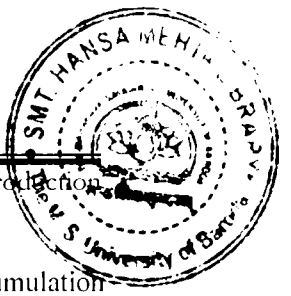
CHAPTER 1

INTRODUCTION

Introduction



1.1 INTRODUCTION

Hepatic fibrosis is a reversible wound healing response characterized by accumulation of extracellular matrix (ECM) or "scar" tissues that follows chronic but not self-limited liver disease. The ECM components in fibrotic liver are similar regardless of the underlying cause. Hepatic fibrosis has evolved in the past 20 years from a pure laboratory discipline to an area of great bedside relevance to practicing hepatologists. This evolution reflects growing awareness not only of the molecular underpinnings of fibrosis, but also of its natural history and methods of detection in chronic liver disease. These advances have culminated in clear evidence that cirrhosis can be reversible, and in realistic expectations that effective antifibrotic therapy will significantly alter the management and prognosis of patients with liver disease.

In view of this remarkable progress, clinicians must now view liver fibrosis in a new light as a clinical problem in its own right amenable to specific diagnostic tests and therapies that are independent of the etiology. In that spirit, it is necessary to integrate current knowledge about the nature and prognosis of fibrosis in different forms of chronic liver disease with recent advances in elucidating its pathophysiology. These advances form the basis for rational treatment of hepatic fibrosis (Friedman, 2001; Gressner et at., 2002; Mann and Smart, 2002).

Cirrhosis can be defined as the end stage consequence of fibrosis of the hepatic parenchyma resulting in nodule formation and altered hepatic function. A notable omission from this contemporary definition is that cirrhosis is irreversible, since ample evidence now demonstrates that reversal of cirrhosis is often possible. Fibrosis and cirrhosis represent the consequences of a sustained wound healing response to chronic liver injury from a variety of causes including viral, autoimmune, drug induced, cholestatic and metabolic diseases. The clinical manifestations of cirrhosis vary widely, from no symptoms at all, to liver failure, and are determined by both the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis. Up to 40% of patients with cirrhosis are asymptomatic and may remain so for more than a decade, but progressive deterioration is inevitable once complications develop including ascites, variceal hemorrhage or encephalopathy. In such patients there is 50% 5-year mortality, with approximately 70% of these deaths directly attributable to liver disease (Fattovich et al., 1997). In asymptomatic individuals, cirrhosis may be first suggested during routine examination or diagnosed at autopsy.

although biopsy is still required to establish the diagnosis antemortem. The overall prevalence of cirrhosis in the United States is estimated at 360 per 100,000 population, or 900,000 total patients, the large majority of whom have chronic viral hepatitis or alcoholic liver disease.

Cirrhosis affects hundreds of millions of patients worldwide. In the US, it is the most common non-neoplastic cause of death among hepatobiliary and digestive diseases, accounting for approximately 30,000 deaths per year. In addition 10,000 deaths occur due to liver cancer, the majority of which arise in cirrhotic livers, with the mortality rate steadily rising (El-Serag and Mason, 2000; Befeler and Di Bisceglie, 2002).

The molecular composition of the scar tissue in cirrhosis is similar regardless of etiology and consists of the extracellular matrix constituents, collagen types I and III (i.e. 'fibrillar' collagens), sulfated proteoglycans, and glycoproteins (Schuppan et al., 2001). These scar constituents accumulate from a net increase in their deposition in liver and not simply collapse of existing stroma. Although the cirrhotic bands surrounding nodules are the most easily seen form of scarring, it is actually the early deposition of matrix molecules in the subendothelial space of Disse so called 'capillarization' of the sinusoid – that more directly correlates with diminished liver function.

In normal liver, hepatic stellate cells (HSCs) are nonparenchymal, quiescent cells whose main functions are to store vitamin A and probably to maintain the normal basement membrane-type matrix. However, numerous in vivo and in vitro studies indicate that in response to liver injury, HSCs undergo an "activation" process in which they lose vitamin A, become highly proliferative, and synthesize "fibrotic" matrix rich in type I collagen. This understanding has helped to identify underlying mechanisms, and will likely lead to new therapies for fibrotic diseases of many organs, including liver (Van Waes and Lieber, 1977).

