chapter 1

genral introduction
The liver

The liver is localized in the abdominal cavity between the digestive tract and the spleen. Functionally, it is in a strategic position between the gastro-intestinal tract and the general blood circulation. The organ has a double blood supply; most of the total blood influx is provided by the portal vein bringing nutrient-rich blood from the digestive tract while the hepatic artery delivers blood supplemented with oxygen. Within the liver, the blood passes through a network of micro-vessels, called sinusoids, after which it is collected in the hepatic central veins and finally drained by the inferior vena cava.

The liver plays a central role in the metabolism of carbohydrates, proteins and fats, among other substances, and is thereby important for the maintenance of homeostasis in the body. The liver synthesizes most of the plasma proteins, such as albumin and globulins. Another function of the liver is detoxification, namely the biotransformation of xenobiotic compounds, pollutants and drugs into water-soluble compounds which then can be excreted either in bile or in urine. Importantly, the liver also eliminates particulate substances such as bacteria and viruses and different kinds of macromolecules from the blood stream.

At the microscopical level the liver is anatomically organised in functional units called lobules, built up from the three major liver cell types, the parenchymal cells or hepatocytes, the sinusoidal endothelial cells lining the sinusoids and a resident macrophage population, called Kupffer cells. The lobules have a hexagonal shape with the terminal hepatic vein in the centre and the portal triads at the corners of the hexagon. Each portal triad consists of a portal vein, a hepatic artery and a common hepatic bile duct. The parenchymal cells are structured in cords that are symmetrically distributed around the central vein and separated by liver sinusoids in which blood flows from portal triads towards the central vein.

The metabolic functions of the liver are maintained mainly by the parenchymal cells, which represent the major population of cells in the liver. In the narrow space between the endothelial cells and the hepatocytes, called the space of Disse, a fourth population of cells is localised, the hepatic stellate cells. This population of non-parenchymal cells normally stores vitamin A and is involved in the production of extracellular matrix proteins. Other non-parenchymal cells that can be found in the liver are lymphocytes, including natural killer cells also known as pit cells, and biliary epithelial cells that line the bile ducts.

In the healthy liver, extracellular matrix (ECM) constituents account for 0.5 % of the liver weight. In spite of its relatively small volume, the composition of the ECM in the space of Disse is of great importance for the functioning of the liver cells. Hepatocytes, endothelial cells and hepatic stellate cells require a proper ECM composition to express their phenotypes.

The hepatocytes

Hepatocytes represent about 65 % of all liver cells in number and more than 90 % of liver volume and are the main functional unit of this organ. Although they have a polyhedral shape, functionally two major domains can be distinguished with regard to the outer plasma membranes of these cells. The bile canicular (i.e. apical) surface is specialised in transport of bile salts, bilirubin, cholesterol, phospholipids as well as xenobiotics into the bile. The contact of hepatocytes with the blood occurs at the basolateral domain, bordering the space of Disse. Numerous microvilli on this membrane domain allow
for a substantial increase in exchange surface. In addition, the plasma membrane of
the basolateral domain contains many transmembrane transport proteins and also has
endocytotic properties. Thus, this domain is rich in receptors such as the asialoglycopro-
tein receptor, the transferrin receptor and several lipoprotein receptors.
Hepatocytes are crucial for the glucose homeostasis in the body and can store as well as
release glucose. Thus, their cytoplasm contains the storage form of glucose, the glycogen
granules. Beside, hepatocytes harbour specific metabolic pathways such as the urea
cycle, regulating the blood levels of amino acids and ammonia derived from the intestine,
and the specific lipogenic and lipolytic enzyme systems involved in the synthesis and
metabolism of a variety of serum and intestinal lipoproteins. Also the formation of
bilirubin from heme and the excretion of cholesterol and its conversion into bile acids are
specific hepatocytic processes.

Liver sinusoidal cells
Kupffer cells, endothelial cells and hepatic stellate cells are major cells of hepatic sinusoid
(Fig. 1). Each of these cell populations has its own specific characteristics and function-
ality. Although these cells form a minority of the total liver cell population, they are
essential for the proper functioning of the organ.

Liver endothelial cells
Liver endothelial cells (LEC) comprise approximately one fifth of all liver cells and
represent about half of the non-parenchymal cells in number. They form the endothelial
lining of the hepatic sinusoids and as such posses unique features allowing them to
provide a selective barrier between the blood stream on the one hand and the hepatocytes
and hepatic stellate cells on the other. Liver endothelial cells distinguish themselves from
other vascular endothelial cells because they contain pores or fenestrations varying in
diameter from 150–175 nm that are grouped in clusters (1). Furthermore, a basal lamina
underneath the all other endothelial cells in the body is lacking in the sinusoids of the liver.
This architecture of the sinusoidal capillaries allows substances smaller than the diameter
of the fenestrations to freely exchange between the blood and the space of Disse.
LEC also play an important functional role in a variety of liver-associated processes. High
endocytotic activity of these cells enables them to clear the blood from different macro-
molecular waste products. Moreover, LEC are known to secrete cytokines, such as tumor
necrosis factor – α (TNF-α) (2) and interleukin 6 (IL-6) (3) and express adhesion molecules
such as intercellular adhesion molecule – 1 (ICAM-1) and vascular cell adhesion molecule
– 1 (VCAM-1) (4) which are crucial in the interaction with leukocytes and neutrophils. In
addition LEC express several scavenger receptors, which bind and take up negatively
charged proteins and lipoproteins from the blood and are involved in the regulation of
lipoprotein metabolism as well as in antigen presentation.
In the healthy liver, LEC play a role in the turn-over of ECM. They secrete collagen type
IV and laminin (5) and clear hyaluronan (6), (pro)collagen and fibronectin (7), degradation
products of ECM.
Kupffer cells

