Addressing liver fibrosis with lipid-based drug carriers targeted to hepatic stellate cells

Joanna E. Adrian
referred to as liver cirrhosis, or reverse under certain conditions and then the normal function of the liver may be restored.

The progression of fibrosis can reverse or slow down in response to removal of the primary cause of damage. However, for complete cure of advanced liver fibrosis, medical intervention is required. A number of different antifibrotic strategies are tested in laboratories and in clinical trials, but so far no effective treatment other than liver transplantation has been established. In the USA and Europe, liver cirrhosis is the most common non-neoplastic cause of death among hepatobiliary and digestive diseases (17). The urge to improve current strategies for the treatment of liver fibrosis is growing. Liver transplantation is a complex and costly operation that also is hampered by a lack of donor livers. The number of patients waiting for liver transplantation is approximately four times higher than the number of available donor livers. In addition, epidemiological prognoses indicate that, in the coming years the prevalence of liver fibrosis will significantly increase due to increasingly occurring cases of hepatitis C infections (increased number of infected patients and aging of already infected) (18). The growing group of patients with NASH is also significantly contributing to the rising number of patients with liver cirrhosis. The underlying pathology of NASH is not fully understood, but it is strongly linked with food intake and composition, physical activity (referred as “lifestyle”) as well as central obesity, insulin resistance and genetic factors (19).

The clinical manifestation of liver cirrhosis is determined by the nature and severity of the primary cause of the disease and the stage of fibrosis in the liver tissue and varies from the absence of symptoms to complete liver failure. Liver fibrosis is often a long term disease; up to 40 % of patients is asymptomatic and may have no manifestation for more than a decade (20). This aspect of liver fibrosis urges us also to develop appropriate diagnostic tools, especially reliable early markers of the fibrotic process.

**Mechanism of liver fibrosis**

Hepatic stellate cells are considered to play a central role in liver fibrosis although development of this disease is a complex process, involving several other types of liver cells. Two main phases can be distinguished in the progression of the disease: inflammation and fibrogenesis. In the initial steps of liver fibrosis, various hepatotoxic factors induce the production of mediators which cause an inflammatory reaction in hepatic cells. For example, acetaldehyde, the oxidative metabolite of alcohol, and the bacterial toxin, LPS released from the gut stimulate Kupffer cells to secrete reactive oxygen species (ROS), TGF-β, TNF-α and IL-6 in livers of alcohol abused patients. Decreased excretion of bile (cholestasis) results in accumulation of bile acids in the liver, which promotes biliary epithelial cells to secrete TNF-α, ET-1 and PDGF. Hepatitis C infection primarily causes damage to hepatocytes and subsequent production of ROS, TGF-β, TNF-α, EGF and IGF by these cells. In this stage of fibrosis development, cytokines released by the injured hepatic cells provide an additional stimulus to Kupffer cells, which further enhances the inflammation phase. Liver endothelial cells, activated by Kupffer cell derived TNF-α, express adhesion molecules, including ICAM-1, enabling leukocyte infiltration in the areas of injured liver tissue. In addition, activated LEC produce cytokines and growth factors like PDGF, VEGF, IL-1, TGF-β, ET-1 and molecules such as NO and ROS (21). Microarray analysis of endothelial cell gene expression in normal and cirrhotic rat livers revealed up-regulation of endothelial genes involved in inflammation, ECM production
**Front cover:** schematic representation of hepatic sinusoid structure in a healthy (top) and injured (bottom) liver (detailed description in chapter 1, figure 2).

**Back cover:** schematic representation of liposome structure. Properties of liposomes can be modulated by coupling different ligands to their surface. Drug molecules can be incorporated into the lipid bilayer or encapsulated in the inner part of the vesicle.

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Addressing liver fibrosis with lipid-based drug carriers targeted to hepatic stellate cells

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After all, science is essentially international, and it is only through lack of the historical sense that national qualities have been attributed to it.

Maria Curie-Sklodowska
scope of the thesis
Liver fibrosis is a chronic disease that results from hepatitis B and C infections, alcohol abuse or metabolic and genetic disorders. Ultimately progression of fibrosis leads to cirrhosis, a stage of the disease characterised by failure of the normal liver functions. Currently, there are no efficacious antifibrotic drugs available and the treatment of liver fibrosis is mainly based on the removal of the underlying cause of the disease, which will, however, not cure the disease or reverse it. Liver transplantation is the only treatment for patients with advanced fibrosis, but this is a highly complicated surgical operation, not even mentioning serious such as lack of donors and very high costs.

Hepatic stellate cells (HSC) are considered to be key players in the development of liver fibrosis. Chronically activated HSC produce large amounts of extracellular matrix which leads to impairment of the structure and function of the liver. In addition, HSC enhance fibrosis by secreting a broad spectrum of cytokines that exert pro-fibrotic action in other cells, and in an autocrine manner maintain their own activation. Therefore, therapeutic interventions which inhibit activation of HSC and HSC pro-fibrotic activity are currently under investigation world-wide. The ideal antifibrotic drug and/or drug delivery system should be well tolerated, accumulate preferentially in the liver and have limited side effects in other organs. Because of the complexity of the disease, the ideal therapy for liver fibrosis should simultaneously inhibit pro-fibrotic processes in HSC and pro-inflammatory actions of Kupffer cells and liver endothelial cells.

Liposomes are versatile drug carriers, that are already clinically applied for the delivery of cytostatic compounds to tumours as well as for the delivery of anti-fungal compounds. Liposomes are made of phospholipids and are considered non-immunogenic, non-toxic and biodegradable. In addition, liposomes can accommodate compounds with different chemical properties either in the aqueous interior or in the lipid bilayers.

In this project we applied liposomes and explored their potential as drug carriers to HSC in the fibrotic liver. To specifically target them to HSC, liposomes were surface grafted with human serum albumin that, in turn, was derivatized with mannose 6-phosphate groups (M6P-HSA). The research described in this thesis focuses on the characterization of the interaction of M6P-HSA liposomes with HSC and other liver cells, such as Kupffer cells and liver endothelial cells, in vitro using cultured cells and in vivo in experimental model of liver fibrosis. Receptors involved in these processes were tentatively identified and features of M6P-HSA liposomes as drug carriers, including organ distribution and pharmacokinetic properties were studied.

The primary role of drug carriers is to deliver therapeutic compounds specifically to the diseased tissue. However, carriers, prepared from bioactive compounds may possess additional therapeutic properties against a disease. We prepared liposomes from dilinoleoylphosphatidylcholine (DLPC), which has been shown to have antifibrotic properties and studied the effects of DLPC containing liposomes on the activation of HSC and the progression of liver fibrosis in a rat model of this disease.

Changes in gene expression in HSC in fibrotic livers allow gene-therapeutic approaches to modulate HSC function and to inhibit the fibrotic process in the liver. Virus-mediated gene delivery of matrix metalloproteinases or proteins which interfere with signalling pathways of pro-fibrotic cytokines was shown to reduce experimental fibrosis. Specific transfection of HSC may improve the efficacy of delivered genes and minimize adverse effects caused by the expression of these genes in other cell types. In this thesis M6P-HSA
liposomes were used to create a targeted viral vector by fusing them with the Hemagglutinating Virus of Japan.

In summary, the aim of the research described in this thesis was to explore the potential of liposomes targeted to HSC as drug carriers in antifibrotic therapy and to investigate effects of bioactive compounds delivered by these liposomes on the progression of liver fibrosis. To our knowledge, this is the first study demonstrating that lipid-based drug carriers can be selectively delivered to HSC in the fibrotic liver.
HSC at day 3. The two Thursdays after a new isolation of stellate cells, being the 3rd and 10th day, are the standard moments to test their properties. Thursday quickly became the regular ‘experiment day’.
chapter 1

general 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 microvessels, 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 canalicular (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 asialoglycoprotein 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 functionality. 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 macromolecular 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.
Chapter 1

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 hemochromatosis, 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...
and degradation, oxidative stress, cell proliferation and apoptosis (22), demonstrating the important function of LEC in modulating both inflammation and the progression of fibrosis in the liver.

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 reduction of collagen deposition. 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><strong>Inhibition of HSC activation</strong></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:</td>
<td>positive data (89)</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>camostat mesilate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>dilinoleoylphosphatidylcholine</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:</td>
<td>positive data (95-97)</td>
<td>positive data (98,99)</td>
</tr>
<tr>
<td></td>
<td>losartan</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cariporide</td>
<td>positive data (100)</td>
<td>not tested</td>
</tr>
<tr>
<td><strong>Induction of HSC apoptosis</strong></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><strong>Antioxidants</strong></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</td>
<td>BDL</td>
</tr>
<tr>
<td>adenovirus (AxCARIFN) (114)</td>
<td>rat interferon-α</td>
<td>rat</td>
<td>DMN</td>
</tr>
<tr>
<td>adenovirus (AdSMMP-1) (115)</td>
<td>human pro-matrix metalloproteinase-1</td>
<td>rat</td>
<td>TAA</td>
</tr>
<tr>
<td>adenovirus (AdMMMP8) (116)</td>
<td>human neutrophil collagenase (matrix metalloproteinase-8)</td>
<td>rat</td>
<td>CCl4 and BDL</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</td>
<td>BDL</td>
</tr>
<tr>
<td>adenovirus (AdSmad7) (118)</td>
<td>mouse Smad 7</td>
<td>rat</td>
<td>BDL</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</td>
<td>BDL</td>
</tr>
<tr>
<td>recombinant adeno-associated virus (rAAV/HO-1) (50)</td>
<td>rat heme oxygenase-1</td>
<td>rat</td>
<td>CCl4</td>
</tr>
<tr>
<td>electroporation (pUC-SR α/human HGF) (120)</td>
<td>human hepatocyte growth factor</td>
<td>rat</td>
<td>DMN</td>
</tr>
<tr>
<td>electroporation (pCYIL-10) (121)</td>
<td>human interleukin-10</td>
<td>rat</td>
<td>TAA</td>
</tr>
<tr>
<td>electroporation (pCMV-ACTH 1-17) (122)</td>
<td>recombinant α-melanocyte-stimulating hormone</td>
<td>mice</td>
<td>TAA</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 HSA 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, acetylated 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


general introduction


54. Beljaars L, Olinga P, Molema G, de Bleser P, Geerts A, Groothuis GM, Meijer DKF, Poelstra K. Characteristics of the hepatic stellate cell-


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.
chapter 2

Interaction of targeted liposomes with primary cultured hepatic stellate cells: involvement of multiple receptor systems

Joanna E. Adrian
Klaas Poelstra
Gerrit L. Scherphof
Grietje Molema
Dirk K.F. Meijer
Catharina Reker-Smit
Henriëtte W.M. Morselt
Jan A.A.M. Kamps
Abstract

**Background/Aims:** For designing a versatile liposomal drug carrier to hepatic stellate cells (HSC), the interaction of mannose 6-phosphate human serum albumin (M6P-HSA) liposomes with cultured cells was studied. Methods: M6P-HSA was covalently coupled to the liposomal surface and the uptake and binding of 3H-labelled M6P-HSA liposomes by primary rat HSC and liver endothelial cells was determined. The targeting ability of M6P-HSA liposomes to HSC was tested in bile duct ligated rats using immunohistochemical method.

**Results:** The association of M6P-HSA liposomes with HSC was 4-fold higher than of control liposomes. An excess of M6P-HSA inhibited this association by 58%, indicating M6P receptor specificity. The scavenger receptor competitor polyinosinic acid abolished association of M6P-HSA liposomes with HSC. M6P-HSA liposomes also amply associated with endothelial cells, which abundantly express scavenger receptors. Endocytosis of M6P-HSA liposomes by HSC was temperature dependent and could be inhibited by monensin. In the fibrotic liver M6P-HSA liposomes co-localised with HSC.

**Conclusions:** Coupling of M6P-HSA to liposomes strongly increases the in vitro uptake of these liposomes by HSC and endothelial cells. Both the mannose 6-phosphate receptor and the scavenger receptors are involved in the uptake process. M6P-HSA liposomes are potential drug carriers to HSC in the fibrotic liver.
Introduction

In the healthy liver, hepatic stellate cells (HSC) express a quiescent phenotype and are responsible for storage of vitamin A (1). During liver fibrosis, HSC become activated and transform into proliferating fibroblast-like cells, which produce large amounts of collagen (2). Since HSC are identified as key players in the development of fibrosis, they are attractive targets for antifibrotic therapy.

Human serum albumins (HSA) modified with sugar moieties or cyclic peptides that recognise receptors on HSC were shown to selectively accumulate in HSC in a rat model of liver fibrosis. In particular, mannose 6-phosphate groups attached to HSA (M6P-HSA) exerted specificity for the mannose 6-phosphate/insulin like growth factor II (M6P/IGF II) receptors present on HSC (3-5). The expression of these receptors is increased during HSC transformation from the quiescent into the activated phenotype (6, 7). In the fibrotic liver M6P/IGF II receptors facilitate the activation of transforming growth factor β (TGF-β) (8, 9) which is a potent cytokine that stimulates the production of collagen by HSC. Binding of latent TGF-β to the M6P/IGF II receptors mediated by two M6P groups on a short peptide enables plasmin-mediated cleavage of the peptide and generation of active TGF-β (6).

Intracellular M6P receptors are responsible for sorting of lysosomal enzymes (10, 11). Approximately 10 - 20% of the total number of M6P/IGF II receptors is present on the surface of the cells (12) where they bind diverse M6P- and non M6P-containing ligands such as TGF-β, proliferin, IGF II, retinoic acid and urokinase-type plasminogen activator receptor (13).

In the present study, we coupled M6P-HSA to the surface of liposomes as a homing ligand to HSC. Liposomes have a high capacity for encapsulation of various drugs, but so far this drug delivery system had not been specifically targeted to HSC in fibrotic livers. We performed in vitro studies in primary cultures of HSC and in vivo experiments using a rat model of liver fibrosis to determine whether M6P-HSA modified liposomes specifically associate with HSC. Furthermore, the mechanism of this interaction was characterised. Since modification of HSA by M6P groups introduces negative charges to the protein, we also examined the interaction of M6P-HSA liposomes with primary cultures of liver endothelial cells (LEC) that express scavenger receptors (ScR) (14).

It is conceivable that ScR also play a role in the association of M6P-HSA liposomes with LEC and HSC.

Materials and Methods

Materials

Cholesterol (Chol), N-succinimidyl-S-acetylthioacetate (SATA), cis-aconitic anhydride, monensin and polyinosinic acid (poly I) were from Sigma (St. Louis MO, USA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(maleimidophenyl)butyramide] (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster AL, USA). [3H]cholesteryloleyl ether (‘H-COE) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human serum albumin fraction V was from the Central Laboratory of the Red Cross (Amsterdam, The Netherlands). Dulbecco’s modified Eagles Medium (DMEM), RPMI-1640, L-glutamine were obtained form Invitrogen (Paisely, Scotland, UK), foetal calf serum (FCS) from
BioWhittaker Europe (Verviers, Belgium), penicillin, streptomycin from Sigma. All other chemicals were analytical grade or the best grade available.

**Animals**

Specified pathogen free (SPF) male Wistar and Wag/Rij rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to food and water. The local committee for care and use of laboratory animals approved the presented study.

**Preparation of modified HSA**

HSA was modified either with mannose 6-phosphate moieties (4) or with cis-aconitic anhydride yielding cis-aconitylated HSA (AcoHSA) (15) as described before.

**Preparation of liposomes**

Liposomes were composed of POPC, CHOL, MPB-PE in molar ratio of 23:16:1, labelled with a trace amount of \(^3\)H-COE, a non-degradable bilayer marker and prepared as described before (16). The phospholipid phosphorus content of each liposome preparation was assessed by phosphate assay (17). The lipid concentration was calculated taking into account the amount of cholesterol in the preparation of liposomes. Size and size distribution were determined by dynamic laser light scattering with a Nicomp submicron particle analyzer (NICOMP 380 ZLS, Santa Barbara, CA, USA). The average diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. M6P-HSA and AcoHSA were subsequently coupled to liposomes by the SATA method described before (15). The phospholipid phosphorus, protein content (18) and particle size of both M6P-HSA and AcoHSA liposomes were determined. Control liposomes were prepared similarly but they were not coupled with protein. Liposomes were stored under argon at 4°C and used within three weeks after preparation.

**Isolation of HSC and LEC**

HSC were isolated from livers of male Wistar rats (550-600 g) (Harlan) as described before (19). Isolated HSC were cultured in DMEM containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. One day before the experiments, HSC were trypsinized and seeded in 24 wells plates (Costar).