Stellate cell "activation" is a key event in liver injury, and refers to the transition from a quiescent vitamin A-rich cell to a highly fibrogenic cell. Cells with features of both quiescent and activated states are often called "transitional cells." Proliferation of stellate cells occurs in regions of greatest injury, and is typically preceded by an influx of inflammatory cells and associated with subsequent ECM accumulation.

The rate of progression of fibrosis in an individual patient with chronic liver disease cannot be predicted with certainty. Accurate assessment of the extent of fibrosis is

essential in guiding management and predicting prognosis in patients with chronic liver injury. Histologic assessment of a liver biopsy specimen remains the gold standard for quantifying fibrosis, with increasing interest in the use of noninvasive markers to allow more frequent sampling and avoid the risks of percutaneous biopsy.

In reason of their role in hepatic fibrogenesis and of the several pro-fibrogenic mechanisms identified, HSC represents a major focus of anti-fibrotic research. Indeed, the well-described pathway of HSC activation, subsequent fibrogenesis, with the potential for apoptosis and reversibility, provides a logical framework to define sites of intervention. Consequently the search for effective antifibrogenic strategies is based on the knowledge gained in the area of HSC biology, including the biology of the factors (growth factors, cytokines, etc.) conditioning their profibrogenic attitude [10,80]. Although this major progress in understanding is fairly recent and, hence, still difficult to be translated into practical strategies, more and more articles published in top specialized journals report on the potent anti-fibrogenic action of old and new drugs, including single agents or mixtures derived from traditional herbal medicine. As any treatment aimed at curing the chronic disease, any potential anti-fibrotic agent should fulfill several criteria: (1) the treatment should be well tolerated, in view of a long duration and of multiple administrations; and (2) the active moiety of the drug should reach a sufficient concentration within the liver, possibly with some cell specific targeting (e.g. HSC, and other ECM-producing cells). The liver is an advantageous destination for orally administered drugs and those with efficient first pass metabolism will have inherent liver targeting. However, while this statement is true for a healthy liver, several limitations apply when liver tissue is affected by progressive scarring and initial or advanced derangement of the normal angio-architecture, as often happens when the patient reaches clinical attention.

Obviously, the best anti-fibrogenic treatment would be represented by any strategy able to eliminate the primary cause of parenchymal damage, metabolic overload or excessive oxidative stress. Once this primary requirement is fulfilled, the association with an anti-fibrogenic drug would be relevant for stabilizing the cure and favor optimal remodeling. Since the fibrogenic process is in its essence a compensatory phenomenon aimed at maintaining sufficient tissue continuity and cohesion in the presence of continuous microscopic parenchymal collapse, it would be erroneous to

attempt to cure fibrogenic chronic liver diseases (CLDs) only with anti-fibrogenic drugs once some effective compounds will become available for clinical use.

Along with the elucidation of the cellular and molecular mechanisms responsible for hepatic fibrogenesis, an impressive amount of experimental data proposing the anti-fibrogenic effect of several compounds has been accumulating. In general, all reports suggest that the compound under investigation is able to reduce or abolish the pro-fibrogenic potential of HSC in culture, and/or prevent and even reverse the fibrogenic evolution in animal models. These positive results need, however, to be subjected to some objective criticism before being translated into clinical applications for human CLDs. In vitro studies performed on activated HSC in their myofibroblast like phenotype provide thoughtful insights on the biology of this cell type, on the intracellular mechanisms regulating their pro-fibrogenic role, and on the effects of a drug added to the cell culture.

So far no effective treatment has been established other than removal of primary cause of the disease and liver transplantation for severe fibrosis. Therefore, research is being carried out on therapeutic agents who inhibit activation and proliferation of HSC, reduce ECM production by HSC, neutralize HSC contractile responses or stimulate HSC apoptosis (Wu and Zern, 2000; Lee et al., 2008; Bataller and Brenner, 2001; Adrian et al., 2007).