Kupffer cells account for 15% of the total liver cell population. Together with macrophages of the spleen they are a major part of the so-called mononuclear phagocyte system (MPS). Kupffer cells are predominantly located in the sinusoids, where they are directly exposed to the blood stream while being anchored to the endothelial cells. In addition to their strategic location, Kupffer cells are characterised by their high phagocytic activity and they are responsible for the removal of circulating microorganisms, immune complexes, dead cells and other debris from the blood stream as well as the detoxification of bacterial endotoxins. In addition to performing these phagocytic processes, Kupffer cells take up different substances from the circulation via receptor mediated endocytosis. There are several receptors which facilitate this process, for example: mannose receptors, fucose receptors, Fc receptors, CD14 receptors (7) and scavenger receptors class A I, A II and B I (8).

As part of the innate immune system, KC play a central role in the regulation of inflammatory processes and other immunological reactions in the liver. In response to stimuli, for example by bacterial endotoxin, Kupffer cells produce various cytokines, such as TNF-α, IL-1, IL-6 (9) eicosanoids and reactive oxygen species (ROS), which promote chemotaxis, phagocytosis and ROS production by other inflammatory cells, as well as stimulating different reactions of other liver cells. On the other hand, Kupffer cells are also capable to release factors which have an anti-inflammatory action, like IL-10 (3).

Figure 1. Schematic representation of hepatic sinusoid. Endothelial cells (EC) shape the walls of sinusoids in which Kupffer cells (KC) are localised. In the space of Disse, between endothelial lining and parenchymal cells (PC), hepatic stellate cells (HSC) are situated. Fenestration in the endothelium provide a selective barrier between the blood stream and the space of Disse.
Hepatic stellate cells

Hepatic stellate cells (HSC) represent 5 to 8% of all liver cells in the healthy organ. A characteristic feature of these cells is the expression of two different phenotypes; quiescent in the normal liver, and activated in the diseased. As a consequence of this ability of transformation, hepatic stellate cells also change their functions. Quiescent HSC have a star-like shape and their cytoplasm contains vitamin A droplets, for which they were formerly also known as fat-storing cells (other former names are Ito cells, lipocytes). Storage and controlled release of retinoids is a major function of HSC in the healthy liver. In the digestive tract esterified retinol is incorporated into chylomicrons which enter the blood stream after being secreted into the lymphatic system. After having been depleted from part of their triglyceride-load in the peripheral vasculature, the resulting chylomicron remnants are taken up from the blood by the hepatocytes and from there the retinol is transported to HSC with the help of retinol binding proteins (RBP), where it is stored as retinyl ester. From HSC, retinol bound to RBP can be secreted into the circulation or transported back to the hepatocytes.

The crucial process of ECM turnover in the space of Disse is also controlled by HSC. They secrete limited amounts of ECM proteins such as collagen type III, collagen type IV and laminin (5). Furthermore, HSC express several matrix metalloproteinases (MMP), such as MMP-2, MMP-3, MMP-10, MMP-13 and MMP-14, as well as their inhibitors (tissue inhibitors of matrix metalloproteinase -1 (TIMP-1) and TIMP-2) to control the matrix degradation processes (10).

Because of their anatomical position, it is likely that quiescent HSC also are involved in controlling the blood flow through the hepatic sinusoids. In fact, HSC encircle the sinusoid with their long cellular processes in a cylindrical manner and can produce vasoactive proteins, including substance P, neuropeptide Y and somatostatin (11).

Quiescent HSC are producers of hepatocyte growth factor (HGF) (12), which stimulates hepatocyte proliferation, and vascular endothelial growth factor (VEGF) (13), a stimulus for growth of sinusoidal and vascular endothelial cells. In addition, molecules such as endothelin-1 (14), transforming growth factor-β (TGF-β) (15), neuotrophins and erythropoietin (16) are secreted by HSC in the normal liver. All these mediators tightly control homeostasis within sinusoids and pathological processes within the liver.