LEC were isolated from livers of male Wag/Rij rats (200-250 g) (Harlan) as described before (16). LEC were cultured in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 ng/ml endothelial cell growth factor (Boehringer, Mannheim, Germany) for 2 days in 24 wells collagen-coated plates (Greiner) before being used in the experiments.

**Association of M6PHSA liposomes and AcoHSA liposomes with HSC and LEC**

To study association of M6P-HSA liposomes and AcoHSA liposomes in cultures of HSC or LEC, the cells were pre-incubated for 1h with FCS-free medium. Next, 3h incubation with \(^3\)H-COE-labelled liposomes (160 nmol/ml, at 37°C unless stated otherwise) in culture medium without FCS was performed. When appropriate, other agents were added to the incubation mixture as indicated. Incubation was stopped by placing the culture plate on ice and cells were washed with ice-cold PBS pH 7.4. Subsequently, cells were lysed.
using 0.4 M NaOH. Cell associated radioactivity was determined by liquid scintillation counting of the lysed cell suspension and radioactivity was normalized for protein content measured according to Lowry (20).

Detection of M6P/IGF II receptors expression by the cells

**RNA isolation and reverse-transcription polymerase chain reaction**

Total RNA was isolated from HSC and LEC using Absolutely RNA Microprep kit (Stratagene, Amsterdam, The Netherlands) according to the protocol of the manufacturer. Subsequently, synthesis of first strand cDNA from total cellular RNA was performed with SuperScript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and 40 units RNaseOut (Invitrogen) in a volume of 20 µl containing 250 ng random primers (Promega). One µl of cDNA (10 ng/µl) was used for polymerase chain reaction in a total volume of 30 µl containing 1 unit Taq polymerase (T. aquaticus; Amersham Bioscienses, Roosendaal, The Netherlands) and 10 µM primers. Primers for M6P/IGF II rat receptor were as follows: forward 5’-GTTGCCCTCTGGGTGGACT-3’, reverse 5’-CTCCTCCTCTGCTGACCTTTG-3’ (Sigma Genosys). GAPDH (forward 5’- CGCTGGTGCTGATATGTCG -3’, reverse 5’- CTGTGGTCATGAGCCCTTC-3’) (Sigma) was used as house keeping gene. Thirty cycles of reaction for M6P/IGF II receptor primers at 94°C for 5 min, 94°C for 30 s, 57°C for 45 s, 72°C for 45 s and 72°C for 7 min, and 26 cycles for GAPDH primers were carried out using GeneAmp® PCRSystem 9700 (Applied Biosystem). The polymerase chain reaction product was visualized on 1.5 % agarose gel containing ethidium bromide.

**Immunohistochemical analysis**

HSC and LEC were isolated and cultured as described above. After fixation in an acetone: methanol (1:1) solution, cells were incubated with a goat polyclonal antibody against M6P/IGF II receptor (K-21, Santa Cruz Biotechnology, Inc.). Subsequently, endogenous peroxidase was inhibited with H₂O₂, followed by incubation with peroxidase conjugated rabbit-anti-goat IgG (RaGPo, Dako Cytomation, Denmark) in the second step and step with peroxidase conjugated goat-anti-rabbit IgG (GaRPo, Dako) in the third. Antibody associated peroxidase was visualised with 3-amino-9-ethyl-carbazole (AEC).

**In vivo accumulation of M6P-HSA liposomes**

Liver fibrosis was induced by ligation of the bile duct in male Wistar rats (250 g) under anaesthesia. Three weeks after bile duct ligation (BDL 3), rats developed a severe liver fibrosis and were used for experiments (4). Two µmol M6P-HSA liposomes per 100 g of body weight were injected into BDL 3 rats via the penile vein. One hour after injection of liposomes, livers were harvested and snap-frozen in isopentane. Cryostat sections of livers (4 µm) were fixed in acetone. Double immunohistochemical staining was performed to assess whether M6P-HSA liposomes were taken up in HSC as described previously (4). Briefly, M6P-HSA liposomes were stained with a rabbit polyclonal antibody directed against HSA (Cappel, Organon Teknika, Turnhout, Belgium) followed by incubation with mouse monoclonal antibodies directed against desmin (Organon Teknika), an established marker of HSC. Next, the endogenous peroxidase was inhibited with H₂O₂ and the sections were incubated with two secondary antibodies, GaRPo and RaGPo (Dako). The antibody against desmin was detected with alkaline phosphatase conjugated rabbit-anti-mouse
IgG (Dako) and visualised with Naphtol AS-MX/Fast Blue reaction at 37°C. Peroxidase conjugated antibodies were visualised with AEC.

**Statistical analysis**

Data were normalized to the control value and presented as mean ± SEM. Statistical significance of differences was evaluated by a two-tailed unpaired Student’s t-test.

**Results**

**Liposome characterization**

M6P-HSA used for the preparation of targeted liposomes contained 27 ± 7 molecules of M6P per molecule HSA. In AcoHSA an average of 54 out of the 60 ε-amino groups of HSA groups were aconitylated.

The preparation of M6P-HSA liposomes and AcoHSA liposomes was reproducible and yielded liposomes that were comparable in size and in amount of coupled protein (Table 1).

**Table 1. Characterization of liposomes.**

<table>
<thead>
<tr>
<th>Type of liposomes</th>
<th>coupled protein [μg protein/μmol TL]</th>
<th>size [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6P-HSA liposomes</td>
<td>52 ± 12</td>
<td>101 ± 9</td>
</tr>
<tr>
<td>AcoHSA liposomes</td>
<td>47 ± 11</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>control liposomes</td>
<td></td>
<td>80 ± 5</td>
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</table>

Size of liposomes and protein coupled to the liposomes was determined as described in section Materials and Methods. Data are presented as a mean ± S.D of 6 to 11 liposome preparations.

**Expression of M6P/IGF II receptor on HSC and LEC**

Primary rat HSC were used, 3 or 10 days after isolation (referred to as day-3 or day-10 HSC). Day-3 HSC displayed the quiescent phenotype, as assessed with light microscopy. They showed a compact shape and contained lipid droplets. After 10 days of culturing, HSC transformed into an activated cell type, which is characterised by a stretched shape and loss of lipid droplets (21).

The expression of M6P/IGF II receptors by day-3 and day-10 HSC as well by LEC was checked at the mRNA level, as well as at the protein level. In day-3 HSC, a relatively low level of M6P/IGF II receptor mRNA (Fig. 1 A) was detected, while increased expression was observed in day-10 HSC. These results were confirmed by immunohistochemical staining which revealed higher expression of the M6P/IGF II receptor on day-10 HSC (Fig. 1C) than on day-3 HSC (Fig. 1B). In LEC, the mRNA of M6P/IGF II receptor was also detectable (Fig. 1A), but immunohistochemical staining showed no expression of the receptor at the protein level (Fig. 1D).

**Association of M6P-HSA liposomes with day-3 and day-10 HSC**

Coupling M6P-HSA to the surface of liposomes resulted in 5-fold and 3.5-fold higher association with day-3 and day-10 HSC, respectively, when compared to control liposomes.
Figure 1. Expression of M6P/IGF II receptors by HSC and LEC. mRNA expression of M6P/IGF II receptors by day-3 HSC, day-10 HSC and LEC was determined using RT-PCR with GAPDH as a housekeeping gene (A). Protein expression of M6P/IGF II receptors by day-3 HSC (B), day-10 HSC (C) and LEC (D) was assessed by immunohistochemical staining for M6P/IGF II receptor. Red cells express M6P/IGF II receptors. At a protein level M6P/IGF II receptor expression could only be detected in day-10 HSC. Original magnification x200.

Figure 2. Association of M6P-HSA liposomes with HSC. HSC 3 days (A) and 10 days (B) after isolation were incubated with 80, 160, 320 nmol/ml $^3$H-COE labelled M6P-HSA liposomes (closed bars) and control liposomes (open bars). The association of control liposomes at the concentration 160 nmol/ml was taken as 100%. Control values for day-3 HSC and day-10 HSC were 11.7 ± 2.9 and 4.2 ± 0.91 nmol lipid per mg of cell protein, respectively. Data are presented as mean ± S.E.M of 3 to 4 experiments. *P<0.05, **P<0.001 versus control liposomes.
(Fig.2). In absolute amounts, day-3 HSC bound and took up five times as much M6P-HSA liposomes as day-10 cells (Fig. 3).

The specificity of the interaction of M6P-HSA liposomes was assessed by incubating HSC with these liposomes and an excess of free M6P-HSA. M6P-HSA reduced the association with day-3 HSC by 49% ± 8 (Table 2), while a similar effect was observed for day-10 HSC, with an inhibition of 68% ± 5. Unmodified HSA did not inhibit the association of M6P-HSA liposomes, for neither day-3 nor day-10 HSC (data not shown).

### Table 2. Effect of M6P-HSA on the association of M6P-HSA liposomes with HSC.

<table>
<thead>
<tr>
<th></th>
<th>M6P-HSA liposomes</th>
<th>+ M6P-HSA</th>
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<tbody>
<tr>
<td>day-3 HSC</td>
<td>100 ± 2.6</td>
<td>51.0 ± 8.4</td>
</tr>
<tr>
<td>day-10 HSC</td>
<td>100 ± 4.0</td>
<td>32.0 ± 4.8</td>
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Day-3 and day-10 HSC were incubated with ³H-COE labelled M6P-HSA liposomes in the presence of 0.1.mg/ml M6P-HSA. Data are expressed as % of the control (³H-COE labelled M6P-HSA liposomes). Control values for day-3 and day-10 HSC were 78.2 ± 27.1 and 7.6 ± 1.4 nmol lipid per mg protein, respectively, mean ± S.E.M of 3 experiments.

Endocytosis of M6P-HSA liposomes by HSC

The natural ligands for the M6P/IGF II receptor undergo endocytosis after binding to the receptor. In order to investigate whether binding of M6P-HSA liposomes is followed by endocytosis in HSC, the effects of temperature and monensin, a widely used inhibitor of endocytosis were examined (22). HSC were incubated with M6P-HSA liposomes at 37°C and 4°C. At 4°C (no internalisation) HSC bound 5-fold less M6P-HSA liposomes than at 37°C. Monensin had no effect on the association of control liposomes, but inhibited the intracellular uptake of M6P-HSA liposomes by 45% ± 6 (Fig. 4B).

Effect of polyanions on the association of M6P-HSA liposomes and AcoHSA liposomes with HSC and LEC
The discrepancy in the expression of the M6P/IGF II receptors by day-3 and day-10 HSC and the data on the association of M6P-HSA liposomes with these cells suggests that receptors other than M6P/IGF II must also be involved in the binding and uptake of these liposomes. To study whether ScR might mediate this interaction, HSC and LEC were incubated with M6P-HSA liposomes and AcoHSA liposomes in the presence or absence of free M6P-HSA, AcoHSA and polyinosinic acid, by virtue of its polyanionic character a potent antagonist of ScR activity (23, 24). AcoHSA is a highly negatively charged molecule (15), known to be taken up by cells via the ScR (25). Additionally, LEC express ScR (14), but no M6P receptors (Fig. 1). Association of M6P-HSA liposomes with LEC was 113 nmol lipid/mg protein, while association of AcoHSA liposomes was 69 nmol lipid/mg protein. Furthermore, AcoHSA liposomes associated with day-3 and day-10 HSC (42 and 7 nmol lipid/mg protein, respectively) at comparable levels as M6P-HSA liposomes (56 and 9 lipid/mg protein, respectively) (Fig. 5).

Excess AcoHSA reduced the association of M6P-HSA and AcoHSA liposomes with LEC, day-3 and day-10 HSC stronger than excess of M6P-HSA (Fig. 5). In addition, polyinosinic acid substantially blocked the uptake of M6P-HSA and AcoHSA liposomes with all types of cells (Fig. 5).

**Uptake of M6P-HSA liposomes by HSC in fibrotic livers**

To test the targeting ability of M6P-HSA liposomes to HSC in the fibrotic liver, we injected these liposomes in BDL 3 rats. Accumulation of M6P-HSA liposomes in HSC was determined by a double immunostaining method as described before (4). M6P-HSA liposomes and HSC were detected simultaneously with specific antibodies against albumin and desmin, respectively. As Figure 6 shows, double positive cells were found 1 h after injection, indicating accumulation of M6P-HSA liposomes in HSC.
Control values of AcoHSA liposomes for day-3 HSC, day-10 HSC and LEC were 41.6 ± 9.4, 7.4 ± 1.2 and 68.6 ± 8.8 nmol lipid per mg of cell protein, respectively. Control values of M6P-HSA liposomes for day-3 HSC, day-10 HSC and LEC were 56.5 ± 14.9, 8.6 ± 1.9 and 112.6 ± 11.8 nmol lipid per mg protein, respectively.

Figure 5. Comparison of the association of M6P-HSA liposomes and AcoHSA liposomes with HSC and LEC. Day-3 HSC (A), day-10 (B) HSC and LEC (C) were incubated with ³H-COE labelled M6P-HSA liposomes (closed bars) and AcoHSA liposomes (open bars) in the absence or presence of 0.1 mg/ml M6P-HSA, AcoHSA or 10 µg/ml poly I. The association of M6P-HSA liposomes and AcoHSA liposomes without treatment was taken as 100%. Control values of M6P-HSA liposomes for day-3 HSC, day-10 HSC and LEC were 56.5 ± 14.9, 8.6 ± 1.9 and 112.6 ± 11.8 nmol lipid per mg protein, respectively. Control values of AcoHSA liposomes for day-3 HSC, day-10 HSC and LEC were 41.6 ± 9.4, 7.4 ± 1.2 and 68.6 ± 8.8 nmol lipid per mg of cell protein, respectively. Mean ± S.E.M of 3 to 4 experiments. *P<0.05, **P<0.001 versus M6P-HSA liposomes without treatment, #P<0.05, ##P<0.001 versus AcoHSA liposomes without treatment.

Figure 6. Accumulation of M6P-HSA liposomes in HSC in the fibrotic liver. Double immunostaining using specific antibodies directed against HSA (red staining) to detect M6P-HSA liposomes and against desmin (blue staining) to identify HSC was performed as described in Materials and Methods. Arrows indicate double positive cells. (A) original magnification x200, (B) original magnification x1000 of the selected area from picture (A).
Discussion

In the present study, we demonstrated that coupling M6P-HSA to the surface of liposomes significantly increased binding and intracellular uptake of these liposomes by cultured primary HSC. Surprisingly, a higher association of the M6P-HSA liposomes was observed for day-3 HSC than for day-10 HSC, yet the results of RT-PCR and immunohistochemical staining showed abundant presence of the M6P/IGF II receptor on day-10 only. This latter observation is consistent with previous data (9, 26), showing that the mRNA and protein levels of the M6P/IGF II receptor increased in HSC cultured for more than 6 days. We hypothesise, therefore, that two receptor systems might contribute to the recognition of M6P-HSA liposomes. The M6P/IGF II receptors bind M6P-HSA liposomes via M6P groups attached to HSA. The negative charge of M6P-HSA, caused by the clustering of M6P groups on the protein core, could also qualify M6P-HSA liposomes as a ligand for scavenger receptors. Competition studies with free M6P-HSA indicate a specific interaction of M6P-HSA liposomes with both day-3 and day-10 HSC, even though the M6P/IGF II receptor is hardly detectable on day-3 cells. Liposomes coupled with aconitylated HSA have been shown to associate readily with LEC through the ScR (25). In our studies, AcoHSA liposome association with HSC was comparable to that of M6P-HSA liposomes. Association of M6P-HSA liposomes and AcoHSA liposomes with LEC were both in the same order of magnitude. We did find expression of the M6P/IGF II receptor by LEC at the mRNA, but not at the protein level, which is consistent with data reported earlier (9). Given the high association of M6P-HSA liposomes with LEC, recognition of M6P-HSA liposomes by ScR seems to be implied. The observed differences in competition between activated and non-activated HSC by M6P-HSA and AcoHSA, respectively, correspond with an increased M6P/IGF II receptor expression on activated HSC. However, to discriminate between the relative contribution of M6P/IGF II receptors and ScR in the uptake of M6P-HSA liposomes by HSC, blocking antibodies against these receptors are required. Little is known about the expression of ScR on HSC. Schneiderhan et al. reported the expression of ScR class B on activated human HSC and demonstrated that oxidatively modified lipoproteins stimulate extracellular matrix synthesis through this receptor in HSC (27). In our experiments, though, the inhibitory effect of polyinosinic acid on the uptake of M6P-HSA liposomes and AcoHSA liposomes by HSC suggests that not ScR class B, but rather ScR class A contributes to the association of these liposomes (13). Although ScR class A is mainly expressed by macrophages, it is also present on endothelial cells, smooth muscle cells and fibroblasts (14, 28). A variety of factors regulate expression of ScR class A in different types of cells; TGF-β and platelet-derived growth factor upregulate ScR class A on smooth muscle cells (29). In macrophages, interferon gamma and tumor necrosis factor α decrease ScR class A expression (30). The presence of a wide range of growth factors and cytokines in fibrotic livers, as well as particular stage of activation of the HSC may influence the expression of ScR class A by these cells. Our data suggest that HSC might express the ScR class A, but additional studies will be required to provide direct proof.