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors (Green and Wahli, 1994). PPAR forms heterodimers with the retinoid X receptor and binds to specific response elements to induce transcription in response to a variety of endogenous and exogenous ligands, including fatty acids, arachidonic acid metabolites, and synthetic drugs (Forman et al., 1996). Of the PPAR isoforms, PPAR-γ is the most widely studied (Auwerx, 1999). Previous studies indicated that expression of PPAR-γ inhibited PDGF-induced proliferation and migration of vascular smooth muscle cells (Fu et al., 2001). Three recent studies independently demonstrated that the level of PPAR-γ and its trans-activating activity were diminished during HSC activation in vitro, whereas NF-κB and activator protein- 1 (AP-1) activities were increased (Galli et al., 2000; Marra et al., 2000). PPAR-γ ligands inhibited cell proliferation and collagen- α1(I) expression in primary HSC (3–4 days) (Miyahara et al., 2000). The dramatic reduction in the abundance of PPAR-γ results in a significant decline in response to exogenous PPAR-γ ligands in

activated HSC. These findings implied a potential therapeutic value of PPAR-γ ligands in treatment of liver fibrosis if the expression of PPAR-γ can be induced in activated HSC.

Angiotensin II (Ang II) plays a central role in the regulation of systemic blood pressure and fluid homeostasis. The action of Ang II is mediated by mainly two subtypes of receptors, angiotensin II type 1 (AT1) receptors and type 2 (AT2) receptors, which are distributed in many kinds of organs and tissues. Recently, several lines of evidence have suggested that the rennin–angiotensin system (RAS) plays an important role in the pathogenesis of organ fibrosis (Brilla, 2000; Sun et al., 2000). In mesangial cells and other cell types, Ang II has been shown to promote the proliferation and collagen synthesis (Ray et al., 1991; Wolf et al., 1992; Kagami et al., 1994; Weber et al., 1994; Tharaux et al., 2000). Moreover, the expression of transforming growth factor-b (TGF-b), the key cytokine in the development of cardiac and renal fibrosis, is increased by Ang II (Weber, 1997). Blockade of the RAS by angiotensin converting enzyme (ACE) inhibitors or by AT1 antagonists has been shown to improve the progression of organ fibrosis (Ishidoya et al., 1995; Kim et al., 1995; Molteni et al., 2000). In the liver, Ang II is considered to play a role in the regulation of intrahepatic circulation (Schneider et al., 1999). Recently, it has been reported that Ang II induces proliferation and contraction of human HSCs, and TGF-b expression in rat HSCs, which are mainly mediated by AT1 receptors (Bataller et al., 1999; Yoshiji et al., 2001), and that ACE inhibitors or AT1 antagonists attenuate the progression of liver fibrosis in vivo (Ramos et al., 1994; Jonsson et al., 2001; Ohishi et al., 2001; Paizis et al., 2001; Yoshiji et al., 2002). These reports suggest that Ang II and RAS might play an important role in the pathogenesis of liver fibrosis.

Liposomes are vesicles composed of a phospholipid bilayer in which pharmaceutical agents can be contained. Liposomal drug delivery allows controlled release, target specificity, prolonged half life of drugs with its unique membrane properties, and resemblance of the membrane structure to cell membranes makes liposomes non-immunogenic and diversifies intake methods. Liposomes have tremendous potential as a carrier because they are nontoxic, non-immunogenic, and biodegradable and have a high loading capacity for a variety of therapeutic agents and have been investigated for long period of time. Particulate carriers such as liposomes have many attractive features, one of the perceived benefits of liposomes as a drug carrier is based on their

ability to alter favorably the pharmacokinetic profile of the encapsulated species and thus provide selective and prolonged pharmacological effects at the site of administration. However, effectiveness of these conventional liposomes is often limited due to the lack of the target specificity. Liposomes can be surface modified in many ways by conjugating cell specific ligands to target wide variety of cells. In liver, galactosylated (Managi et al., 2005; Hattori et al., 2000; Sliedregt et al., 1999) or asialofetuin (Dasi et al., 2001) coated liposomes target hepatocytes, aconitylated human serum albumin coated (Kamps et al., 1997) liposomes recognize endothelial cells and mannosylated liposomes (Opanasopit et al., 2002; Kawakami et al., 2000) identify kupffer cells as their targets (Adrian et al., 2007).