Liver fibrosis

Fibrosis in the liver is a consequence of liver damage. Under normal circumstances the underlying process of collagen deposition is instrumental in the healing of wounds. Chronic activation of this healing mechanism may lead however, to liver pathology. This can be due to a variety of causes, including: chronic viral infection by hepatitis B and C, chronic alcoholism and/or exposure to some drugs and toxins, non-alcoholic steatohepatitis (NASH), inherited metabolic diseases such as hemachromatosis, Wilson’s disease and alfa 1- antitrypsin deficiency, and autoimmune diseases like primary biliary cirrhosis and autoimmune hepatitis. As a result of major liver tissue injury, formation of connective tissue through progressive accumulation of extracellular matrix (ECM), mainly consisting of interstitial collagen, is promoted. Liver fibrosis is a dynamic process, in which remodelling of ECM takes place in addition to excessive ECM formation and reduced degradation. Eventually, fibrosis may either proceed towards the fatal end stage.
Mediators of inflammation activate HSC, in a concerted action leading to changes in the ECM composition. In the course of the activation process, HSC transform into myofibroblast-like cells, which are characterised by several specific features. Although it is still not clear whether retinoid loss is required for HSC activation, activated HSC lose their vitamin A droplets. Stimulated by TGF-β, activated HSC also start to produce markedly increased amounts of ECM proteins, mainly of collagen type I and III. The main source of TGF-β in the fibrotic liver are HSC, but liver endothelial cells and Kupffer cells also contribute to the production of this growth factor (15). Importantly, TGF-β is secreted as a biologically inactive protein that is bound to a non-covalently linked latency-associated peptide (LAP), which has to be cleaved off to yield the active protein (23). The proteolytic activation of the LAP-TGF-β complex is through tissue plasminogen activator (tPA) or metalloproteinases. In addition, activation of TGF-β requires other proteins, such as the mannose 6-phosphate/insulin-like growth factor II (M6P/IGF) receptor, which binds the LAP-TGF-β and exposes it for cleavage (24). Up-regulation of the M6P/IGF II receptor was found on activated HSC in fibrotic livers (25;26).

Simultaneously with the accelerated ECM production by HSC, the degradation of the ECM is impaired in the chronically injured liver. During fibrosis, the low density matrix that is normal for the healthy liver is degraded and replaced by an excess of scar tissue. Calcium-dependent enzymes, matrix metalloproteinases (MMP), disrupt both collagen and non-collagenous compounds of ECM (27). There are several categories of MMP, that can be distinguished according to their substrate specificity. In the fibrotic liver healthy sinusoidal ECM is degraded mainly by MMP-2, which is also produced by activated HSC. This process perpetuates the deposition of collagen type I and III. In addition, MMP that are able to degrade interstitial collagen, like MMP-1 or MMP-13, are inhibited by tissue inhibitors of matrix metalloproteinases (TIMP). The level of TIMP during fibrosis increases markedly and HSC are found to be a major source of TIMP.

Apart from the activation, HSC also proliferate and their number is significantly increased in the fibrotic liver. PDGF was found to be a particularly potent stimulus for HSC proliferation (28). Kupffer cells, endothelial cells and the HSC themselves are major producers of this cytokine (21). Consequently elevated levels of PDGF and its receptor are observed in the fibrotic livers (29). ET-1 and IGF were identified as additional mitogens of HSC (30;31). Activated HSC migrate towards regions of injury and accumulate around damaged tissue. The same cytokines that are mitogens for HSC play the role of chemoattractants for these cells. However, only activated HSC show a chemotactic response to these substances. Activated HSC also secrete chemoattractants such as monocyte chemotactic protein (MCP-1) (32), which further attract activated HSC and also promote recruitment of monocytes and leukocytes. Other cytokines released by HSC that can amplify inflammation include colony stimulating factor and cytokine-induced neutrophil chemoattractant (CINC)/IL-8 (33). Additionally, activated HSC express adhesion molecules, such as ICAM-1 and VCAM-1 (34), suggesting an active involvement of HSC in the recruitment of inflammatory cells during tissue injury.

The occurrence of portal hypertension is a common clinical manifestation of fibrosis in the liver. There is a growing body of evidence that activated HSC contribute to the
increased portal resistance (35;36). After the transformation from the quiescent to the activated phenotype, HSC express the cytoskeleton protein α-smooth muscle actin (α-SMA) abundantly, equipping the cells with a contractile apparatus. HSC thus may constrict individual sinusoids and contract the cirrhotic liver as a whole too. A balance between two compounds, ET-1 and NO, regulates the contractile activities of HSC (37). The first is the key contractile stimulus of HSC, the latter is an antagonist of ET-1 produced by HSC, Kupffer cells and liver endothelial cells. In the fibrotic liver, a decrease in the production of NO and a simultaneous increase in ET-1 release is observed.

Activation of HSC and transformation to myofibroblasts are considered to be the fibrogenetic phase of the fibrotic process in the chronically injured liver. Key cytokines, such as TGF-β, PDGF, ET-1 and MCP-1 are produced by HSC itself and therefore regulate activation of these cells in an autocrine manner. Consequently, fibrogenesis can proceed independently from the other hepatic cells through this mechanism. During disease progression, closure of endothelial fenestrations (38) and replacement of parenchymal tissue by scar-like ECM lead to deterioration of hepatocellular functions and ultimately to the clinical manifestation of the disease.