We designed M6P-HSA liposomes as a novel drug carrier to HSC, for selective targeting of antifibrotic drugs. Our in vivo data showed that M6P-HSA liposomes are able to reach HSC in the fibrotic liver. In a subsequent study, the in vivo properties of M6P-HSA liposomes as a drug delivery system to HSC will be determined in a rat model of liver fibrosis, including the contribution of the other liver cell types in the uptake of these
HSC play a crucial role in the pathogenesis of liver fibrosis, producing large amounts of collagen as well as cytokines and growth factors, all of which perpetuate the fibrotic process in a concerted action (31). Targeting antifibrotic compounds such as inhibitors of collagen production, to these cells might therefore improve current therapies with cell non-specific drugs.

In conclusion, we showed that coupling M6P-HSA to liposomes strongly enhanced the binding and uptake of these liposomes by cultured primary HSC. In addition, it leads to accumulation of the liposomes in HSC in the fibrotic liver opening possibilities to deliver anti-fibrotic drugs to this cell type. The M6P/IGF II receptor and the ScR mediate the association of M6P-HISA liposomes with HSC. We demonstrated that M6P-HSA liposomes are internalised by these cells, which should enable them to deliver enclosed drugs into the cell. Liposomes targeted to these crucial cells may provide a new mean to attenuate fibrotic processes in the liver.

References


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interaction of liposomes with HSC


31. Reeves HL, Friedman SL. Activation of hepatic stellate cells--a key issue in liver fibrosis. Front Biosci 2002;7:d808-d826.
The scientific world can be compared to traffic, where it is always wise to have limited trust in unknown participants. Published sequences of primers did not give the expected PCR products and finally turned out not to encode the claimed rat gene for
A novel lipid-based drug carrier targeted to the non-parenchymal cells, including hepatic stellate cells in the livers of bile duct ligated rats
Abstract

In fibrotic livers, collagen producing hepatic stellate cells (HSC) represent a major target for antifibrotic therapies. We designed liposomes with surface-coupled mannose 6-phosphate modified human serum albumin (M6P-HSA) to target HSC via the M6P receptor. In this study we determined the pharmacokinetics and target specificity of M6P-HSA-liposomes in a rat model of liver fibrosis.

Ten minutes after injection of [3H]-M6P-HSA-liposomes 90 % of the dose has cleared from the circulation. The blood elimination of these liposomes was counteracted by free M6P-HSA and polyinosinic acid, a competitive inhibitor of scavenger receptors. The M6P-HSA-liposomes accumulated in HSC. However, also Kupffer cells and endothelial cells contributed to the uptake of M6P-HSA-liposomes in the fibrotic livers. Polyinosinic acid inhibited the accumulation of the liposomes in Kupffer cells and liver endothelial cells, but not in HSC. PCR analysis revealed that cultured HSC express scavenger receptors. This was confirmed by Western blotting, although activation of HSC diminishes scavenger receptor protein expression.

In conclusion, in a rat model for liver fibrosis M6P-HSA-liposomes can be efficiently targeted to non-parenchymal cells, including HSC. M6P receptors and scavenger receptors are involved in the cellular recognition of these liposomes, allowing pharmacological interference in different pathways involved in the fibrosis.
Introduction

The potential of liposomes as a drug carrier system has been extensively investigated. Various types of targeted liposomes have been developed aiming at specific populations of liver cells. Hepatocytes can be targeted using galactosylated liposomes (1-3) or asialofetuin grafted vesicles (4). Liposomes that are modified with aconitylated human serum albumin predominately accumulate in liver endothelial cells (LEC) (5) and mannosylated liposomes are recognised by Kupffer cells (KC) (6;7). So far, no studies have been reported on targeting of liposomes to hepatic stellate cells (HSC), which, in addition to KC and LEC, comprise an important population of non-parenchymal cells in the liver.

In healthy organs, HSC are mainly responsible for storage of vitamin A (8). In contrast, during chronic liver injury, HSC become activated and transdifferentiate into myoﬁbroblast-like cells. Activated HSC are characterised by loss of vitamin A and a high rate of proliferation (9). Moreover, they migrate towards the site of injury and on long run produce an excessive amounts of interstitial collagen, mainly type I and III, which leads to impairment of liver functions. HSC acquire also contractile properties that allow them to play a role in the control of sinusoidal blood flow (10). Thus, HSC contribute to the portal hypertension occurring in cirrhotic patients. Current antifibrotic therapies are often not effective because of lack of cell specificity. Therefore, drug carriers targeted to these pivotal cells represent an attractive prospect for future therapies.

Liposomes are effective drug carriers because they can carry relatively large payloads. We designed a liposomal drug carrier to HSC by coupling mannose 6-phosphate-modified human serum albumin (M6P-HSA) to the surface of liposomes. In a rat model of liver fibrosis M6P-HSA was shown to accumulate in HSC (11) and the uptake of this protein by HSC was demonstrated to be mediated by mannose 6-phosphate/insulin like growth factor II (M6P/IGF II) receptor (12). In ﬁbrotic livers, HSC express an increased amount of the M6P/IGF II receptor (13) that is involved in the activation of transforming growth factor β (TGF-β) (14;15), a key profibrotic cytokine. Previously, we demonstrated a high degree of association of M6P-HSA liposomes with cultured HSC and we showed that these liposomes can be targeted to HSC in ﬁbrotic livers (16).

In the current in vivo studies, using bile duct ligated (BDL) rats as a model of liver ﬁbrosis, we determined the pharmacokinetic properties and the biodistribution of M6P-HSA liposomes in diseased animals. Subsequently, we analyzed whether next to HSC, KC and LEC play a role in the accumulation of M6P-HSA liposomes in BDL rats and tentatively characterised the receptors responsible for the in vivo uptake of these liposomes.

Materials and Methods

Chemicals

Cholesterol (Chol), N-succinimidyl-S-acetylthioacetate (SATA) and polyinosinic acid (poly I) were from Sigma (St. Louis MO, USA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-4-(maleimidophenyl)butyramide (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster AL, USA). (3H)cholesteryloleyl ether (3H-COE) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human serum albumin fraction V was from Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade or the best grade available.

Animals
Specified pathogen free (SPF) male Wistar or Wag/Rij rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to food and water. The presented studies were approved by the local committee for care and use of laboratory animals.

**Preparation of M6P-HSA**

Human serum albumin N-substituted with mannose 6-phosphate (M6P) groups was prepared and characterised as described before (11). The average number of M6P groups per albumin molecule was 29.

**Preparation of liposomes**

To prepare liposomes, appropriate volumes of POPC, CHOL and MPB-PE stock solutions in chloroform/methanol (9:1) were mixed to obtain a molar ratio 23:16:1. When indicated the non-degradable bilayer marker, \( ^3\text{H}-\text{COE} \) (0.25 µCi/µmol total lipid) was added. Liposomes were prepared as described before (17). The phospholipid phosphorus content of each liposome preparation was assessed by a phosphate assay (18). The total lipid concentration was calculated taking into account the amount of cholesterol in the lipidosomal preparation. Size and size distribution were determined by dynamic laser light scattering with a Nicomp submicron particle analyzer (NICOMP 380 ZLS, Santa Barbara, CA, USA). The mean diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. Liposomes without coupled protein were used in the experiments as control liposomes. M6P-HSA was coupled to liposomes prepared as described above by a coupling method using SATA, as reported before for aconitylated HSA (19). Phospholipid phosphorus, protein content (20) and particle size of M6P-HSA liposomes were analyzed again after protein coupling. Liposomes were stored under argon at 4°C and used within two weeks after preparation.

**In vivo experiments**

**Animal model of liver fibrosis**

Liver fibrosis was induced by bile duct ligation of male Wistar rats (500 g or 250 g) under anaesthesia with \( \text{O}_2/\text{N}_2/\text{O} \) (isoflurane, Abbott Laboratories Ltd., Queensborough, UK) according to standard procedures (21). When indicated, rats were used for in vivo experiments either one week (BDL-1) or three weeks (BDL-3) after bile duct ligation.

**Serum disappearance and organ distribution**

BDL-3 rats (250 g) were injected with \( ^3\text{H}-\text{COE} \) labelled liposomes at a dose of 1 µmol per 100 g of body weight via the penile vein under \( \text{O}_2/\text{N}_2/\text{O} \) anaesthesia. When indicated, rats were injected with unlabelled M6P-HSA or HSA (13 mg/kg of body weight) or poly I (16 mg/kg of body weight), 5 min before injection of liposomes. Blood samples were taken 5, 10, 20, 30 min and 1 h after injection of liposomes. The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = [0.0219 x body weight (g)] + 2.66 (22). The pharmacokinetic parameters were derived from measured serum concentrations using the program Multifit (23). The serum concentrations of uncoupled liposomes were fitted to a mono-exponential function and the appropriate pharmacokinetic parameters were derived. To calculate the pharmacokinetic parameters for serum elimination of targeted
targeting liposomes to HSC in vivo

liposomes, we assumed that the preparation of the targeted liposomes contains small amounts of uncoupled liposomes, which, after rapid clearance of targeted liposomes from the blood, could be still detected in the serum samples for 1h after injection. Therefore the serum concentrations of the targeted liposomes were fitted to a bi-exponential function and the pharmacokinetic parameters of the targeted liposomes were derived from the fastest exponential, assuming that the uncoupled liposomes were responsible for the slow second phase of the serum disappearance profile.

At the last time point, rats were sacrificed and liver, spleen, kidneys, lungs and heart were taken out for measurement of radioactivity as described before (19).

**HSC isolation**

BDL-1 rats of 500 g were used to ensure harvesting of sufficient amounts of HSC from the livers. Rats were injected with $^3$H-COE labelled liposomes at a dose of 2 μmol per 100 g of body weight via the penile vein under $O_2/N_2/O/Forene$ anaesthesia and after 1 h HSC were isolated as described before (24). Radioactivity associated with the isolated cells was determined by liquid scintillation counting. The purity of the isolated HSC was assessed by immunohistochemical staining with antibodies directed against specific markers for HSC. Briefly, aceton/methanol (1:1) fixed cells were simultaneously incubated with mouse monoclonal antibodies directed against desmin and α-smooth muscle actin (both from Sigma). Details of the staining procedure are described below. Positively stained cells were counted in ten microscopic fields, magnification x 400 using Image J program (http://rsb.info.nih.gov/ij/).

**Immunohistochemical analysis of liver sections**

To identify the cells that bind M6P-HSA liposomes in fibrotic livers, 2 μmol of M6P-HSA liposomes per 100 g of body weight were injected into BDL-3 rats (250 g) via the penile vein under $O_2/N_2/O/Forene$ anaesthesia. When indicated, rats were injected with poly I (16 mg/kg of body weight), 5 min prior to the administration of liposomes. Livers were harvested 20 min after injection of liposomes, snap frozen in isopentane and stored at -80°C until further processing.

Cryostat sections of fibrotic livers (4 μm) were fixed in acetone. Immunohistochemical staining was performed as described earlier (11). Briefly, M6P-HSA liposomes were stained with a rabbit polyclonal antibody directed against HSA (Cappel, Organon Teknika, Turnhout, Belgium). Endogenous peroxidase was inhibited with $H_2O_2$ and sections were incubated, first with peroxidase conjugated goat-anti-rabbit IgG (Dako Cytomation, Denmark), and then with peroxidase conjugated rabbit-anti-goat IgG (Dako). Antibody associated peroxidase was visualized with 3-amino-9-ethyl-carbazole (AEC). Sections were counterstained with Mayer’s hematoxylin.

When double immunostaining was performed, incubation of acetone fixed liver sections with antibody directed against HSA was followed by incubation with mouse monoclonal antibodies directed against cell proteins: desmin (Cappel), ED2 (Serotec Oxford, UK) and HIS 52 (a kind gift from Prof G. Molema UMCG, Groningen, The Netherlands) which are established markers for HSC, KC and LEC, respectively. After inhibition of endogenous peroxidase with $H_2O_2$ and incubation with two secondary peroxidase conjugated antibodies for HSA detection as described above, liver sections were incubated with alkaline phosphatase conjugated rabbit-anti-mouse IgG (Dako). Antibody-associated
alkaline phosphatase was visualized with Naphtol AS-MX/Fast Blue (30 min at 37 °C) and peroxidase activity with AEC (10 min at RT).

**Detection of SR-A expression by HSC**

**RNA isolation and RT-PCR**

HSC were isolated from livers of male Wistar rats (550-600 g) as described before (26). Isolated HSC were cultured in DMEM containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin for 3 (quiescent phenotype) or 10 days (activated phenotype). LEC and KC were isolated from livers of male Wag/Rij rats (200-250 g) as described before (16). LEC and KC were cultured in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and in case of LEC 10 ng/ml endothelial cell growth factor (Boehringer, Mannheim, Germany) for 2 days before being used in the experiments.

Total RNA from cultured HSC was isolated using Absolutely RNA Microprep kit (Stratagene, Amsterdam, The Netherlands) and from liver tissue of healthy and BDL-3 rats with RNeasy® Mini Kit (QIAGEN, Venlo, The Netherlands) both according to the protocol of the manufacturer. RNA was measured by NanoDrop® ND-1000 Spectrophotometer (Wilmington, DE, USA) and analysed qualitatively by gel electrophoresis. Subsequently, synthesis of first strand cDNA from total RNA was performed with SuperScript™ III RNase H - Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and 40 units RNaseOut inhibitor (Invitrogen) in a volume of 20 µl containing 250 ng random hexamers (Promega). The cDNA thus obtained was diluted with Millipore water to a concentration of 10 ng / µl. For the PCR reaction, 1 µl of primary-cell cDNA and 10 µl of liver-homogenate cDNA was applied in a total volume of 30 µl containing 2x ReddyMix™ PCR Master Mix (ABgene®, UK) and 10 µM primers. Primers for SR-A rat receptor were designed with the aid of Primer Designer from Scientific and Educational Software, based on the mRNA sequence for the rat scavenger receptor type A obtained from Genbank (Accession number XM_573919). The sequence of SR-A primers was as follows: forward 5'- ACTATAAATGGCTCCTCCGTTCA -3', reverse 5'- AGACTCTGCAAGGACTTGGAAT -3' (Biolegio BV, Nijmegen, The Netherlands), giving a 440 bp product. GAPDH (forward 5'-ACGGAAGGCCATGCCAGTGA-3', reverse 5'-ACATGTTCCAGTATGACTCT-3') (Biolegio BV) was used as house keeping gene (590 bp product). For isolated cells 26 cycles of reaction were carried out at 94°C for 5 min, 94°C for 30 s, 57°C for 45 s, 72°C for 45 s and 72°C for 7 min, and for liver homogenates 30 cycles were carried out using the GeneAmp® PCRSytem 9700 (Applied Biosystem). The polymerase chain reaction product was visualized on 1 % agarose gel containing ethidium bromide.

**Western Blot**

HSC and liver tissue from healthy and BDL-3 rats were homogenised in lysis buffer (0.02 M Tris-HCl, 5 mM EDTA, 2 mM EGTA, 0.1 M NaCl, 0.05% SDS, 0.5% NP-40, 10 µg/ml Protease Inhibitor Cocktail (Sigma)), separated by 10 % polyacrylamide gel electrophoresis (30 µg protein of HSC per lane and 60 µg of liver homogenates per lane) and electroblotted onto nitrocellulose membrane (Bio-Rad). SR-A (Santa Cruz Biotechnology Inc.) primary antibody was used to detect scavenger receptor class A. Blots were developed using a chemiluminescence detection system (Lumi-LightPLUS Western Blotting substrate, Roche Diagnosti).
Statistical analysis

Statistical analysis of differences was performed by a two-tailed unpaired Student’s t-test. Differences were considered significant when p < 0.05.

