)	0.66	0.506
Magnesium (mmol/L)	0.95 (0.63–1.36)	0.90 (0.53–1.37)	-1.72	0.086

Table 5. Serum concentrations of trace elements in cirrhotic patients with and without ascites

Trace elements	Without ascites (N=45) median and 5 - 95 percentiles	With ascites (N=60) median and 5 - 95 percentiles	Statistics	
			z	p
Zinc (μmol/L)	0.97 (0.36–1.57)	0.69 (0.18–1.78)	1.77	0.077
Copper (μmol/L)	20.25 (11.84–30.47)	22.42 (10.74–30.82)	-0.28	0.778
Manganese (μmol/L)	1.80 (0.01–11.20)	4.10 (0.01–31.80)	-3.43	< 0.001
Magnesium (mmol/L)	0.92 (0.64–1.13)	0.94 (0.54–1.46)	-0.58	0.564

5. Discussion

Mechanisms linked on ethanol metabolism, especially oxidative stress, redox potentials and acetaldehyde, participate in the emergence of liver damage. Trace elements play an important role in oxidative stress and redox potentials. A possible role of zinc, copper, manganese and magnesium in pathogenesis of liver cirrhosis and its complications is still subject of researches.

In our research the serum levels of zinc were significantly lower in patients with liver cirrhosis in comparison to controls (Table 1, median 0.82 μmol/L in patient with liver cirrhosis and 11.22 μmol/L in controls, $p<0.001$). The results confirm Kugelmans' research (Kugelmas et al., 2000), which explained low zinc levels with low ingestion due to protein reluctance, increased loss in gastroenterological system due to diarrhea or intestinal malabsorption and increased urinary losses. The assumption is also based on the research of McClain (McClain et al., 1991) and Extremera (Extremera et al., 1990). Protein deficiency occurs frequently due to the poor dietary intake. Our results confirm findings of decreased serum concentrations of zinc in patients with liver cirrhosis. Possible explanations for the decreased zinc levels in cirrhotic patients are mentioned above.

In Celik's research (Celik et al., 2002) the decrease in both serum and ascites zinc content was found in patients with liver cirrhosis. The interaction between zinc and copper in their intestinal absorption and their competition for binding sites on the carrier proteins and cellular uptake may be regulators of their homeostasis. Maybe this can explain inverse concentrations of zinc and copper**.** Zinc binds on albumin, transferrin and metalloproteins in the cell, so relative concentrations of these proteins might regulate the serum concentration of zinc (Celik et al., 2002; Mertz, 1981).

The serum copper content was found significantly increased in patients with liver cirrhosis in comparison to the control group (Table 1, median 21.56 μmol/L in patient with liver cirrhosis and 13.09 μmol/L in controls, $p<0.001$). It could be explained with copper's role in the redox process. Redox cycling between Cu^{2+} and Cu^{1+} can catalyze the production of toxic hydroxyl radicals (Askwith et al., 1998; Harrison et al., 2000). It is a well known fact that redox processes and oxidative stress play an important role in the pathogenesis of liver cirrhosis.

Serum concentrations of manganese were significantly higher in cirrhotic patients in comparison to the controls (Table 1, median 2.50 μmol/L in cirrhotic patients and 0.02 μmol/L in controls, $p<0.001$). Higher serum levels of manganese in Krieger's research

(Krieger et al., 1995) as well as in research of Layrargues and co. (Layrargues et al., 1998) were also found in cirrhotic patients.

Moscarella did not find any significant difference in the concentrations of manganese between the cirrhotic patients and the controls (Moscarella et al., 1994). After all, it seems that serum levels of manganese are higher in patients with liver cirrhosis than in healthy people. Manganese is secreted in bile so the concentration of manganese increases in cholestatic liver disease, which could be one of the possible explanations why manganese accumulation is common in liver cirrhosis (Krieger et al., 1995).

It has been suggested that a possible mechanism responsible for manganese accumulation in the pallidum of patients include a decrease in biliary excretion and increased systemic availability due to the portosystemic shunting.

Intrahepatic shunting or portosystemic shunting also have an additional effect on manganese accumulation. In the study of Rose and co. (Rose et al., 1999) pallidal manganese concentrations were the highest in shunted rats, which confirms that shunting is a major determinant of manganese accumulation in the brain. Manganese accumulation in the brain was confirmed by several clinical studies (Krieger et al., 1995; Rose et al., 1999; Hauser et al., 1996, Spahr et al., 1996).

The difference between serum concentrations of magnesium in cirrhotic patients and controls was not significant (Table 1, median 0.94 mmol/L in cirrhotic patients and 0.88 mmol/L in controls, p= 0.132). Results are opposite to Kosch's research (Kosch et al., 2000). In that research serum levels of magnesium were lower in patients with liver cirrhosis in comparison to patients with liver steatosis and controls. In addition, the research of Rocchi (Rocchi et al., 1994) and Suzuki (Suzuki et al., 1996) confirmed the same. Our research did not confirm lower concentrations of magnesium in patients with liver cirrhosis. That partially could be explained with influence of spironolactone on magnesium levels. Namely, in Stergiou's research (Stergiou et al., 1993) spironolactone in health subjects decreased urine excretion of magnesium and in cirrhotic patients antagonized magnesiuric effect of furosemide. Our patients with liver cirrhosis mostly have spironolactone in their standard therapy. However, there were no differences in serum concentration of magnesium between patients who were taking spironolactone and those who were not taking spironolactone.