**Dual role of Kupffer cells in progression of liver fibrosis**

A growing number of reports show that apart from a profibrotic role of Kupffer cells in liver fibrosis, these cells can also exert significant antifibrotic actions. Kupffer cells do influence ECM degradation in the fibrotic liver. When treated with gadolinium chloride, they produce interstitial collagenase MMP-13, which reduces ECM deposition in experimental fibrosis (39). In addition, activated Kupffer cells can effectively kill HSC by a
caspase 9-dependent mechanism and possibly through the involvement of TNF-Related Apoptosis-Inducing Ligand (TRAIL) (40). Kupffer cell depletion during liver repair after cholestatic injury impairs collagen metabolism, inhibits the resolution of fibrosis and promotes infiltration of inflammatory cells (41). Moreover, shortly after bile duct obstruction, cytokines secreted by the Kupffer cells, including IL-6, play a critical role in abrogating cholestatic liver injury, as was demonstrated in IL-6 deficient mice (42). Kupffer cells are also a source of IL-10, another important cytokine known to have anti-inflammatory and antifibrotic effects, especially in the early stages of fibrosis and during acute liver injury. While decreasing collagen production, IL-10 up-regulates collagenase secretion, resulting in a reduced deposition of collagen. In line with this idea, specific targeting of the anti-inflammatory drug dexamethasone, which reduces nitric oxide, TNF-α, TGF-β and ROS production, to Kupffer cells in bile duct ligated rats using mannosylated human serum albumin, caused an increased collagen I and III deposition as well as enhanced TIMP-1 mRNA expression (43). These reports clearly indicate that Kupffer cells play a crucial role in the suppression of inflammation and fibrosis apart from their well known pro-inflammatory activities.

Resolution of fibrosis

The fate of activated HSC in the fibrotic liver is not fully understood yet, but during restoration of normal liver tissue their number decreases. This indicates that activated HSC either reverse to the quiescent phenotype or that they undergo apoptosis. Currently, there is no evidence from in vivo experiments which supports the first possibility, although culture-activated HSC are able to revert to quiescent cells. In contrast, apoptosis of activated HSC is well documented both in vitro and in vivo, and correlates with the regression of experimental fibrosis (44). Activated HSC express cell-death receptors like Fas (45) and nerve growth factor receptor (NGFR) (46). Stimulation of these receptors causes apoptosis. Factors including IGF-I and TNF-α that are abundantly secreted during chronic liver injury promote survival of activated HSC. The ECM degradation process also seems to influence the balance between survival and apoptosis of activated HSC in the fibrotic liver. TIMP-1 blocks apoptosis of activated HSC even when they are stimulated with pro-apoptotic factors such as serum deprivation, cycloheximide and nerve growth factor (47). The anti-apoptotic effects of TIMP-1 most likely involve inhibition of MMP activity while the level of activity of MMP-2 may in turn depend on HSC apoptosis.

Antifibrotic strategies

For long (advanced) liver fibrosis has been thought to be an irreversible disease. However, recent clinical and experimental data indicate that advanced fibrosis and even cirrhosis can be reversed (48). Importantly, regression of either fibrosis or cirrhosis after the effective elimination of the primary cause of the disease was observed in the entire spectrum of chronic liver diseases, including iron overload, NASH, secondary biliary cirrhosis, chronic hepatitis C and B and alcohol-induced liver injury. However in most cases treatment of the underlying disease alone is not sufficient to cure hepatic fibrosis. Also, significant regression of fibrosis may take years, depending on the primary cause of the disease and its severity. Therefore, the development of antifibrotic therapies that would stop progression of fibrosis and/or accelerate regression of the disease would improve
current treatment protocols significantly. Proper antifibrotic drugs would have to fulfil the requirements of being well tolerated, even when administrated over years, and having good targeting properties to the liver, as well as having minimal adverse effects on other tissues. Depending on the underlying cause of the disease, the predominant mechanism leading to and promoting fibrosis is different. For example, in addition to fibrogenesis, chronic inflammation occurs in hepatitis B and C infections. In alcohol-induced injury and NASH, oxidative stress together with inflammation predominantly stimulate fibrosis. The dominant features in biliary cirrhosis are derangement of the normal epithelial mesenchymal interactions accompanied by chronic inflammation. Therefore, an ideal antifibrotic treatment should be a combination of drugs that exert synergistic effects on different disease related processes in the fibrotic liver.

Antifibrotic drugs
Currently, a large variety of drugs is being investigated for antifibrotic effects (Table 1). These compounds can be classified according to their therapeutic effects, including reduction of inflammation, antioxidant properties or promotion of ECM degradation. Many of the compounds aim at HSC to inhibit their activation and proliferation, reduce ECM production by HSC, neutralize HSC contractile responses or stimulate HSC apoptosis.