Results

Blood elimination and organ distribution

Blood disappearance of M6P-HSA liposomes in fibrotic rats 3 weeks after bile duct ligation (BDL-3) was compared to that of control liposomes of the same lipid composition but without surface grafted M6P-HSA. The average size of injected M6P-HSA liposomes was 102 ± 13 nm and per μmol of total lipid 46 ± 15 μg of M6P-HSA was coupled (n=9). Control liposomes had a diameter of 80 ± 2 nm (n=5). As early as 10 min after injection more than 90 % of the injected dose of M6P-HSA liposomes had disappeared from the blood circulation, while of control liposomes only 45 % was cleared at that time point (Fig. 1). One hour after injection, still 27 % of the control liposomes was found in the blood while M6P-HSA liposomes had disappeared almost completely.

In order to study the specificity of the fast blood elimination of M6P-HSA liposomes in BDL rats, free M6P-HSA was injected into rats prior to administration of liposomes. Blood clearance of M6P-HSA liposomes was significantly attenuated by an excess of M6P-HSA (Fig. 1). This resulted in an increase in the half life ($t_{1/2}$) of M6P-HSA liposomes in the circulation and in a decrease in the plasma clearance (CL) in M6P-HSA pre-treated rats (Table 1). An excess of M6P-HSA had no effect on the blood elimination of control liposomes. When BDL rats were injected with free HSA prior to the M6P-HSA liposome

Figure 1. Blood disappearance of M6P-HSA liposomes (●, ▼, ■) and control liposomes (○, Δ) in BDL rats that were either untreated (●, ○) or preinjected with M6P-HSA (13 mg/kg of body weight) (▼, Δ) or with HSA (13 mg/kg of body weight) (■). $^3$H-COE labelled liposomes were injected into BDL-3 rats, at indicated time points blood samples were taken and the radioactivity was measured as described in Materials and Methods. Data are presented as means ± SEM of 3 to 4 rats.
administration, the blood disappearance of these liposomes was not influenced (Fig. 1).

Table 1. Pharmacokinetic parameters of M6P-HSA liposomes and control liposomes in BDL-3 rats.

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<th>$t_{1/2}$ (min)</th>
<th>CL (ml/kg/min)</th>
<th>V (ml/kg)</th>
<th>AUC$_{(0 - \infty \text{ min})}$ (min $\times \mu$mol/ml)</th>
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<td>M6P-HSA liposomes</td>
<td>2.6 ± 0.2</td>
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<td>+ M6P-HSA</td>
<td>43.6 ± 7.4</td>
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<td>+ HSA</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>control liposomes</td>
<td>46.1 ± 1.5</td>
<td>0.65 ± 0.01</td>
<td>43.5 ± 0.7</td>
<td>15.3 ± 0.3</td>
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<tr>
<td>+ M6P-HSA</td>
<td>42.8 ± 2.2</td>
<td>0.71 ± 0.02</td>
<td>43.5 ± 1.2</td>
<td>14.2 ± 0.5</td>
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$^3$H-COE labelled M6P-HSA liposomes and control liposomes were administered at a dose of 10 μmol / kg body weight into anaesthetized BDL-3 rats. M6P-HSA or HSA (13 mg / kg of body weight) was injected 5 min before M6P-HSA liposomes. Blood samples were taken 5, 10, 20, 30 and 60 min after administration of liposomes and analyzed as described in Materials and Methods. Data are from fits over the mean of 3 rats ± SE. $t_{1/2}$ - half time; CL, plasma clearance; V, volume of distribution; AUC, area under the fitted concentration – time curve.

The organ distribution of M6P-HSA liposomes in BDL rats was determined 1 h after injection of liposomes. At this time point 71.0 ± 3.5 % of the injected dose of M6P-HSA liposomes had been taken up by the liver. The spleen accounted for 2.6 ± 0.2 % of the injected dose of M6P-HSA liposomes, while the lungs contained 3.2 ± 0.6 %. In other
targeting liposomes to HSC in vivo

organisms examined (heart and kidneys) uptake of M6P-HSA liposomes was less than 1%. Control liposomes showed higher uptake in the spleen (8.5 ± 1.2 % of the dose), but 50 % less uptake was observed in the liver when compared to M6P-HSA liposomes (Fig. 2).

In rats preinjected with free M6P-HSA, liver uptake of M6P-HSA liposomes was 15 % lower than in untreated rats while increased accumulation of M6P-HSA liposomes was observed in lungs of M6P-HSA pre-injected rats (Fig. 2).

Cellular localisation of M6P-HSA liposomes in fibrotic livers

The localisation of M6P-HSA liposomes was determined in livers of BDL-3 rats using immunohistochemical methods. The elongated morphological appearance of the immunostaining pattern observed following injection of M6P-HSA liposomes, with an antibody directed against HSA, indicated that these liposomes accumulate in non-parenchymal cells (Fig. 3A). By double-immunostainings we subsequently determined which of the non-parenchymal cell types contribute to the uptake of M6P-HSA liposomes. Liver sections were simultaneously stained for HSA and cell-specific markers for KC (ED 2), LEC (HIS 52) or HSC (desmin). Immunohistochemical analysis revealed that M6P-HSA liposomes co-localised with HSC (Fig. 3B), LEC (Fig. 3C) as well as with KC (Fig. 3D). In addition, to quantify the uptake of M6P-HSA liposomes in HSC in fibrotic livers, these cells were isolated from BDL-1 rats injected with radioactively labelled liposomes. Uptake of M6P-HSA liposomes in HSC-enriched fractions was almost 7-fold that of control

![Figure 3. Intrahepatic distribution of M6P-HSA liposomes in livers of BDL rats. M6P-HSA liposomes taken up by the liver were immunohistochemically stained with a specific antibody directed against HSA (A, red staining) as described in Materials and Methods. Identification of the liver cells responsible for M6P-HSA liposome uptake was done by double immunostaining using antibodies directed against HSA (red staining) to detect M6P-HSA liposomes and against specific cell markers (blue staining) to identify either HSC (desmin, B), LEC (HIS 52, C) or KC (ED 2, D). Arrows indicate double positive cells, original magnification of A x 100, of B, C, D x 400; cv, central vein. (E) Uptake of M6P-HSA liposomes and control liposomes in HSC enriched cell fractions isolated from fibrotic livers was determined one hour after injection of 3H-COE labelled liposomes into BDL-1 rats. HSC were isolated from the livers and the radioactivity was measured as described in Material and Methods. Data are presented as means ± SD of 3 experiments. *p< 0.05.]

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liposomes (Fig. 3 E). 70% of the cells in the HSC-enriched cell fraction were HSC, while KC and LEC accounted for the remainder of the cells in this HSC fraction, as assessed by immunohistochemical staining against ED2 and HIS 52 (data not shown).

Effect of polyinosinic acid on blood elimination and intrahepatic distribution of M6P-HSA liposomes in BDL rats

![Image of photomicrographs and graph]

The uptake of M6P-HSA liposomes in poly I treated rats compared to untreated rats (A vs. B, C vs. D). Note the reduced HSA/ED2 and HSA/HIS 52 double staining in poly I-treated rats compared to untreated rats (A vs. B, C vs. D), whereas HSA/desmin double staining is not affected by poly I injection (E vs. F).

(G) Effect of poly I on blood elimination and the pharmacokinetics of M6P-HSA liposomes in BDL-3 rats. 3H-COE labelled M6P-HSA liposomes were administered at a dose of 10 μmol / kg body weight into anaesthetized BDL-3 rats. Poly I (16 mg/kg of body weight) was injected 5 min before M6P-HSA liposomes. Blood samples were taken at indicated time points and analyzed as described in Materials and Methods. Data of blood elimination of M6P-HSA liposomes in poly I treated rats are presented as means ± SEM of 3 rats. Pharmacokinetic parameters of M6P-HSA liposomes in poly I treated rats are stemming from fits over the mean of 3 rats and given as ± SE. t_{1/2} - half life; CL, plasma clearance; V, volume of distribution; AUC, area under the fitted concentration – time curve.
liposomes by cultured HSC is mediated by M6P/IGF II receptors and most likely also by scavenger receptors (ScR) (16). Firstly, the M6P/IGF II receptor can recognise the M6P moieties of the modified albumin. Secondly, the phosphate residues of the sugar group confer multiple negative charges to M6P-HSA while positive charges of albumin are cancelled out. The polyanionic character of the molecule may classify it as a ligand for ScR class A. We hypothesised that accumulation of M6P-HSA liposomes in LEC and KC may be mediated through ScR class A, since they do not express significant levels of M6P/IGF II receptor (14;25) but are known to express different types of ScR, including ScR class A (26). To examine this, we pre-injected poly I, which is a competitive inhibitor of ScR class A (27), into BDL-3 rats injected with M6P-HSA liposomes. Blood elimination of liposomes was strongly inhibited in the poly I treated rats, compared to untreated animals (Fig. 4G). As a result, $t_{1/2}$ of M6P-HSA liposomes was significantly increased and CL decreased (Fig. 4G) compared to M6P-HSA liposomes in rats that were not pre-injected with poly I (Table 1). Immunohistochemical analysis of liver sections, 20 min after injection of M6P-HSA liposomes, revealed a distinct inhibition of the uptake of these liposomes by poly I in the liver that, however, did not lead to an increased uptake of liposomes elsewhere (data not shown). Further investigations into the cell types responsible for the uptake of M6P-HSA liposomes in poly I treated rats showed that accumulation of these liposomes in KC (Fig. 4A, B) and LEC (Fig. 6C, D) is strongly inhibited by poly I. We did not observe a prominent difference in the uptake of M6P-HSA liposomes by HSC between poly I treated and untreated rats (Fig. 4E, F).

The expression of ScR class A by quiescent HSC (qHSC) and activated HSC (aHSC)
was checked at the mRNA and protein level. In qHSC and aHSC levels of ScR class A mRNA were comparable to that of KC and LEC (Fig. 5 A). However, Western blot analysis showed that qHSC abundantly express ScR class A while this protein was absent in aHSC (Fig. 5 B). ScR class A could also be detected in liver homogenates of healthy and fibrotic organs. The ratio of ScR class A to β-actin used as house keeping protein for qHSC was 0.72 while for aHSC 0.002.

Discussion

In the present study we characterised the targeting properties of M6P-HSA liposomes in a rat model of liver fibrosis induced by bile duct ligation. We showed that M6P-HSA liposomes are rapidly cleared from the blood circulation in diseased rats and mainly accumulate in the liver. This fast elimination of M6P-HSA liposomes from the blood by the target organ and the limited accumulation in other organs demonstrate that the organ distribution profile of M6P-HSA liposomes is highly suitable to deliver drugs to a diseased liver. The prolonged blood circulation time of M6P-HSA liposomes in animals treated with M6P-HSA in combination with lung injury that has been reported in BDL rats may have caused increased lung uptake in these rats (28). Particularly, the development of pulmonary intravascular macrophages (PIM) in the lungs of BDL rats may play a role in this elevated accumulation. Similarly, a shift in uptake from the liver to the lungs caused by PIM was observed in BDL rats injected with adenoviral particles (29;30). The rapid blood clearance of M6P-HSA liposomes was blocked by pre-injection of M6P-HSA, but not by HSA, indicating a specific recognition of mannose 6-phosphate groups by cells within the liver.

In the livers of BDL rats, collagen deposition predominately surrounds the proliferating bile ducts and to a lesser extent is localized in zones 2 and 3 (periportal and portal area) of the liver. M6P-HSA liposomes mainly distribute to zones 2 and 3 i.e. the areas containing relatively low amounts of collagen. The regions of the liver characterized by massive deposition of extracellular matrix proteins were clearly less accessible to liposomes. Changes in the hepatic architecture associated with fibrosis most likely interfere with the distribution of M6P-HSA liposomes within the liver. In the healthy liver, endothelial cells are fenestrated with pores of 150 to 175 nm in diameter (31), that enable a close contact between blood and the space of Disse where HSC are situated. In the fibrotic liver, endothelial cells at least partly lose their fenestrations (32) and, in addition, the contraction of HSC causes an increased vascular resistance in the sinusoids (10). As a result, the highly fibrotic areas of the liver may not be readily accessible to macromolecules. The size of M6P-HSA liposomes (approximately 100 nm) allows them to pass fenestrations in endothelial cells in those parts of the fibrotic livers where they are still present and functional.

The high accumulation of M6P-HSA liposomes, but not of control liposomes in HSC-enriched fractions isolated from BDL rat livers, demonstrates that M6P-HSA liposomes are taken up by HSC in fibrotic livers, whereas untargeted liposomes are not. These data are in agreement to our previous study in which we demonstrated a 3,5-fold higher uptake of M6P-HSA liposomes as compared to control liposomes in cultured activated HSC (16).

Immunohistochemical examination revealed that M6P-HSA liposomes also accumulate in two other major non-parenchymal cells populations: liver endothelial cells (LEC)
targeting liposomes to HSC in vivo

and Kupffer cells (KC). It is known that activated HSC express an increased number of M6P/IGF II receptors (13;14). In contrast, no significant levels of M6P/IGF II receptor expression were observed on LEC (14) and KC (25). Therefore, in the recognition of M6P-HSA liposomes by KC and LEC scavenger receptors (ScR) might be involved. ScR are known to bind anionic (macro)molecules and are abundantly expressed by KC and LEC. Ongoing studies in our laboratory, showed that polyinosinic acid (poly I), an established competitive inhibitor of ScR class A strongly prevented the rapid blood clearance of M6P-HSA liposomes and exerted an inhibitory effect on the association of these liposomes with KC and LEC in fibrotic livers. Uptake of M6P-HSA liposomes by HSC was still observed in poly I treated rats (Fig. 5 F), which indicates that liposomes are recognised by other type of receptor on these particular cells, i.e. M6P/IGF II receptors.

However, we cannot exclude the presence of scavenger receptor class A on HSC. We reported before, that cultured HSC take up liposomes modified with polyanionic albumin, aconitylated HSA and that this process can be inhibited by poly I (16). In the present study, we demonstrated that at the mRNA level ScR class A was present in both quiescent and activated HSC while at the protein level the expression of ScR class A decreased in the activated cells. The molecular weight of ScR class A found in the Western blot analysis was slightly lower (64 kDa) than indicated by manufacturer of the antibody (75 kDa). However, different molecular masses (72 and 65 kDa) of mature bovine ScR class A type II receptor have been reported (33). Although M6P/IGF II receptors and scavenger receptors considerably contribute to the uptake of M6P-HSA liposomes, involvement of other receptors cannot be ruled out.

The development of liver fibrosis is a complex process, in which different types of cells are involved. The initial step, the impairment of hepatocytes, results in the release of reactive oxygen species and fibrotic and inflammatory cytokines stimulating in turn KC, LEC and HSC, as well as in recruitment of cells of the immune system. KC, LEC and inflammatory cells further promote activation of HSC, leading to transformation of these cells into an activated phenotype characterised by a high proliferation rate, production of collagen and mediators of fibrosis, which in an autocrine manner maintain the activated status of stellate cells (9). The uptake of M6P-HSA liposomes by KC and LEC, next to the uptake in HSC has to be taken into account when an antifibrotic drug is chosen for encapsulation into M6P-HSA liposomes. Basically, there are two possibilities to deal with this matter. Firstly, one could ensure that the compounds that are encapsulated in M6P-HSA liposomes do have therapeutic effects on HSC, but are not harmful to LEC or KC. Since HSC are the major producers of collagen in the fibrotic liver, inhibitors of its production would seem to be suitable candidates for incorporation into M6P-HSA liposomes. Secondly, one could aim at encapsulating a drug that influences the profibrotic and proinflammatory functions of HSC, KC and LEC altogether. Therefore, M6P-HSA liposomes that selectively accumulate in all the relevant regulatory cells of the fibrotic process could be suitable carriers for anti-inflammatory and antifibrotic drugs. Antioxidants such as quercetin and resveratrol inhibit proliferation and activation of HSC, and in addition decrease the production of nitric oxide (NO) and tumor necrosis factor α (TNF-α) by KC (34). Alternatively, thiazoldinediones, which are peroxisome proliferator-activated receptor-γ agonists, inhibit HSC collagen production (35) as well as proliferation and chemotaxis of these cells (36). In KC, these compounds reduce TNF-α and NO production (37). This strategy for attenuation of liver fibrosis may be efficient, since the complexity of the disease suggests that a simul-
taneous interference with multiple pathways of the fibrotic process may be the most successful antifibrotic approach.

In conclusion, we have shown that liposomes coupled with M6P-HSA are effective drug carriers, targeted to the non-parenchymal cells, including HSC, which play a crucial role in the development of liver fibrosis. The recognition of M6P-HSA liposomes by HSC, LEC and KC in the fibrotic liver is M6P-HSA specific, involving both M6P/IGF II and scavenger receptors and opens up new possibilities for pharmacological interference in liver fibrosis.