Mannose 6-phosphate/ insulin like growth factor II (M6P/IGF II) receptor are over expressed on the surface of HSCs during liver fibrosis. Mannose 6-phosphate modified human serum albumin (M6P-HSA) is selective to M6P/IGF II receptor and thus accumulates in activated HSCs of fibrotic liver. M6P-HSA as such has been investigated as a carrier for a number of drugs, including pentoxyfiline, mycophenolic acid, doxorubicine and gliotoxin. M6P-HSA conjugated liposomes can be used as HSCs selective carrier of antifibrotic drugs to improve the efficacy of drugs at the same time to reduce their adverse effects. Liposomes with bioactive lipid dilinoleoylphosphatidylcholine (DLPC) into the membrane as a major constituent act as a bioactive drug carrier which can deliver drugs and simultaneously have beneficial antifibrotic effects (Beljaars et al., 1999; Beljaars et al., 2001; De Bleser et al., 1996; De Bleser et al., 1995; Adrian et al., 2006; Cao et al., 2002).

An attempt was made to develop liposomal formulation by thin film hydration method and optimized for drug: total lipid ratio, Phospholipid: cholesterol ratio and total solid: hydration medium ratio to maximize the percentage drug entrapment (PDE) and to minimize percentage reduction (PR) in PDE after 10 days by 3^3 full factorial design. M6P-HSA was synthesized, characterized and conjugated to optimized liposomal formulations to provide targeting ability. *In vitro* drug diffusion studies were ascertained to identify release kinetics of developed formulations. *In-vivo* pharmacokinetic and pharmacodynamic properties of prepared formulation were evaluated in carbon tetrachloride (CCL_4) induced rat liver fibrosis model for exploitation of the findings of the studies in developing relevant product for effective treatment of liver fibrosis.

1.2 RESEARCH ENVISAGED

The research project focuses on the different aspects of pharmaceutical development and optimization of liposomal formulations of selected drugs [PPAR-γ ligand (Rosiglitazone) and AT1 receptor antagonist (Candesartan)]; surface conjugation with M6P-HSA to provide targeting potential; characterization, evaluation of prepared formulation for *in vitro* drug diffusion studies and for *in vivo* pharmacokinetic and pharmacodynamic properties.

1.3 PROPOSED PLAN OF WORK

I. Literature reviews covering various aspects of liver fibrosis, treatment options for liver fibrosis, liposomes as drug delivery carrier, targeting liposomes to hepatic stellate cells, liposomes surface conjugation technologies, *in vitro* and *in vivo* evaluation techniques and profiles of selected drugs like Rosiglitazone and Candesartan cilexetil.

II. Preparation of liposomes using thin film hydration method using lipids such as dilinoleoyl phosphatidylcholine, hydrogenated soya phosphotidylcholine, distearoyl phosphoethanolamine and cholesterol. Optimization of liposomal formulations for drug: total lipid ration, phospholipid: cholesterol ration and total solid: hydration medium to maximize the percentage drug entrapment (PDE) and to minimize percentage reduction (PR) in PDE after 10 days employing 3^3 full factorial design. Characterization of optimized liposomes with respect to: particle size and size distribution, zeta potential etc.

III. Lyophilization of optimized liposomal formulations using appropriate cryoprotectants and anti adherents to stabilize the formulations.

IV. Preparation and characterization of M6P-HSA.

V. Conjugation of M6P-HSA to optimized liposomes.

VI. *In-vitro* drug diffusion studies.

VII. Stability studies of potential formulations with respect to percentage drug retention, particle size, zeta potential and physical changes like caking and discoloration.

VIII. Comparative evaluation of the developed formulations for *In vivo* pharmacokinetic and pharmacodynamic properties in carbon tetrachloride (CCL_4) induced rat liver fibrosis model.

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