Gene therapy for liver fibrosis
Several reports show that experimental liver fibrosis can be attenuated using tools for gene therapy (Table 2). Plasmid DNA containing therapeutic genes or antisense oligonucleotides were delivered in the fibrotic liver with the help of adenoviruses. Recently, recombinant adeno-associated viruses (rAAV) were applied as well, which, in contrast to adenoviral vectors, provide stable transgene expression. As a non-viral method for gene transfer into the fibrotic liver, electroporation was applied. Enhanced degradation of ECM achieved by overexpression of MMP or modulation of the inflammatory process with gene transfer of genes encoding interferon-α or IL-10 showed reduction of liver fibrosis in experimental models. Antisense oligonucleotides proved to be successful in blocking the signalling pathways of TGF-β and production of this cytokine as well as in inhibiting of PDGF production. Because of their natural tropism, adenoviruses predominantly infect hepatocytes in the liver. To achieve specific expression of genes carried by the adenoviral vectors into HSC, virus re-targeting strategies, selective gene expression methods or other vectors need to be developed. Recently, adenovirus-mediated selective overexpression of the transcriptional repressor of the TGF-β/Smad signalling pathway was demonstrated in the collagen-producing cells of the fibrotic liver, using a tissue specific enhancer of the mouse α2(I) collagen gene, COL1A2 (49). High association of rAAV was found in the fibrotic areas of the liver which were characterised by an up-regulated expression of fibroblast growth receptor-1α (FGFR-1α), a rAAV coreceptor (50). In addition, it was confirmed that activated HSC express FGFR-1α, and the expression of the transgene carried by rAAV was high in cells isolated from fibrotic livers. Although most of the studies performed with adenoviral vectors report successful attenuation of liver fibrosis, special safety precautions have to be taken for application in humans. In cirrhotic rats, adenoviral vectors also readily accumulate in lungs due to the presence of pulmonary intravascular macrophages (PIM) during the progression of fibrosis (51). As a result, adenoviruses in the lungs of cirrhotic rats cause strong immune
Table 1. Examples of antifibrotic compounds tested for treatment of liver fibrosis.

<table>
<thead>
<tr>
<th>Main mechanism of action</th>
<th>Name of compound</th>
<th>Effects in experimental fibrosis</th>
<th>Effects in clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>colchicine</td>
<td>limited data (77)</td>
<td>inconsistent results (78-80)</td>
</tr>
<tr>
<td></td>
<td>pentoxifylline</td>
<td>limited data (81,82)</td>
<td>limited negative data (83)</td>
</tr>
<tr>
<td></td>
<td>halofuginone</td>
<td>inconsistent/conflicting results (84-86)</td>
<td>not tested</td>
</tr>
<tr>
<td>Inhibition of HSC activation</td>
<td>prolyl 4-hydroxylase inhibitor: HOE 077 and S4682</td>
<td>positive data (87,88)</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>serine protease inhibitor: camostat mesilate</td>
<td>positive data (89)</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>dilaenoylethanolphosphatidylcholine</td>
<td>positive data (90,91)</td>
<td>not proven in patients with alcohol-induced fibrosis (92)</td>
</tr>
<tr>
<td></td>
<td>PPARγ antagonist: rosiglitazone</td>
<td>positive data (93)</td>
<td>positive effects in NASH patients (94)</td>
</tr>
<tr>
<td></td>
<td>angiotensin II receptor inhibitor: losartan</td>
<td>positive data (95-97)</td>
<td>positive data (98;99)</td>
</tr>
<tr>
<td></td>
<td>cariporide</td>
<td>positive data (100)</td>
<td>not tested</td>
</tr>
<tr>
<td>Induction of HSC apoptosis</td>
<td>gliotoxin</td>
<td>positive data (101;102)</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>α-tocopherol</td>
<td>limited data (103;104)</td>
<td>isolated reports on NASH patients (105;106)</td>
</tr>
<tr>
<td></td>
<td>S-adenosyl-methionine</td>
<td>positive data (107)</td>
<td>effective in alcohol-induced fibrosis (108)</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Sho-saiko-to</td>
<td>positive data (109;110)</td>
<td>limited data</td>
</tr>
<tr>
<td></td>
<td>quercetin</td>
<td>limited data (111;112)</td>
<td>not tested</td>
</tr>
</tbody>
</table>
Table 2. Summary of experimental antifibrotic therapies using gene delivery.