Acknowledgement

The authors thank Dr. H. Proost (University of Groningen) for helpful discussions and suggestions concerning the pharmacokinetics analysis and H. M. Morselt for isolation of Kupffer cells and endothelial cells.

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Active compounds that are stable in pure, chemically graded solutions might change into a nightmare when put in a biological environment. For quercetin, the potential for beneficial effects on the activation of stellate cells never materialized because of problems with the estimation of its concentration and its accommodation within liposomes.
chapter 4

Effects of bioactive liposomes on cultured hepatic stellate cells and liver fibrosis in bile duct ligated rats

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Abstract

Purpose: To evaluate effects of liposomes containing the bioactive lipid dilinoleoylphosphatidylcholine (DLPC) on hepatic stellate cells (HSC) activation and liver fibrosis.

Methods: To target DLPC-liposomes to HSC, human serum albumin modified with mannose 6-phosphate (M6P-HSA) was coupled to the surface of these liposomes. In vitro, the effects of the carrier were determined in primary cultures of HSC, Kupffer cells and liver endothelial cells using real-time RT-PCR. In vivo DLPC-liposomes were tested in bile duct ligated rats.

Results: Targeted M6P-HSA-DLPC-liposomes and DLPC-liposomes significantly reduced gene expression levels for collagen 1α1, α-smooth muscle actin (α-SMA) and transforming growth factor-β (TGF-β) in cultured HSC. In fibrotic livers, DLPC-liposomes decreased gene expression for TGF-β and collagen 1α1 as well as α-SMA and collagen protein expression. In contrast, M6P-HSA-DLPC-liposomes enhanced expression of profibrotic and proinflammatory genes in vivo. Up-regulation of inflammatory genes was also observed in cultured Kupffer and endothelial cells. Both types of liposomes increased hepatocytes glycogen content in fibrotic livers.

Conclusions: DLPC-containing liposomes attenuate activation of cultured HSC. In fibrotic livers, M6P-HSA mediated activation of Kupffer and endothelial cells probably counteracts this beneficial effect of DLPC-liposomes. We conclude that these bioactive drug carriers modulate the activity of all liver cells during liver fibrosis.
Introduction

Acute or chronic liver injury may lead to development of fibrosis, a process in which hepatic stellate cells (HSC) play a major role. As a result of liver injury, HSC, which in the healthy organ store vitamin A, undergo a process of activation that is mediated by the concerted action of resident hepatic cell-types such as Kupffer cells (KC), liver endothelial cells (LEC) and hepatocytes. The phenotype of activated HSC resembles that of myofibroblasts and is characterised by α-smooth muscle actin (α-SMA) expression, intensive synthesis of extracellular matrix proteins, mainly collagen type I and type III and a high rate of proliferation (1;2). In addition to this, activated HSC secrete profibrotic and proinflammatory mediators which, in an autocrine manner, perpetuate the activated state of HSC and attract immune cells from the bloodstream (3;4). Also, the contractile features of activated HSC are the basis for their pivotal role in the portal hypertension, which is a major clinical characteristic of this disease (5).

Any antifibrotic therapy that would uniquely and specifically address the HSC population would signify a major breakthrough in the therapeutic treatment of this disease. Although current antifibrotic drugs which aim at HSC often show promising effects in vitro, their effectiveness is rather limited when tested in vivo. A strategy to enhance the delivery of such drugs to the relevant cells within the liver can be expected to lead to considerable improvement of the success of such treatments. Liposomes, biodegradable lipid nanospheres which can encapsulate drugs with high efficiency, are considered to represent a suitable and versatile drug carrier system. Recently, we showed efficient targeted delivery of liposomes to HSC in the fibrotic liver by coupling human serum albumin modified with mannose 6-phosphate groups (M6P-HSA) to the liposomal surface (6).

It has been reported that dilinoleoylphosphatidylcholine (DLPC) has beneficial effects on cultured HSC. DLPC reduces the activation and proliferation of HSC (7-9). In addition, DLPC decreases tumour necrosis factor-α (TNF-α) production by Kupffer cells (10;11). Liposomes with DLPC as a major constituent might therefore function as a bioactive drug carrier which can deliver drugs and simultaneously have beneficial antifibrotic effects. In the present study we therefore incorporated DLPC into the lipid bilayer of liposomes. In order to target them to HSC, the surface of DLPC-containing liposomes was modified with M6P-HSA. We evaluated the antifibrotic properties of this new drug carrier on cultured HSC, by testing its effects on the expression of profibrotic genes. In addition, we investigated whether DLPC-containing liposomes can modulate gene expression in cultured KC and LEC. The influence of DLPC-containing liposomes on the fibrotic process in the liver was evaluated in a bile duct ligation rat model of liver fibrosis.

We provide evidence that a DLPC-containing liposome is endowed with antifibrotic properties and thus may, while serving as a carrier for other antifibrotic compounds, be itself bioactive.

Material and Methods

Chemicals

Cholesterol (Chol) and N-succinimidyl-S-acetyltioacetate (SATA) were from Sigma (St. Louis MO, USA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(maleimidophenyl)butyramide] (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster AL, USA). Human serum albumin fraction V was from
Sanquin (Amsterdam, The Netherlands). Dulbecco’s modified Eagles Medium (DMEM) and L-glutamine were obtained from Invitrogen (Paisley, Scotland, UK), foetal calf serum (FCS) from BioWhittaker Europe (Verviers, Belgium), penicillin and streptomycin from Sigma. All other chemicals were of analytical grade or the best grade available.

Animals

Specified pathogen free (SPF) male Wistar rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to food and water. The presented studies were approved by the local committee for care and use of laboratory animals.

Preparation of M6P-HSA

Synthesis of human serum albumin modified with mannose 6-phosphate groups was performed as described before (12).

Preparation of liposomes

Liposomes composed of either POPC/CHOL/MPB-PE or DLPC/CHOL/MPB-PE in a molar ratio 23:16:1 were prepared as reported before (13). Briefly, the lipids were taken from chloroform : methanol (9:1) stock solutions, organic solvent was evaporated under nitrogen, cyclohexan was added and the lipid solution was lyophilised overnight. The dried powdery lipids were hydrated in HN buffer (10 mM HEPES, 135 mM NaCl) pH 6.7 for 1 h at room temperature. Liposomes were sized by extrusion through polycarbonate filters of 50-nm pore size. Size of liposomes was measured using dynamic laser light scattering with a Nicomp submicron particle analyzer (NICOMP 380 ZLS, Santa Barbara, CA, USA). The diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. The lipid concentration of each preparation of liposomes was determined by phosphate assay (14). The total lipid concentration was calculated taking into account the amount of cholesterol in the liposomal preparation. Coupling of M6P-HSA to liposomes was performed according to the method described before (15). Shortly, SH groups were introduced into the M6P-HSA using the SATA. Liposomes were incubated with SATA-modified protein for 4 h at room temperature; the coupling reaction was stopped by adding N-ethylmaleimide (Sigma). Uncoupled M6P-HSA was removed by ultracentrifugation (2 x 2h at 40 000 rpm, 4°C) in Opti-PrepTM (Axis-Shield PoCAS, Oslo, Norway) and dialysed overnight against HN buffer pH 7.4. After coupling M6P-HSA, liposomes were again characterised by size measurement and lipid concentration. The amount of coupled protein was determined according to Peterson-Lowry (16). Liposomes were stored at 4°C under argon and used for experiments within 3 weeks after preparation.

Isolation and culture of HSC, KC and LEC

HSC were isolated from livers of male Wistar rats (550-600 g) as described before (17). Isolated HSC were cultured in DMEM containing 10 % FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Directly after isolation, for experiments 3 days after cell isolation with HSC that have a quiescent phenotype (HSC d-3), cells were seeded on plates as indicated below. For experiments performed 10 days after isolation (activated phenotype,
HSC d-10), cells were cultured in the 75 cm² culture flasks (Corning) and two days before start of the experiments cells were trypsinized and seeded in plates as indicated below. KC and LEC were isolated from livers of male Wag/Rij rats (200-250 g) (Harlan) as described before (13). KC and LEC were cultured in RPMI-1640 medium supplemented with 20 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and in case of LEC with 10 ng/ml endothelial cell growth factor (Boehringer, Mannheim, Germany). KC and LEC (initially 350000 cells/cm²) were cultured for 2 days on 12-wells plates (Costar) and 24-wells collagen-coated plates (Greiner) respectively, before being used in the experiments.

Effects of DLPC liposomes or linoleic acid on gene expression levels in cultured cells
HSC were seeded on 12-wells plates (HSC d-3, 200 000 cells/well and HSC d-10, 125 000 cells/well), KC and LEC were plated as described in the preceding paragraph. On the day of the experiment, cells were washed with culture medium without FCS and subsequently incubated for 24 h with culture medium containing 5 % FCS supplemented with liposomes at a concentration of 320 nmol lipid/ml or 30 µg/ml M6P-HSA or with 0.1mM linoleic acid (Sigma) diluted in ethanol. The incubation was stopped by washing the cells twice with PBS and cells were lysed with Lysis Buffer provided with the Absolutely RNA MicroPrep kit (Stratagene, Amsterdam, The Netherlands). Collected samples were stored at -80°C until further analysis.

Viability assay
HSC were seeded in 96-wells plates (HSC d-3, 10 000 cells/well, HSC d-10, 5 000 cells/well). On the day of the experiment, cells were washed with culture medium without FCS and liposomes (final concentration of 320 nmol/ml) or 30 µg/ml (M6P-HSA) were added to the culture medium containing 5 % FCS. After 4 h of incubation, AlamarBlueTM reagent (Serotec Ltd) was added (10 % of the total volume) and the incubation was continued for another 20 h. Subsequently, the incubation mixture was transferred into a white 96-wells plate (Costar) and fluorescence was measured on Perkin Elmer LS 50B (Perkin-Elmer Ltd. Beaconsfield, Buckinghamshire, England)

Effects of DLPC liposomes on liver fibrosis in BDL rats
To induce liver fibrosis, bile duct ligation (BDL) was performed in male Wistar rats (250 g) under anaesthesia with O₂/N₂O/Forene (isoflurane, Abbott Laboratories Ltd., Queensborough, UK) according to standard procedures (18). Ten days after bile duct ligation, rats were treated with M6P-HSA DLPC-liposomes, DLPC-liposomes, M6P-HSA POPC-liposomes or POPC-liposomes at a dose of 3 µmol of lipid per 100 g of body weight. Control animals were injected with PBS. After 24 h, rats were killed and samples of the livers were snap frozen in isopentane and stored at -80°C for further immunohistochemical and real-time RT-PCR analysis.

Histological analysis
Immunohistochemical staining
Sections of the livers (4 μm) were fixed with acetone, rehydrated in PBS and incubated either with primary mouse antibodies directed against α-smooth muscle actin (Sigma) or with primary goat antibodies directed against collagen type I and type III (Southern
Biotechnology Associate Inc.). Endogenous peroxidase activity was inhibited by a 0.075% solution of hydrogen peroxide. Sections were incubated with peroxidase-conjugated secondary antibodies; rabbit-anti-mouse (Dako Cytomation, Denmark) for α-SMA and rabbit-anti-goat (Dako) for collagen type I and III. Sections were subsequently incubated with peroxidase-conjugated second secondary antibody; goat-anti-rabbit (Dako). Antibody-associated peroxidase activity was visualized with 3-amino-9-ethyl-carbazole (AEC) (Sigma) and sections were counterstained with Mayer’s hematoxylin. Semi-quantitative analysis of liver sections immunostained for either collagen type I and III or α-SMA was performed using the custom Matlab code (www.mathworks.com). For analysis, 10 power fields of a liver section of each rat at magnification x 40 were analyzed. The total stained area was set to be equal to the fraction of pixels above a threshold value, typically between 0.950 and 0.965, that left some room for visual matching to the observed staining patterns.

**PAS staining**

Frozen sections of the livers (4 μm) were fixed in a methanol: formalin (9:1) solution. Subsequently, sections were incubated in 1% periodic acid and Schiff’s reagent (Merck). Nuclei were counterstained with hematoxylin.

PAS staining of liver sections was scored semi-quantitatively in a double blind test, where the following scores were given for the percentage of stained hepatocytes: 1 when 0 – 25%; 2 when 25 – 50%; 3 when 50 – 75% and 4 when 75 – 100% of the liver section was stained for PAS.

**RNA isolation and real-time RT-PCR analysis**

Total RNA from cultured HSC was isolated using the Absolutely RNA Microprep kit (Stratagene, Amsterdam, The Netherlands) and from liver tissue with the RNeasy® Mini Kit (QIAGEN) both according to the protocol of the manufacturer. The amount of RNA was measured by NanoDrop® ND-1000 Spectrophotometer (Wilmington, DE) and analysed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with SuperScript™ III RNase H - Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and 40 units RNaseOut inhibitor (Invitrogen) in a volume of 20 µl containing 250 ng random hexamers (Promega). Obtained cDNA was diluted with Millipore water to a concentration of 10 ng / µl and 1 µl was applied for each PCR reaction. The following primers purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands) were used for real-time PCR: collagen 1α1 (Rn01463848_m1), TIMP-1 (tissue inhibitor of matrixmetalloproteinase-1, Rn00587558_m1), TGF-β (Rn00572010_m1), TNF-α (Rn00562055_m1). α-SMA primer was ordered as Assays-by-Design (Applied Biosystems). GAPDH was used as a house keeping gene (Rodent GAPDH Control Reagent, Applied Biosystems). The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA) with a final concentration of 200 nM GAPDH primers and 250 nM for primers of the other genes studied. The amplification reaction was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C and 40 two-steps cycles of 15 s at 95°C and 60 s at 60°C. For each sample, the real-time PCR reaction was performed in triplicate and the averages of the obtained threshold cycle values (Ct) were processed for further calculations according to
the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com). Briefly, gene expression levels were normalized to the expression of the housekeeping gene GAPDH giving the ΔCt value. Then the average value of ΔCt obtained from non-treated HSC or PBS treated rats was subtracted from the average of the ΔCt value of each sample, yielding the ΔΔCt value. Finally, the gene expression level was calculated as $2^{-\Delta\Delta C_t}$ giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied genes.

**Statistical analysis**

Statistical analysis of differences was performed by a two-tailed unpaired Student’s t-test. Differences were considered significant when $p < 0.05$.

**Results**

**Liposomes characterisation**

In M6P-HSA used for the preparation of targeted liposomes, 29 out of the 60 ε-amino groups of human serum albumin were modified with mannose 6-phosphate moieties. Liposomes containing POPC were used as control liposomes. Therefore, the lipid composition and lipid molar ratio was the same as in DLPC liposomes, except that DLPC was replaced with POPC. Preparations of DLPC and POPC containing liposomes were reproducible and yielded liposomes comparable in size as well as in amount of protein-coupled (Table 1).

**Effect of DLPC liposomes on the profibrotic gene expression levels in HSC**

<table>
<thead>
<tr>
<th>Type of liposomes</th>
<th>coupled M6P-HSA [µg protein/µmol TL]</th>
<th>size [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6P-HSA-DLPC-L</td>
<td>25 ± 17</td>
<td>81 ± 11</td>
</tr>
<tr>
<td>DLPC -L</td>
<td></td>
<td>75 ± 9</td>
</tr>
<tr>
<td>M6P-HSA-POPC-L</td>
<td>40 ± 10</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>POPC-L</td>
<td></td>
<td>76 ± 7</td>
</tr>
</tbody>
</table>

*Size of liposomes and the amount of M6P-HSA coupled to the liposomes was determined as described in the Materials and Methods section. Data are presented as the mean ± S.D of 6 liposome preparations. L, liposomes; TL, total lipid.*

The effects of DLPC-containing liposomes on profibrotic gene expression levels were studied in quiescent and activated HSC. During culturing on plastic, HSC spontaneously activate (19). Therefore we performed experiments with cells cultured for 3 days (HSC d-3) or 10 days (HSC d-10), representing cells with a quiescent or activated phenotype, respectively. The phenotype of the cells was routinely checked to confirm their state of activation. The expression of collagen 1α1 in HSC d-10 increased 4.8 ± 0.94 fold when compared to HSC d-3 while TGF-β expression was 1.5 ± 0.24 fold higher compared to the expression in HSC d-3. However, mRNA levels of α-SMA were the same in HSC d-10 and HSC d-3.
Figure 1. Effects of DLPC-containing liposomes on HSC. Gene expression of TGF-β, collagen 1α1 and α-SMA was determined in HSC d-3 (A) and HSC d-10 (B) using real-time RT-PCR after 24 h incubation with M6P-DLPC-L, DLPC-L, M6P-POPC-L, POPC-L at a concentration of 320 nmol lipid/ml or 30 µg/ml M6P-HSA as described in Materials and Methods. Expression of the genes by the cells incubated without any addition was set to 1. Data are presented as ± SEM of 3 to 4 experiments, * p < 0.05, ** p < 0.01, *** p < 0.001 versus control. L, liposomes
Both in HSC-3 and HSC-10 substantial inhibition of all three genes that reflect HSC activation was achieved only with the liposomal formulations containing DLPC (Fig. 1). The highest levels of inhibition by DLPC were observed for the M6P-HSA coupled DLPC liposomes, especially for the collagen 1α1 and α-SMA genes. Some inhibition of the expression of these genes in the HSC-3 cells was also observed with the M6P-HSA liposomes without DLPC. In these cells, free M6P-HSA had a small but not significant effect on the expression of collagen 1α1 and α-SMA. Expression of the TGF-β gene was least affected in all cases. Plain liposomes without DLPC had no effect whatsoever.