There was a slight decrease in serum zinc concentrations in patients with more severe clinical state of liver cirrhosis according to Child-Pugh classification but these differences in our research were not significant.

As zinc is bound to albumin in the serum, it has been thought that the serum zinc concentration would decrease with advancing grades of hepatic fibrosis (Hatano et al., 2000). Yoshida found that patients with decompensated liver cirrhosis have lower levels of zinc than patients with compensated cirrhosis (Yoshida et al., 2001). However, in Hatano's research (Hatano et al., 2000) serum zinc levels did not differ significantly between grades of hepatic fibrosis.

Copper levels in our research were similar in all three Child-Pugh groups (Table 3), as well as in Hatano's research.

Serum levels of manganese were higher in patients with Child-Pugh C liver cirrhosis in comparison to those in Child-Pugh A and B cirrhosis. Our results are contrary to Spahr's research (Spahr et al., 1996) who found similar concentrations of manganese in all three Child-Pugh groups. It seems that manganese concentrations are higher in patients with severe liver cirrhosis possible due to the advanced intrahepatic and portosystemic shunting.

In our study magnesium levels were similar in all Child-Pugh groups. Moscarella's research (Moscarella et al., 1994) also confirms similar levels in compensated and decompensated liver cirrhosis. However, Wang found that magnesium deficiency occurs more frequently in severe liver disease (Wang et al., 2004). Significantly lower zinc levels were found in cirrhotic patients with hepatic encephalopathy

(Table 4, median 0.54 μmol/L in patients with encephalopathy and 0.96 μmol/L without encephalopathy, p= 0.002), which was confirmed in other studies (Grungreiff et al., 2000; Riggio et al. 1992). There are some findings that zinc supplementation can cause increased releasing of glutamine from skeletal muscle and also activate glutamine synthetasis, which can decrease the level of ammonia and improve hepatic encephalopathy (Grungreiff et al., 2000). That can be explained with the fact that zinc supplementation increases the hepatic activity of ornithine transcarbamoylase, key enzyme of the urea cycle, which consecutively increases urea formation and decreases ammonia levels (Riggio et al., 1992). The rationale for use of zinc is also its ability to induce intestinal and hepatic metallothionein synthesis. Zinc decreases copper absorption by increasing the formation of Cu-metallothionein in intestinal epithelial cells (Friedman, 2004). However, Riggio found that short-term zinc supplementation has no influence on hepatic encephalopathy (Riggio et al., 1991).

Considering all, zinc supplementation could have a positive influence on hepatic encephalopathy but before the implementation of this result in the treatment, further researches are necessary.

The levels of manganese were not significantly different between patients with liver cirrhosis and hepatic encephalopathy and patients without encephalopathy (Table 4, p=0.506), which is opposite to the researches of Hauser (Hauser et al., 1996) and Krieger (Krieger et al., 1995). They found increased concentrations of manganese and suggested a beneficial effect of prevention of accumulation or decreasing manganese concentration in patients with liver cirrhosis. Rose and Layrargues found increased concentrations of manganese in basal ganglia of cirrhotic patients in comparison to the controls (Rose et al., 1999; Layrargues et al., 1998).

Manganese concentrations were significantly higher in cirrhotic patients with ascites in comparison to those without ascites (Table 5, median 4.10 μmol/L in patients with ascites and 1.80 μmol/L in patients without ascites, p<0.001). The levels of zinc, copper and magnesium were within reference range. Our results are contrary to the research of Pasqualetti and co. who found significantly lower magnesium concentrations in patients with ascites (Pasqualetti et al., 1987). Therefore, it is necessary to research the possible role of manganese in emergence of ascites in patients with liver cirrhosis.

Finally, decreased serum concentrations of zinc and increased levels of manganese in patients with liver cirrhosis could have an important role in the pathogenesis of liver cirrhosis and its complications, especially in hepatic encephalopathy. The supplementation of zinc could improve hepatic encephalopathy. The decrease in manganese levels could also have a beneficial effect on the neurological status in patients with liver cirrhosis and hepatic encephalopathy. Increased concentrations of manganese in cirrhotic patients with ascites inspire further researches about a possible role of manganese in the pathogenesis of ascites in patients with liver cirrhosis. Maybe, decreasing of manganese levels might also have beneficial effect on prevention or volume of ascites.

6. Conclusion

Considering all that, the correction of serum trace elements concentrations would have a beneficial effect on some complications of liver cirrhosis and maybe on progression of the disease, so it would be recommendable to provide laboratory analysis of trace elements in patients with liver cirrhosis as a routine.

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Chapter Sources

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