<table>
<thead>
<tr>
<th>Method of delivery</th>
<th>Gene/antisense oligonucleotides</th>
<th>Model of liver fibrosis</th>
<th>Effects on liver fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenovirus (Ad5-Mn-SOD) (113)</td>
<td>mitochondrial superoxide dismutase (SOD)</td>
<td>rat BDL</td>
<td>Reduction of oxygen radical formation, decreased production of TNF-α, TGF-β and collagen I mRNA level.</td>
</tr>
<tr>
<td>adenovirus (AxCA-rIFN) (114)</td>
<td>rat interferon-α</td>
<td>rat DMN</td>
<td>Reduction of collagen deposition and α-SMA expression, down-regulation of MMP and TIMP-1 mRNA levels.</td>
</tr>
<tr>
<td>adenovirus (AdSMMP-1) (115)</td>
<td>human pro-matrix metalloproteinase-1</td>
<td>rat TAA</td>
<td>Reduction of collagen deposition and number of α-SMA positive cells, stimulation of hepatocyte proliferation.</td>
</tr>
<tr>
<td>adenovirus (AdMMMP8) (116)</td>
<td>human neutrophil collagenase (matrix metalloproteinase-8)</td>
<td>rat CCl4 and BDL</td>
<td>Reduction of hepatic fibrosis, down-regulation of TGF-β and up-regulation of hepatocyte growth factor and MMP-9 as well as MMP-2.</td>
</tr>
<tr>
<td>adenovirus (Ad5-CMV-AS-TGF-β1) (117)</td>
<td>antisense complementary to the 3'-portion of rat TGF-β1 mRNA</td>
<td>rat BDL</td>
<td>Reduction of collagen deposition and α-SMA protein expression.</td>
</tr>
<tr>
<td>adenovirus (AdSmad7) (118)</td>
<td>mouse Smad 7</td>
<td>rat BDL</td>
<td>Reduction of collagen deposition, α-SMA protein expression and hydroxyproline content in livers. Arrested transformation of quiescent HSC into activated phenotype in cultured cells.</td>
</tr>
<tr>
<td>adenovirus (Ad5-CMV-asPDGF) (119)</td>
<td>antisense mRNA complementary to the 5’-coding sequence of PDGF B-chain</td>
<td>rat BDL</td>
<td>Reduction of PDGF-B protein expression, collagen deposition and α-SMA expression.</td>
</tr>
<tr>
<td>recombinant adeno-associated virus (rAAV/HO-1) (50)</td>
<td>rat heme oxygenase-1</td>
<td>rat CCl4</td>
<td>Down-regulation of TGF-β, collagen I mRNA levels and inhibition of HSC proliferation.</td>
</tr>
<tr>
<td>electroporation (pUC-SR α/human HGF) (120)</td>
<td>human hepatocyte growth factor</td>
<td>rat DMN</td>
<td>Long-term survival of rats with DMN-induced liver fibrosis. Reduction of fibrous regions and pseudomodule formation.</td>
</tr>
<tr>
<td>electroporation (pCYIL-10) (121)</td>
<td>human interleukin-10</td>
<td>rat TAA</td>
<td>Reduction of collagen deposition and down-regulation of TGF-β1, TNF-α, collagen I, cell adhesion molecules and TIMP-1.</td>
</tr>
<tr>
<td>electroporation (pCMV-ACTH 1-17) (122)</td>
<td>recombinant α-melanocyte-stimulating hormone</td>
<td>mice TAA</td>
<td>Reduction of collagen deposition and down-regulation of TGF-β1, collagen I, cell adhesion molecules and TIMP-1 and up-regulation of MMP mRNA levels.</td>
</tr>
</tbody>
</table>
reactions that may lead to fatal pulmonary hemorrhagic edema (52). Since the same pathological changes in the lungs also occur in patients suffering from liver cirrhosis, the intravascular administration of adenoviral vectors in such patients might be potentially dangerous.

**Drug targeting to HSC**

A large number of currently tested antifibrotic drugs aim, either direct or indirect, at activated HSC. In vivo, however, effectiveness of these compounds is often limited due to the lack of the specificity for HSC. Selective delivery of compounds to the HSC in the fibrotic liver by means of specific drug carriers is an alternative for traditional treatments. Due to selective accumulation in the HSC, high drug concentrations can be achieved, while at the same time adverse effects will be avoided in other tissues and cells. In order to target HSC, protein based carriers were developed using chemically modified human serum albumin (HSA) with specific groups that are recognised by receptors expressed on the cell membrane of activated HSC. The introduction of mannose 6-phosphate (M6P-HSA) groups (53;54) into the albumin molecule yielded a carrier which was recognised by the mannose 6-phosphate/insulin like growth factor II receptor that is abundantly expressed on activated HSC. The best homing properties of this carrier were achieved when around 30 M6P groups were attached per albumin molecule. In two other carriers, specific sequences of cyclic peptides, that mimic the binding-sites of natural ligands such as PDGF (55) and collagen type VI (56) to their receptors, were attached to HSA. Both PDGF receptor and collagen type VI receptor are up-regulated on activated HSC and the association of these carriers with activated HSC was demonstrated. In vivo, it was shown that all these carriers accumulate in HSC in the fibrotic livers of bile duct ligated rats.