To check whether the reduced expression levels of studied genes is not the result of reduced viability of the cells, we studied the influence of DLPC-containing liposomes on the viability of HSC d-3 and HSC d-10. The viability of the cells after 24 h incubation with the various liposomal formulations showed little if any effects by most of liposomal formulations tested (Table II). A viability decrease was found only for the HSC d-10 cells incubated with untargeted DLPC liposomes. Incubation with both the M6P-HSA targeted and the untargeted DLPC-containing formulations caused a small but significant increase in the viability of the HSC d-3. Variation in cell viability can therefore not explain the reduced gene expression levels in these cells.

In a preliminary approach to clarify the mechanism by which the DLPC might exert its down-regulatory effect on profibrotic gene expression, we determined the effect of unesterified linoleic acid on the expression of these genes in HSC d-10. At the concentration of linoleic acid 0.1 mM which is comparable with that of DLPC in liposomes, the fold induction of collagen 1α1, α-SMA and TGF-β compared to vehicle control set at 1 was 0.77 ± 0.16, 0.93 ± 0.19 and 0.79 ± 0.19, respectively.

### Table 2. Effects of DLPC-containing liposomes on viability of HSC.

<table>
<thead>
<tr>
<th>type of liposomes</th>
<th>viability of the cells [% of control]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HSC d-3</td>
</tr>
<tr>
<td>control cells</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>POPC-L</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>M6P-HSA-POPC-L</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>DLPC-L</td>
<td>112 ± 3*</td>
</tr>
<tr>
<td>M6P-HSA-DLPC-L</td>
<td>128 ± 4***</td>
</tr>
</tbody>
</table>

Effects of M6P-HSA-DLPC-L, DLPC-L, M6P-HSA-POPC-L and POPC-L (320 nmol lipid/ ml) on HSC d-3 and HSC d-10 viability were determined after 24 h incubation as described in Material and Methods. Viability of the cells incubated without addition of liposomes was set at 100%. Data are presented as ± SEM of 3 experiments, * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) versus control. L, liposomes.
Effects of DLPC-containing liposomes on liver fibrosis in BDL rats

To examine whether liposomes containing DLPC attenuate the fibrotic processes within the liver, we performed a study using bile duct ligated rats as a model of liver fibrosis. Measurements of bilirubin, alanine aminotransferase, aspartate aminotransferase and γ-glutaryl transpeptidase serum concentrations confirmed that all rats equally developed an intrahepatic disease process (data not shown). Analysis of profibrotic gene expression levels 24 h after injection of liposomes revealed that in rats treated with untargeted DLPC-containing liposomes the expression levels of TGF-β and collagen 1α1 were reduced by 70 % and 20 %, respectively (Fig 2). In contrast, M6P-HSA DLPC liposomes slightly up-regulated the expression of collagen 1α1 and α-SMA while TGF- β expression was not affected.

Expression of collagen types I and III as well as of α-SMA in BDL rats treated with liposomes was also investigated at the protein level. Immunohistochemical analysis of liver sections showed a reduction of collagen type I and III and α-SMA by 40 % and 30 % respectively, in rats treated with untargeted DLPC liposomes as compared to PBS-treated animals. None of the other liposomal formulations significantly affected the expression level of either collagen or α-SMA (Fig. 3).

Since prolonged damage to hepatocytes initiates the fibrotic process in the liver, we investigated the effect of different liposomes on these cells in the fibrotic livers of BDL rats. Detection of glycogen stored in hepatocytes showed that the number of hepatocytes containing glycogen in the livers of rats treated with DLPC liposomes and M6P-HSA-DLPC liposomes was higher than in control rats injected with PBS (Fig. 4). Control liposomes did not induce any effect on glycogen storage in hepatocytes.

Figure 2. Expression of profibrotic genes in livers of BDL rats treated with DLPC-containing liposomes.
Rats were injected with M6P-HSA DLPC-L, DLPC-L, M6P-HSA POPC-L and POPC-L at a dose of 3 µmol of liposomes per kg of body weight. Expression of TGF-β, collagen 1α1 and α-SMA in livers was determined using real-time RT-PCR 24 h after injection of liposomes as described in Materials and Methods. Gene expression levels in rats treated with PBS were set at 1. Data are presented as ± SEM of 4 to 5 rats, * p < 0.05, *** p < 0.001 versus control. L, liposomes.
Figure 3. Effects of DLPC-containing liposomes on protein expression of collagen type I, III and α-SMA in livers of BDL rats. Photomicrographs represent liver sections immunostained for collagen type I and III (A – E) and α-SMA (G – K) of rats treated with PBS (A, G), M6P-HSA-DLPC-L (B, H), DLPC-L (C, I), M6P-HSA-POPC-L (D, J) or POPC-L (E, K). Immunostaining of collagen type I, III (F) and α-SMA (L) was semi-quantitatively analysed as described in Materials and Methods. Rats treated with PBS were set at 100 % and results are presented as % of stained area per section from 3 to 5 rats ± SEM. Original magnification x 40. L, liposomes.
chapter 4

Effects of DLPC-containing liposomes on proinflammatory genes expression in livers of BDL rats and primary cultured Kupffer cells and liver endothelial cells

Our previous studies on the uptake of M6P-HSA targeted liposomes by hepatic cells in fibrotic livers showed that they not only accumulate in HSC but also in Kupffer cells (KC) and liver endothelial cells (LEC). We hypothesised that the accumulation of M6P-HSA targeted DLPC liposomes in KC and LEC may activate these cells and stimulate the fibrotic process. To investigate this, we analyzed the expression of proinflammatory cytokines such as tumour necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1) and interleukin 6 (IL-6) in BDL rats treated with liposomes. Expression levels of MCP-1 and TNF-α were up-regulated 145 % and 25 %, respectively by M6P-HSA-DLPC liposomes (Fig. 5A). Interestingly, in rats injected with DLPC liposomes, mRNA level of TNF-α was down-regulated by 30 % as compared to untreated rats.

We also tested the influence of DLPC-containing liposomes on TNF-α, MCP-1 and IL-6 expression by primary cultured rat KC and LEC. After 24 h incubation of these cells with liposomes, we observed that M6P-HSA-DLPC liposomes had significantly decreased the expression of MCP-1 and TNF-α in KC, while a 4-fold up-regulation of IL-6 was observed in these cells (Fig. 5 B). Similarly, M6P-HSA-DLPC liposomes caused up-regulation of IL-6 in LEC (Fig. 5 C). Expression levels of TNF-α, MCP-1 and IL-6 in KC and LEC were down-regulated by DLPC liposomes. M6P-HSA-POPC liposomes stimulated IL-6 and MCP-1 expression in both KC and LEC, whereas POPC liposomes inhibited expression of the genes studied. So, whereas DLPC and POPC liposomes reduced expression levels of TNF-α, MCP-1 and IL-6 in KC and LEC, coupling of M6P-HSA to these liposomes increased the expression levels most of these proinflammatory genes.

**Figure 4. Effects of DLPC-containing liposomes on hepatocyte glycogen storage in livers of BDL rats.** Liver sections of BDL rats treated with PBS (A), M6P-HSA-DLPC-L (B), DLPC-L (C), M6P-HSA-POPC-L (D) and POPC-L (E) were stained with PAS. Semi-quantitative analysis of stained sections (table) was performed as described in Materials and Methods. Data are presented as the median value ± SEM of 4 to 5 rats, * p < 0.05 versus PBS treated rats. pink – glycogen, blue – nuclei, original magnification x100. L, liposomes.
a bioactive drug carrier to the fibrotic liver

**Discussion**

DLPC is the major compound of polyenylphosphatidylcholines extracted from soybeans and has been shown to be biologically active (20;21). Studies in animal models of liver fibrosis showed that supplementation of the food with polyenylphosphatidylcholines has

![Figure 5. Comparison of the effects of DLPC-containing liposomes on proinflammatory gene expression in fibrotic livers and primary cultures of KC and LEC. (A) Gene expression of MCP-1, IL-6 and TNF-α in livers of BDL rats injected with different liposomal formulations: M6P-HSA-DLPC-L, DLPC-L, M6P-HSA-POPC-L and POPC-L. Gene expression levels in rats treated with PBS were set at 1. Data are presented as ± SEM of 4 to 5 rats, * p < 0.05, *** p < 0.001 versus control. Gene expression of MCP-1, IL-6 and TNF-α was measured in KC (B) and LEC (C) using real-time RT-PCR after 24 h incubation with M6P-HSA-DLPC-L, DLPC-L, M6P-HSA-POPC-L, POPC-L at the concentration of 320 nmol lipid/ml as described in Materials and Methods. Expression of the genes by KC or LEC incubated without liposomes was set at 1. Data are presented as ± SEM of 3 experiments, * p < 0.05, ** p < 0.01, *** p < 0.001 versus control. L- liposomes.
beneficial effects on the fibrotic liver. In addition, it was demonstrated in vitro in rat HSC that DLPC reduces the activation of HSC by down-regulating α-SMA and α1(I) procollagen gene expression (7;9) and that it inhibits HSC proliferation induced by platelet derived growth factor (22). Recently, it was reported that DLPC prevents production of tissue inhibitor of matrix metalloproteinase I (TIMP-1) in a human hepatic stellate cell line stimulated with leptin (23). Moreover, DLPC decreases lipopolysaccharide (LPS) – and acetaldehyde – induced TNF-α production in KC isolated from ethanol fed rats (10;11). In these studies, DLPC was shown to act as an antioxidant and to interfere at the molecular level with mitogen-activated protein kinases (MAPK) signalling pathways in HSC and KC.

By incorporating the bioactive phospholipid DLPC in the bilayer of liposomes we made an attempt to design a drug carrier system which, when properly targeted, could simultaneously deliver an encapsulated antifibrotic drug as well as the bioactive lipid to the HSC population in fibrotic livers. Since liver fibrosis is a complex disease involving activation of HSC via different pathways, most likely more than one therapeutic compound needs to be delivered to the livers. Bioactive drug carriers may provide such and opportunity. To target our carrier to HSC in the fibrotic livers we coupled M6P-HSA to the surface of liposomes (6).

In our experiments, M6P-HSA-DLPC and DLPC liposomes, but not POPC containing liposomes, induced a strong down-regulation of profibrotic genes such as TGF-β, collagen 1α1 and α-SMA in quiescent and activated HSC. This confirmed the reported antifibrotic properties of DLPC and demonstrated that the incorporation into liposomes did not affect DLPC activity. While free linoleic acid attenuated the expression of TGF-β, collagen 1α1 and α-SMA in HSC, it is plausible that the observed effects of the liposomes are due to the presence of DLPC, with linoleic acid as a the main active group.

The mechanism by which DLPC-containing liposomes affect the fibrotic process in vivo is less straightforward. Surprisingly, at the mRNA level, M6P-HSA-DLPC liposomes did not affect (TGF-β) or even slightly enhanced the expression (collagen 1α1 and α-SMA) of profibrotic genes. In contrast, DLPC liposomes induced a significant reduction in gene expression of two important fibrosis markers, TGF-β and collagen 1α1. Immunohistichemical staining of collagen I and III as well as α-SMA in rats treated with DLPC liposomes showed a reduction in protein expression as well. These findings demonstrated that DLPC incorporated into liposomes can modulate the fibrotic process in the liver, while M6P-HSA influenced this effect in vivo.

In our previous studies, we demonstrated that, in addition to HSC, also KC and LEC take up liposomes modified with M6P-HSA via a scavenger receptor mediated pathway. Both KC and LEC are known to play a major role in inflammatory processes in the liver. Injury to hepatocytes causes activation of KC, which respond by producing, among others, pro-inflammatory cytokines like TNF-α, IL-1, IL-6 and TGF-β. In particular this latter cytokine is a key stimulus for HSC to produce extracellular matrix components (24). TNF-α induces expression of cell adhesion molecules in LEC, which triggers the recruitment of inflammatory cells. In addition, TNF-α has a direct effect on HSC and induces secretion of leukocyte chemoattractants such as MCP-1, macrophage-inhibitory protein 2 (MIP-2) (25) and cytokine-induced neutrophil chemoattractant (CINC, rat analogue of IL-8) (26) as well as expression of leukocyte adhesion markers such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (27). Therefore,
we hypothesized that, due to the uptake of M6P-HSA-DLPC liposomes by KC and LEC in fibrotic livers, the activation status of these cells may be modulated and even may promote progression of fibrotic process which would counteract the beneficial effects of DLPC. Comparison of the effects of DLPC-containing liposomes on MCP-1, IL-6 and TNF-α gene expression in fibrotic livers and primary cultures of KC and LEC did not produce a clear picture. Although in fibrotic livers these genes were up-regulated by M6P-HSA-DLPC liposomes, only the levels of IL-6 were elevated in cultured KC and LEC, while the expression of MCP-1 and TNF-α was suppressed. The response of primary cultured KC and LEC is likely to be different from that of activated KC and LEC in fibrotic livers. In addition, cross-talk between different types of liver cells by means of released cytokines and/or growth factors is an important factor modulating behaviour of cells in vivo. Discrepancies between the effects of DLPC-containing liposomes in vitro and in vivo may therefore be readily explained. In order to achieve a more selective liposome delivery to HSC, a different homing ligand to these cells could be coupled to the surface of DLPC-containing liposomes. For example human serum albumin modified with a cyclic peptide that is recognised by platelet derived growth factor receptor on HSC (28) is an alternative option here since uptake of this protein construct in other non-parenchymal cells is minimal.

The loss of hepatocytes in fibrotic livers and the impaired functionality of those that are still present are well known characteristics of liver fibrosis. The ability of these cells to store glycogen reflects their over-all condition. Therefore, estimation of the glycogen content of hepatocytes may serve as a measure of damage within the liver. Reduced glycogen storage has been reported in patients with alcohol-induced and biliary liver cirrhosis (29) as well as in rat CCl₄ (30) and BDL (31) models of the disease. The mechanism leading to the reduced hepatic glycogen storage is not fully understood, but reduced volume of hepatocytes and the altered hepatocellular metabolism of glycogen most probably play a role here. Interestingly, in our experiments we observed a restoration of the hepatocyte glycogen content in fibrotic rats treated with DLPC-containing liposomes. Part of the injected dose of DLPC liposomes may be taken up by hepatocytes, since these are neutral liposomes, smaller than 100 nm, and are therefore able to pass the fenestration in endothelial lining of the sinusoids (32). Yet also M6P-HSA-DLPC liposomes had a clear effect on hepatocytes. Based on our previous studies, we know that M6P-HSA modified liposomes mainly accumulate in non-parenchymal cells and for this reason the effect of M6P-HSA-DLPC liposomes on hepatocytes is more likely to be a secondary response. Lipids derived from liposomes may readily diffuse from the target cells. Linoleic acid, the bioactive molecule of liposomes containing DLPC, belongs to the essential fatty acids, which are precursors for polyunsaturated fatty acids (PUFA). In patients with liver cirrhosis, PUFA deficiency is a well described phenomenon, but so far the cellular and clinical sequence of events has been not fully elucidated (33;34). In addition to serving as a source of energy, PUFA are responsible for the proper functions of the cell membrane by maintaining its fluidity. Moreover, some PUFA are precursors for the prostaglandins and thromboxanes, molecules which have a broad spectrum of biological activities including a role in intercellular communication (35). In addition, linoleic acid was shown to improve cellular respiration and provide protection against interferon-γ-induced cellular injuries such as lactate dehydrogenase release and protein synthesis inhibition in primary cultures of hepatocytes (36). Finally, DLPC was also shown to reduce the ethanol-induced mito-
chondrial liver injury in rats, probably as a result of a high incorporation rate of DLPC into the cellular membranes (37). Overall, these observations suggest that DLPC liposomes, due to their high linoleic acid content display profound effects in hepatocytes leading to hepatoprotective effects in livers of BDL rats.