Modified HSA can function as a drug carrier, as was recently proved by coupling several drugs, including pentoxyfiline (57), mycophenolic acid (58), doxorubicine (59) and gliotoxin (60), to M6P-HSA. These drug-carrier constructs retained their antifibrotic properties, as was demonstrated in cultured HSC, and were also delivered to HSC in the fibrotic liver. Another application of modified HSA would be to serve as a homing ligand for other of drug carrier systems such as liposomes, cationic lipoplexes or polymers.

**Liposomes in drug delivery to liver cells**

Liposomes are formed through hydration of amphiphilic lipids (Fig. 3). In these microscopic vesicles, an aqueous lumen is surrounded by one or more lipid bilayers. Most preparations of liposomes for drug delivery purposes are made from neutral or anionic phospholipids with addition of cholesterol to stabilise the liposomal membrane. This liposomal composition resembles the naturally occurring cell membrane, and thus liposomes are considered to be biocompatible, i.e. they are biodegradable, non-immunogenic and non-toxic. Because the liposomal structure comprises an aqueous phase and a lipid phase, it can accommodate both water- and lipid-soluble substances. Water-soluble compounds can be encapsulated in the aqueous inner part of the vesicle while lipophilic drugs can be accommodated in the lipid bilayer. Additionally, molecules such as antibodies, proteins and sugar groups can be coupled relatively easily to the surface of the liposomes to target them to specific tissues and cells. Polymers such as polyethyleneglycol (PEG) attached to the liposomal membrane prolong the circulation time of these particles in the blood. In
principle, liposomes change the pharmacokinetic properties and the biodistribution of the encapsulated drugs; they often prolong the circulation time in the blood and may enhance the deposition and internalisation at the target site. In addition, liposomes protect the carried drug from degradation in the blood stream while they safeguard the rest of the body from the encapsulated potentially toxic drugs.

Intravenously injected liposomes smaller than 1 μm readily accumulate in the liver, where they end up predominantly in Kupffer cells. However, depending on their size and lipid composition as well as on surface modification, they can be re-targeted to other types of liver cells, including hepatocytes and liver endothelial cells. Therefore, in liver diseases such as fibrosis, showing a pathology that involves all major population of liver cells, delivery of drugs to particular types of the cells, using specifically targeted liposomes, provide the possibility to interfere simultaneously with different processes that occur during disease development.

Liposomes targeted to hepatocytes

To reach hepatocytes from the blood stream, liposomes have to pass the fenestrations in the endothelial cells. This limits the size of the liposomes that can be taken up by hepatocytes to about 150 nm. Indeed, small unilamellar vesicles with diameters around 50 nm and composed of neutral lipids, accumulate predominantly in hepatocytes (61). The interaction of liposomes with hepatocytes might be mediated by the apolipoprotein E (ApoE) remnant receptor, leading to the endocytosis of liposomes, and the scavenger receptor B-1 (also known as the HDL receptor) resulting in selective transfer of lipids (62-64).

Interestingly, liposomes with a relatively large size of 200 – 400 nm, containing the negative lipid phosphatidylserine (PS), accumulated in large amounts in hepatocytes (65). This phenomenon was not observed when PS was replaced by another negatively charged lipid, phosphatidylglycerol (PG). The uptake mechanism of large PS-containing liposomes by hepatocytes is not fully understood, but it is possible that either PS exerts a pharmacological effect on the dimensions of the endothelial fenestrations or that PS-containing liposomes, due to their weak interaction with endothelial cells are squeezed through the fenestrations by the blood cells (66).

The active targeting of liposomes to hepatocytes can be achieved by modifying the liposomal surface with ligands of the asialoglycoprotein receptor (ASGPr), such as galactose and N-acetylgalactosamine (67-69). ASGPr on the surface of hepatocytes mediates the clearance process of desialylated proteins from the blood. The efficiency of the uptake of galactosylated liposomes by hepatocytes depends on the density of galactose groups attached to liposomes. In addition, the size of liposomes modified with galactose moieties seems to be critical for the interaction with hepatocytes through ASGPr. Galactosylated liposomes smaller than 70 nm are taken up by hepatocytes, larger ones do accumulate in Kupffer cells (68). In the optimal interaction with ASGPr, parameters like the clustering of galactose moieties (tetraantennary or monoantennary) and an appropriate spacing of the sugar residues play a role as well (69).

Recently, targeting of liposomes to hepatocytes was demonstrated using a peptide sequence originating from a surface protein of Plasmodium, a protozoan causing infections in humans (70). The 19-amino acid peptide from the circumsporozoite protein contained a heparan sulphate proteoglycan binding sequence which is recognised by the highly
sulphated heparan sulphate proteoglycans located on the basolateral side of hepatocytes. Systemically injected liposomes modified with this peptide mainly accumulated in hepatocytes.