In conclusion, we successfully incorporated DLPC into the membranes of liposomes targeted to HSC. In cultured HSC these bioactive drug carriers strongly reduced the expression of the genes for collagen 1α1 and α-SMA, important markers of fibrosis. Additionally, DLPC-containing liposomes attenuated the fibrotic process in livers of BDL rats. The uptake of M6P-HSA-DLPC liposomes in KC and LEC seems to activate these cells and counteract the positive effects of DLPC in HSC, whereas uptake of DLPC in hepatocytes seems to be beneficial. The use of bioactive drug carrier in fibrotic liver is feasible and promising for a complex disease like fibrosis. However, cell selectivity of the carrier is crucial for this approach.

References


A failed experiment, but an interesting picture... An attempt to immunostain targeted HVJ-liposomes with gold labelled antibody against F-protein as seen with an electron microscope.
chapter 5

Delivery of viral vectors to hepatic stellate cells in fibrotic livers using HVJ envelopes fused with targeted liposomes
Abstract

Hepatic stellate cells (HSC) are a major target for antifibrotic therapies in the liver and in particular gene delivery to these cells would be relevant. Previously, we demonstrated that mannose 6-phosphate human serum albumin (M6P-HSA) coupled liposomes accumulate in HSC in fibrotic livers. Here we prepared a M6P-HSA modified viral vector that allows the targeted delivery of plasmid DNA to HSC. Therefore, UV inactivated Hemagglutinating virus of Japan (HVJ) containing plasmid DNA was fused with M6P-HSA liposomes to yield HVJ liposomes targeted to HSC. These new particles had a diameter of approximately 200 nm, as determined by electron microscopy. In a carbon tetrachloride mouse model of liver fibrosis, M6P-HSA-HVJ-liposomes associated with HSC. In conclusion, our results demonstrate that fusion of M6P-HSA liposomes with HVJ envelopes results in HVJ particles that accumulate in HSC, allowing new possibilities to interfere with fibrosis in the liver.
Introduction

Upon liver injury, Kupffer cells, hepatocytes and sinusoidal endothelial cells are stimulated so as to effectuate the transformation of quiescent HSC into an activated phenotype that is characterised by a high rate of extracellular matrix production and its deposition in the organ. In addition, activated HSC proliferate and migrate towards areas of injury and produce chemokines which attract immune cells from the circulation (1). These actions of HSC cause a perpetuation of the fibrotic process in the liver, eventually leading to cirrhosis. As a result, HSC are considered a major target for therapies against liver fibrosis. A variety of strategies is currently being tested for their antifibrotic potential and among others therapeutic gene modulation is considered as a viable approach towards treatment of fibrosis (2). Viral gene delivery systems might be an alternative to conventional treatment. It has already been demonstrated that delivery of genes encoding proteins, like interferon α (3), superoxide dismutase (4), matrix metalloproteinase-8 (5) and Smad7 (6) as well as antisense mRNA to transforming growth factor-β 1 (7) using adenoviral vectors can attenuate liver fibrosis in rat models of the disease. Because adenoviruses specifically interact with hepatocytes, the vectors used in these studies mostly transfected hepatocytes while delivery them to other cells is difficult.

The hemagglutinating virus of Japan (HVJ), also known as Sendai virus, has an average diameter of 200 – 400 nm. Its lipid membrane contains two glycoproteins: hemagglutinin-neuraminidase (HN) and the fusion protein F. Both proteins are essential for fusion of the virus with the plasma membrane of the host cells, the process by which HVJ infect cells. HN binds to sialoglycoproteins on the surface of the cell membrane. The actual fusion occurs after exposure of a hydrophobic region of the F protein and its interaction with the lipid bilayer of the cell membrane (8).

Earlier we demonstrated that HVJ is able to fuse with conventional liposomes, even though they do not contain sialoglycoproteins on their surface (9). During this fusion process, membrane proteins of HVJ are incorporated into the liposomal membrane. Several reports showed that vectors derived from the fusion of liposomes that contain plasmid DNA or oligonucleotides with inactivated HVJ form an effective gene transfection system, both in vitro and in vivo (10-13). However, these HVJ-liposomes lack tissue or cell selectivity. Previously we demonstrated that liposomes that are surface coupled with mannose 6-phosphate modified human serum albumin (M6P-HSA) accumulate in HSC in a rat model of liver fibrosis (14). The combine HSC specific targeting with the effective HVJ gene transfection system may offer the possibility to construct a new vector, characterised by the delivery of therapeutic genes selectively to HSC and an enhanced transfection efficiency of these pivotal cells during fibrogenesis.

In the present study, we therefore tested if fusion can be accomplished between M6P-HSA liposomes and HVJ envelopes containing plasmid DNA (15) and if the HVJ particles thus obtained can be targeted to HSC. The new particles resulting from the fusion process were characterised with regard their size and their phospholipid, protein and DNA content. The transfection efficiency of the targeted HVJ particles was tested in vitro and selective targeting to HSC was assessed in a mouse model of liver fibrosis.

Materials and Methods
Chemicals

Cholesterol (Chol), egg 1,2-diacyl-sn-glycero-3-phosphocholine (PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), N-succinimidyl-S-acetylthioacetate (SATA) were obtained from Sigma (St. Louis, MO, USA). 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide(Polyethylene Glycol)2000] (DSPE-PEG2000-MAL) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Human serum albumin fraction V came from the Sanquin blood bank (Amsterdam, The Netherlands). Luciferase expression plasmid driven by the cytomegalovirus promoter was purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade or the best grade available.

Preparation of HVJ envelope and incorporation of plasmid DNA

The Z strain of Hemagglutinating virus of Japan (HVJ) was propagated in chick eggs and purified by centrifugation as described before (16). HVJ-E was prepared as reported before (16). Shortly, HVJ (10 000 Hemagglutinating units, HAU) was inactivated by UV irradiation (99 mJ/cm²), centrifuged for 10 min at 15 000 rpm. The pellet was resuspended in solution of protamine sulphate (1 mg/ml), plasmid DNA (200 μg) containing luciferase as a reporter gene (pDNA-Luc) and a solution containing 3 % Triton X-100 was added and the mixture was centrifuged for 15 min at 15 000 rpm, 4°C. To remove unincorporated DNA and detergent the pellet was washed with balanced salt solution (10 mM Tris-Cl, pH 7.5, 137 mM NaCl and 5.4 mM KCl). The obtained HVJ envelope was resuspended in PBS and stored at 4°C until use.

Preparation of M6P-HSA

Synthesis of human serum albumin modified with mannose 6-phosphate groups was performed as described before (17). In order to couple M6P-HSA to liposomes, active SH groups were introduced to the protein using the SATA method (18). M6P-HSA used in this study contained three SH groups per molecule. SATA modified M6P-HSA was stored at -20°C until use.

Preparation of liposomes

Liposomes composed of PC/CHOL/DOPE/DSPE-PEG2000-MAL in the molar ratio 21.5:16:2:0.5 were prepared as reported before (19). Briefly, lipids from chloroform-methanol (9:1) stock solutions were pipetted in a glass tube at the indicated molar ratio and organic solvent was evaporated under nitrogen. Dried lipids were dissolved in cyclohexane and freeze-dried over night. The dried lipids were hydrated with HNE (10 mM HEPES, 135 mM NaCl, 0.1 mM EDTA) buffer pH 6.7 and the liposomes thus formed were sized by repeated extrusion through polycarbonate filters (50 nm). The concentration of each liposome preparation was determined by measurement of phospholipid phosphorus (20). The total lipid concentration was calculated taking into account the amount of cholesterol in the liposomal lipid mixture.

M6P-HSA was coupled to the liposomes as described before (21). Briefly, SATA-modified M6P-HSA was incubated with the liposomes for 4 h at room temperature (0.3 mg protein / μmol of liposomal lipid). The coupling reaction was stopped by addition of 10 mM N-ethylmaleimide (Sigma) and uncoupled M6P-HSA was removed by ultracentrifugation (2
x 2h at 40,000 rpm, 4°C) in Opti-Prep™ (Axis-Shield PoCAS, Oslo, Norway). The amount of M6P-HSA coupled to the liposomes was measured by DC Protein assay (Bio-Rad).

**Fusion of HVJ envelope with M6P-HSA liposomes**

HVJ envelopes containing pDNA-Luc were incubated with M6P-HSA liposomes (15,000 HAU per 15 μmol total lipids of M6P-HSA liposomes) on ice for 10 min and subsequently, 1 h at 37°C in a shaking water bath. Fused liposomes were separated from free HVJ and unfused liposomes by discontinuous sucrose density gradient centrifugation (16). From bottom to top the centrifuge tube contained 1 ml 60 % sucrose solution followed by 7 ml 30 % sucrose and 1.5 ml incubation mixture. The tubes were centrifuged for 90 min at 20,000 rpm, 4°C and the fractions obtained after centrifugation were collected and dialysed overnight against HNE buffer pH 7.4.

**Characterisation of fused M6P-HSA-HVJ liposomes**

**Quantitative measurements**

To determine the concentration of fused M6P-HSA-HVJ liposomes, and the overall ratio in which fusion had taken place, independent measurements were done of the protein and phosphate concentrations of M6P-HSA liposomes and HVJ envelopes. Using these, a system of linear equations could be constructed for the M6P-HSA-HVJ liposomes, which was solved to find the separate contributions of these components from measurements on the fused particles. Generally, if \( p_l \) and \( p_e \) are the protein concentrations for M6P-HSA liposomes and HVJ envelopes, respectively, and \( l_l \) and \( l_e \) the lipid concentrations for both, the concentration of liposomes \( C_l \) (μmol Pi) and HVJ envelopes \( C_e \) (HAU) are given by

\[
C_l = \frac{P}{P_l} \frac{l_l}{P_l} - \frac{L}{l_l} \frac{P}{P_l} \frac{l_e}{P_e} + \frac{P}{P_l} \frac{l_l}{P_l} - \frac{L}{l_l} \frac{P}{P_l} \frac{l_e}{P_e}
\]

\[
C_e = \frac{L}{l_l} \frac{P}{P_l} \frac{l_e}{P_e} - \frac{P}{P_l} \frac{l_l}{P_l} - \frac{l_l}{P_l} - \frac{l_e}{P_e}
\]

with \( P \) and \( L \) the measured concentrations of protein and lipid in the fused particles, respectively.

**Electron microscopy**

Electron microscopic examination of the samples was performed after negative staining with a 1.5 % solution of phosphotungstic acid, using Hitachi TEM system (Hitachi, Ibaragi, Japan). The size of the particles was estimated by comparison to scale bar, generated by the TEM system.

**SDS-PAGE analysis**

The proteins of the HVJ envelopes, the M6P-HSA-HVJ liposomes and M6P-HSA (all 2 μg protein/lane) were separated on 10 % SDS-PAGE and stained with a Silver Stain kit (Bio-Rad, CA, USA) according to the protocol of manufacturer.

**Agarose gel electrophoresis**

In order to confirm presence of the plasmid DNA in M6P-HSA-HVJ liposomes, these particles (50 HAU) were analysed on a 1 % agarose gel.
Luciferase gene expression assay
HEK 293 cells (human embryonic kidney cells) were cultured in Minimum Essential Medium (Gibco-BRL, Rockville, MD, USA) containing 10 % foetal calf serum (Bio West, Miami, FL, USA) and penicillin, streptomycin (Nakarai Tesque, Kyoto, Japan). A day before the experiment cells were seeded at a density of 50 000 cells/well in 24 wells plates (Costar) and cultured overnight. Subsequently, cells were incubated with M6P-HSA-HVJ liposomes or HVJ-E at a concentration of 250 HAU for 4 h, after which the incubation medium was removed and the incubation was continued with fresh medium for another 48 h. Incubations were stopped by washing the cells with PBS and cells were lysed with Passive Lysis Buffer (Promega). The luciferase activity in the cell lysates was measured using a Luciferase Assay kit (Promega).

In vivo studies in mice with experimental liver fibrosis
Targeting of M6P-HSA-HVJ liposomes was tested in a mouse CCl\textsubscript{4} model of liver fibrosis. C57 BL/6 mice were injected (i.p) twice a week for 4 weeks with CCl\textsubscript{4} (1ml/kg of body weight), diluted in corn oil (1:10) (22;23). Three days after the last injection, the mice were injected via the tail vein with M6P-HSA-HVJ liposomes (1000 HAU) or HVJ-E (1000 HAU). One hour after injection of the particles the mice were sacrificed and the livers were removed for immunohistochemical analysis.

Immunohistochemical analysis
To detect M6P receptors, liver sections were fixed in acetone and incubated with a goat antibody directed against M6P receptor (Santa Cruz Biotechnology, Inc). Subsequently, endogenous peroxidase was inhibited with H\textsubscript{2}O\textsubscript{2}, followed by incubation with peroxidase conjugated rabbit-anti-goat IgG (RaGoP, Dako) and amplification with peroxidase conjugated goat-anti-rabbit IgG (GaRPo, Dako). Antibody associated peroxidase was visualised with 3-amino-9-ethyl-carbazole (AEC).
To detect HVJ in the liver, sections were fixed with 4% paraformaldehyde using the Vector\textsuperscript{®} M.O.M. Immunodetection kit (Vector Laboratories, Inc, CA, USA) according to the protocol of the manufacturer with minor modifications. Briefly, sections were blocked with M.O.M\textsuperscript{TM} Mouse Ig Blocking Reagent and incubated with a mouse monoclonal antibody against F protein, diluted 1:50 in M.O.M\textsuperscript{TM} Diluent. Subsequently, Alexa-488 conjugated goat anti-mouse antibody (Molecular Probes, Oregon, USA) diluted in M.O.M\textsuperscript{TM} Diluent was used as a secondary antibody and the nuclei of the cells were stained with DAPI. Staining was analysed by fluorescence microscopy and confocal laser scanning microscopy.
To analyse co-localisation of M6P-HSA-HVJ liposomes with HSC liver sections were fixed in acetone and the immunodetection was performed using the Vector\textsuperscript{®} M.O.M. Immunodetection kit according to the protocol of the manufacturer with minor modifications. Sections were blocked with M.O.M\textsuperscript{TM} Mouse Ig Blocking Reagent. Desmin, a HSC marker was detected with an anti-desmin monoclonal antibody (Sigma) followed by a second step with rabbit anti-mouse FITC conjugated antibody (Dako Cytomation, Denmark). HSA was stained with rabbit anti-HSA antibody (Cappel) followed by swine anti-rabbit TRIC conjugated antibody (Dako). All used antibodies were diluted in M.O.M\textsuperscript{TM} Diluent. Stained sections were analysed using a Leica fluorescence microscope.
Results

Quantitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes

HVJ liposomes containing the luciferase gene were fused with M6P-HSA liposomes and the mixture obtained was centrifuged on a discontinuous sucrose density gradient. Thus, three fractions were obtained (Fig. 1A), one (fraction I) near the top of the gradient, another one (fraction II) at the interface between the 30% sucrose and the buffer and the heaviest third one (fraction III) at the interface between 30% and 60% sucrose. The three fractions were quantitatively analysed for phosphorus and protein content and from these values we calculated the relative contribution of the HVJ-E (hemagglutinating units, HAU) and M6P-HSA liposomes (phosphate, Pi) in each fraction, as described in the Materials and Methods section. Fraction III contained 50% of HVJ-E applied to the gradient but did not contain significant concentrations of Pi, indicative of the absence of HVJ-E and M6P-HSA liposome fusion product in this fraction (Table 1). By contrast, fraction I contained 30% of the HVJ-E derived material and around 70% of the initial M6P-HSA liposome-derived material. In the fraction II only low amounts of HVJ-E and liposome-driven material were detected.

Qualitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes

Table 1. Quantitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes.

<table>
<thead>
<tr>
<th>fraction</th>
<th>% of HVJ-E</th>
<th>% of M6P-HSA-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>fraction I</td>
<td>31 ± 9</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>fraction II</td>
<td>12 ± 2</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>fraction III</td>
<td>53 ± 2</td>
<td>6 ± 5</td>
</tr>
</tbody>
</table>

HVJ-E were mixed with M6P-HSA liposomes in a ratio of 1000 HAU of HVJ-E per μmol total lipid of liposomes and after incubation the mixture was centrifuged on a sucrose density gradient, as described in Material and Methods. Three fractions were collected from the gradient as described in Fig. 1A and analysed for the present quantity of HVJ-E material, expressed as % of total added HVJ-E, and of M6P-HSA liposome-derived material, expressed as % of added liposomes. Data are presented as mean ± SEM of 3 preparations.