**Liposomes and Kupffer cells**

Kupffer cells are specialised cells that clear the blood from foreign particles, microorganisms, and senescent blood cells. Also, most of the liposomal preparations injected intravenously are readily taken up by these cells. As a result, researchers tend to make an effort to develop strategies which allow liposomes to escape from Kupffer cell accumulation, rather than actively target liposomes to these cells. Reduction of liposome sizes from 800 nm to around 100 nm decreases Kupffer cell uptake but it does not eliminate accumulation in these cells. The mechanism underlying the fast blood elimination of liposomes by Kupffer cells is called opsonization. Once liposomes enter the blood circulation, they absorb a broad spectrum of plasma proteins. The amount and type of adhered proteins is determined by the physicochemical properties of the liposomes, including the size, lipid composition and surface charge. The adsorbed plasma proteins that mediate the specific interaction of liposomes with receptors on the macrophage are called opsonins. In
principle, opsonins adsorbed on the surface of liposomes mark them for fast recognition and enhanced uptake by Kupffer cells. Liposomal opsonins are classified in two groups: immune opsonins and non-immune opsonins (71). The immune opsonins mainly include complement proteins and immunoglobulins (antibodies), which identify liposomes as foreign particles and mark them for uptake by the MPS. The second class of opsonins are proteins such as fibronectin, α2-macroglobulin and apolipoproteins which are ligands that direct liposomes to specific receptors on the macrophage cell membrane. In order to prevent rapid blood elimination and accumulation in Kupffer cells, liposomes can be surface-grafted with a hydrophilic polymer, polyethylene glycol (PEG). The flexibility of PEG allows a relatively small number of surface-grafted polymer molecules to create a protective layer. Thus PEGylated liposomes are characterised by a significantly extended circulation time. This property is attributed to the decreased adsorption of opsonins from the blood on the liposomal surface, due to the highly flexible hydrophilic steric barrier provided by the polymer brushes. The development of long-circulating liposomes in the early 1990's was considered a breakthrough in the liposomal field, and resulted, among others, in a liposomal formulation of doxorubicin approved for regular clinical use (Doxil/Cealyx) (72).

### Targeting liposomes to liver endothelial cells

Although liver endothelial cells (LEC) have direct contact with blood, significant accumulation of conventional liposomes in these cells does not occur (61). Interesting results were observed when the uptake of negatively charged liposomes containing PS in vivo was compared with that in primary cultures of LEC. The contribution of LEC in the uptake of PS-containing liposomes after intravenous injection was minimal, while accumulation of these liposomes by cultured LEC was almost equal to that of Kupffer cells (73). These in vitro observations were confirmed when uptake of PS-containing liposomes was studied in the serum-free perfused rat liver (74). Polyinosinic acid, a competitive inhibitor of ScR reduced the association of PS containing liposomes with cultured LEC, as well as the uptake in the serum-free perfused livers, suggesting involvement of ScR in this process. These findings demonstrated that LEC have a high capacity to take up liposomes. However, accumulation of PS-containing liposomes by LEC in vivo might be inhibited by “dys-opsonins”, that mask the PS for receptor recognition.

LEC are known to abundantly express different classes of scavenger receptors (ScR) including class A I and II, class B ScR-B I and CD 36, all known to recognise anionic domains. As a matter of fact, massive targeting of liposomes to LEC in vivo was achieved by coupling a poly-anionic molecule, aconitylated human serum albumin (AcoHSA), to the liposomal surface (75). Inhibition of the in vivo uptake of AcoHSA by polyinosinic acid also indicated that this association is specifically mediated by ScR. Application of AcoHSA as a targeting ligand to LEC was successfully applied in the preparation of stabilised lipid coated lipoplexes, that were shown to efficiently deliver functionally active antisense oligonucleotides to LEC in vivo(76).

Injured hepatocytes, activated Kupffer cells and endothelial cells release broad spectrum of cytokines and other substances such as ROS which induce inflammation and oxidative stress as well as activate HSC in the fibrotic liver. These processes perpetuate development of fibrosis and contribute to the liver failure. Liposomal drugs selectively targeted to
hepatocytes, Kupffer cells and endothelial cells may be used for specific inhibition of pro-inflammatory actions in these cells simultaneously with antifibrotic compounds directed to HSC.

Conclusions

The increasing number of patients with a liver disease brings along an increasing urgency to develop more effective treatments for liver fibrosis. Hepatic stellate cells are crucial cells in the process of fibrogenesis and therefore the major aim for antifibrotic therapies. Due to the lack of specificity of experimental antifibrotic treatments towards HSC and toxic side effects, their efficacy in vivo is limited. These obstacles in the design of a therapy against liver fibrosis can possibly be overcome by using liposomes as drug carries to HSC. Specific accumulation of liposomes in HSC can be provided by surface-coupled modified human serum albumin such as mannose 6-phosphate human serum albumin (M6P-HSA). Therefore, liposomes targeted to HSC may increase the specificity of the drug as well as reduce the toxicity, giving possibilities to improve antifibrotic strategies.

References


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Experiments can be abandoned in the lab journal forever, marked as 'strange' or 'unsolved'. After half a year's worth of experiments on the HSC-T6 cell line, these cells inexplicably transformed and the data presented in this figure could not be reproduced any more.