In order to identify the proteins present in each fraction collected from the sucrose density gradients, they were analysed by polyacrylamide gel electrophoresis. As presented in Fig. 1B, fraction III contained the same types of proteins as HVJ-E but hardly any M6P-HSA. In fraction I we found all HVJ-E proteins in addition to a strong band of M6P-HSA. While the appearance of the second fraction on the gel was similar to the first fraction, electron microscopy revealed that fraction I consisted of a rather homogenous population of particles with a size was of around 100 nm (Fig 2 A). Particles in the second fraction were 50-200 nm. In addition, this fraction also contained apparently destroyed particles and debris. The particles in fraction III varied in size from 200 to 400 nm and were identified as HVJ envelopes.
Luciferase gene expression assay

The transfection potential of the particles from the sucrose density gradient was tested on HEK 293 cells using a luciferase gene expression assay. We observed that after 48 h the expression of luciferase in the cells incubated with particles from fraction III was high and almost equal to that in the cells transfected with unmodified HVJ-E alone (Fig. 3A). Significant expression of luciferase activity was also observed in cells transfected with fraction II. However, the cells incubated with the particles of fraction I, which are most
likely to represent real fusion product of envelopes and liposomes did not show significant luciferase expression. The three gradient fractions were also assayed for pDNA-Luc content by means of agarose gel electrophoresis (Fig. 3B). As expected on the basis of its low transfection activity, fraction I contained very little DNA.

Figure 3. Luciferase gene expression assay. HEK 293 cells were incubated for 4 h with particles from gradient fractions I, II and III and HVJ-E at a concentration of 250 HAU/ml. Luciferase expression was measured after 48 h as described in Materials and Methods (A). Content of pDNA-Luc in the particles was analysed by 1% agarose gel electrophoresis (B). To the gel 50 HAU per line was applied.

Targeting of fused particles to hepatic stellate cells in the fibrotic liver
To test whether particles formed during fusion of HVJ-E and M6P-HSA liposomes are able to target HSC, we performed in vivo studies in a CCl₄ mouse model of liver fibrosis. In the livers of these mice, substantial M6P receptor expression was observed in both hepatocytes and non-parenchymal cells (Fig. 4).
We compared the targeting properties of fraction I particles and of HVJ-E by means of immunohistochemical analysis. Immunostaining with an antibody against the F protein of HVJ showed that fraction I particles as well as HVJ-E were abundantly present in the livers as early as 1 h after injection, but with remarkably different sinusoidal distribution patterns (Fig. 5). HVJ-E were detected as bright individual spots (Fig. 5A) while in mice injected with M6P-HSA-HVJ-liposomes the F protein was detected in diffuse elongated shapes, presumably representing the sinusoidal lining (Fig. 5B). For both signals confocal microscopy confirmed that they were cell associated (Fig. 5 C, D). In the livers of mice injected with fraction I particles we were also able to detect separately the liposomes-derived part of the particles by means of immunostaining for HSA (Fig. 5 E). Finally, simultaneous detection of the HSC marker desmin and HSA in the livers of mice injected with fraction I, revealed that the two signals were localised in close proximity indicating that the HSA was localised within HSC (Fig. 5 F).
Figure 4. Expression of M6P receptors in fibrotic livers of CCl₄-treated mice. M6P receptor was detected in control mice injected with only corn oil (A) and in CCl₄ treated mice (B) using immunohistochemical analysis as described in Materials and Methods. Original magnification x 400.

Figure 5. Targeting of fused HVJ liposomes to HSC in fibrotic livers. M6P-HSA-HVJ-liposomes (fraction I) or HVJ-E were injected into mice at a dose of 1000 HAU. In tissue sections of livers obtained 1 h after injection, particles were detected using an antibody against F protein (A, C) HVJ-E, (B, D) M6P-HSA-HVJ-liposomes, magnification x 200. (C) and (D) show representative pictures obtained after examination of sections with a confocal microscope. Note the co-localisation of green fluorescent signal with the nuclei of the cells (arrows). (E) and (F) show representative photographs after immunostaining for the HSA (E, red signal, x 200) and after simultaneous detection of HSA (red staining) and the HSC marker, desmin (F, green signal, x 400). Note the co-localisation of HSA with desmin (arrows). Nuclei of the cells were stained with DAPI (blue).
Discussion

We demonstrated that fusion of HVJ envelopes containing plasmid DNA with M6P-HSA liposomes results in the formation of new particles, M6P-HSA-HVJ-liposomes. These particles were collected as the top fraction from a sucrose density gradient and formed a rather homogeneous population with an average size of around 100 nm. As we reported before, M6P-HSA coupled to the surface of liposomes can target these to HSC in fibrotic livers (14). HSC are situated in the space of Disse, between hepatocytes and endothelial cells, in close contact with the latter. As a result, blood-borne substances can only reach these cells after crossing the 150 - 200 nm fenestrations in the endothelial cells (24). The size of the M6P-HSA-HVJ fusion product particles allows them to pass the fenestrations in the endothelial lining and thus to be taken up by HSC. Indeed, in the mouse CCl₄ model of liver fibrosis, we showed that M6P-HSA-HVJ-liposomes are able to pass the fenestrations and home to the HSC. In contrast, HVJ-E which do not contain M6P-HSA and have a size of over 200 nm, seem to be taken up mainly by Kupffer cells as indicated by the bright punctuate appearance in liver sections tissue. Although M6P receptors expression was observed on hepatocytes as well, we did not observe accumulation of the M6P-HSA-HVJ-liposomes in these cells. In hepatocytes the M6P-binding receptor are present within the cells where they play a role in the shuffling of proteins to lysosomes. These receptors are recognised by antibodies on the fixed liver sections but M6P-containing liposomes can not bind extracellularly to them. The small size of the M6P-HSA-HVJ-liposomes (fraction I) might explain the low transfection efficiency we found in vitro. Analysis of the plasmid DNA content showed that this fraction contained considerably less plasmid DNA than the remaining unfused HVJ-E (fraction III). Smaller size siRNA or antisense oligonucleotides might be incorporated more efficiently in M6P-HSA-HVJ-liposomes than plasmid DNA. HVJ-E have been shown efficient in functional delivery of antisense oligonucleotides (11;25).

An alternative explanation of the lack of transfection activity of fraction I may be that the insertion of the M6P-HSA in the bilayer of the viral envelopes interferes with the natural route by which the native virus enters cells and may, as a consequence, lower the transfection efficiency of the modified HVJ-E. The hemagglutinin-neuraminidase (HN) of the native virus binds to sialoglycoproteins on the cell surface. Subsequently, a hydrophobic region of the F protein is exposed, perturbing the cell membrane and allowing fusion between virus and cell and delivery of the nucleocapsid in the cytoplasm thus circumventing the lysosomal compartment (8). The presence of M6P-HSA on the surface of the modified HVJ-liposomes may direct the particles to enter the cells via M6P receptors followed by delivery into the lysosomal pathway which is the natural route for ligands of these receptors, once inside the cell (26). Although, despite the uptake by receptor-mediated endocytosis, part of the M6P-HSA-HVJ-liposomes and/or its DNA cargo might still escape the endosomes and gain access to the cytoplasm, the bulk of these particles is likely to be degraded in the lysosomes. Further studies will be required to elucidate the mechanism of the intracellular routing of M6P-HSA-HVJ-liposomes.

As a result of tissue damage in fibrotic livers, hepatocytes release reactive oxygen species and cytokines which activate Kupffer cells. The growth factors and cytokines that are subsequently produced by the Kupffer cells activate in turn the HSC, which than differentiate into myofibroblast-like cells. At this stage, the HSC do not only produce large amounts of extracellular matrix proteins, but they also secrete transforming growth factor...
β (TGF-β) and compounds such as free radicals, monocyte chemoattractant protein–1 (MCP-1) and endothelin–1, which in an autocrine manner perpetuate the activated state of HSC and hence the progression of fibrosis (27). Selective intervention aimed at bringing the activation of HSC to halt and preventing the subsequent extracellular matrix production by these cells is considered to be crucial for improvement of current antifibrotic therapies. The delivery to HSC of either specific genes or siRNA, which at the gene expression level could for example inhibit collagen production or interfere with the signalling pathways of TGF-β or platelet derived growth factor (PDGF), may provide means to design new strategies in the treatment of liver fibrosis.

In conclusion, we demonstrated that the fusion of HVJ-E with M6P-HSA liposomes creates new targeted particles, which in an experimental model of liver fibrosis were shown to accumulate in HSC. Since this cell type plays a crucial role in the development of liver fibrosis, these novel M6P-HSA-HVJ-liposomes may provide new opportunities to modulate the expression of essential genes during the development of liver fibrosis.

References


25. Tomita N, Morishita R, Yamamoto K,


The high pressure extruder — ‘beloved’ piece of equipment. A total of 82 preparations of liposomes were done over a period of 4 years.
chapter 6

summarizing discussion and perspectives
chapter 6

Summarizing discussion

The research presented in this thesis focuses on the characterisation of liposomes targeted to hepatic stellate cells (HSC) and their potential use as drug carriers for the treatment of liver fibrosis. In the fibrotic liver, HSC are a major source of extracellular matrix (ECM) constituents. Prolonged activation of HSC and deposition of ECM leads ultimately to liver failure (1). Because of the increasing number of patients with liver diseases which often lead to fibrosis and the lack of an effective therapy, alternative treatments for liver fibrosis are urgently needed. Selective targeting of antifibrotic compounds employing cell specific drug carriers to HSC may be essential to the design of an effective therapy for this disease.

In our approach, we applied liposomes as drug carriers. These highly versatile nanovesicles made of phospholipids, are known to be non-toxic, non-immunogenic and biodegradable. Importantly, liposomal drugs are already successfully used in patients, proving their safety in humans (2). However, to ensure specific accumulation of liposomes in HSC in the fibrotic liver, modification of the liposomal surface is required. It was demonstrated that human serum albumin modified with mannose 6-phosphate groups (M6P-HSA) is recognised by HSC in fibrotic livers (3). Particularly, mannose 6-phosphate/insulin-like growth factor II receptor, whose expression is up-regulated on the surface of activated HSC (4), was demonstrated to be the binding receptor for M6P-HSA (5). Based on this assumption, we reasoned that coupling of M6P-HSA to the surface of liposomes will generate a carrier system with a high drug loading capacity that can be specifically targeted to activated HSC in the fibrotic livers.

In chapter 2 of this thesis, we describe effective coupling M6P-HSA to the surface of liposomes, yielding particles of approximately 100 nm in diameter. This diameter is important because to reach HSC in the liver liposomes have to pass the fenestrations in the endothelial lining which have diameters ranging between 150 and 175 nm (6). The results presented in this chapter demonstrate the accumulation of M6P-HSA liposomes in HSC in the fibrotic livers of bile duct ligated (BDL) rats, which have developed extensive liver fibrosis.

Liposomes with attached M6P-HSA show higher association with cultured HSC than plain liposomes and the uptake of M6P-HSA liposomes by these cells occurs through receptor mediated endocytosis. Interestingly, high association of M6P-HSA liposomes was also observed with quiescent HSC, although these cells do not express the M6P/IGF II receptor. This suggests that, in addition to the M6P/IGF II receptor, there is another kind of receptor that recognises M6P-HSA liposomes. Because the coupling of M6P groups introduces negative charges in the albumin molecule, it is likely that M6P-HSA binds to scavenger receptors, which are known to recognise a variety of polyanionic macromolecules (7). In competition experiments, performed on cultured liver endothelial cells that are known to express scavenger receptors and on HSC, inhibitors of the scavenger receptor did inhibit the association of liposomes modified with M6P-HSA or with polyanionic HSA (acetylated HSA), confirming the recognition of M6P-HSA liposomes by these receptors. These data indicate that also scavenger receptors are present on the HSC membrane.

The experiments described in chapter 3 focus on the characterisation of M6P-HSA liposome properties in a rat BDL model of liver fibrosis. Liposomes surface-grafted with M6P-HSA are rapidly cleared from the circulation of BDL rats and accumulate predominantly in the liver. The blood elimination of M6P-HSA liposomes can be inhibited by
M6P-HSA, but not by HSA alone, indicating that the observed rapid clearance is M6P-HSA specific. Identification of the types of liver cells participating in the uptake of M6P-HSA liposomes revealed that, besides HSC, Kupffer cells and liver endothelial cells participate in the uptake of these liposomes. In addition, polyanionic acid, a competitive inhibitor of scavenger receptors, inhibited both blood elimination of M6P-HSA liposomes and their accumulation in the Kupffer cells and endothelial cells. These results are consistent with the well-established expression of scavenger receptors on Kupffer cells and endothelial cells. In our study, we demonstrate by Western blot and PCR analysis that also HSC express class A scavenger receptors. In activated HSC, expression of the receptor at the protein level is diminished, however.

Results presented in chapter 2 and 3 provide evidence that more than one receptor system is involved in the recognition of M6P-HSA liposomes, most likely M6P/IGF II receptors and scavenger receptors. As a consequence, the selectivity of M6P-HSA liposomes is not limited to HSC alone.

Chapter 4 describes effects of liposomes containing a bioactive lipid, dilinoleoylphosphatidylcholine (DLPC) in cultured HSC and in livers of BDL rats. DLPC was shown to inhibit activation of HSC and reduce the progression of fibrosis in experimental models of this disease (8;9). In our approach we incorporated DLPC in the membrane of M6P-HSA liposomes, what would give possibility to exploit the specific potential of liposomes to encapsulate more than one compound. By encapsulating an established antifibrotic drug such as troglitazone or roglitazone, DLPC-containing liposomes could provide a dual antifibrotic effect. The liposomal encapsulation of these potent anti-inflammatory and antifibrotic drugs, is expected to keep them efficiently away from the hepatocytes, to which they are toxic. In this way the DLPC liposomes, in concerted action with the encapsulated drugs, may effectively reduce inflammation and fibrosis in the liver (10;11). Our in vitro studies demonstrated that both M6P-HSA DLPC-containing liposomes and plain DLPC-containing liposomes have antifibrotic properties. They decreased the expression of profibrotic genes such as collagen I, α-SMA and TGF-β in HSC. However, in vivo studies with DLPC-containing liposomes, using BDL rats as a fibrosis model, did not show a similarly clear antifibrotic activity. Only in animals treated with untargeted DLPC-containing liposomes TGF-β and TNF-α were significantly down-regulated at the mRNA level. In the livers of these animals we also observed a decrease of collagen deposition and α-SMA proteins. However, DLPC-containing liposomes modified with M6P-HSA did not show antifibrotic effects in the livers of BDL rats to the extent that was observed in cultured HSC. In contrast, we even observed pro-inflammatory effects in these livers. Incubations of DLPC-containing liposomes with cultured Kupffer cells and liver endothelial cells indicate that DLPC liposomes modulate inflammatory genes such as IL-6, TNF-α and MCP-1 in these cells. Most likely the effects of DLPC containing liposomes in the fibrotic liver are influenced by the in vivo accumulation of these liposomes in Kupffer cells and endothelial cells, which are both important regulatory cells in inflammatory responses.

Interestingly, DLPC containing liposomes strongly promote the storage of glycogen in hepatocytes of fibrotic livers. During fibrosis, loss of hepatocytes and the impaired functionality of those that are still present are well known characteristics of the disease. Damaged hepatocytes in the fibrotic liver have been shown to lose cytoplasmic glycogen (12;13). The improved glycogen storage observed in BDL rats may indicate that DLPC
containing liposomes have hepatoprotective properties and modulate activities of the non-parenchymal cells. Although in this in vivo study we did not observe a significant regression of fibrosis after treatment with DLPC-containing liposomes, the obtained results justify further investigation of the effects such liposomes may have on progression of the disease. Studies with multiple injections of DLPC-containing liposomes might show more pronounced antifibrotic effects. However, as the bile duct ligated model of liver fibrosis used in these studies is characterised by rapid irreversible development of severe fibrosis, a different animal model of liver fibrosis, such as the CCl₄ model, might be more suitable for the assessment of therapeutic effects.

Specificity of M6P-HSA liposomes for HSC is an important issue for the application of these liposomes as drug carriers. The recognition of M6P-HSA by scavenger receptors causes accumulation of M6P-HSA liposomes in Kupffer cells and endothelial cells. This fact has to be taken into account when the drug to be encapsulated in M6P-HSA liposomes is chosen. For example, compounds which induce apoptosis would likely enhance fibrosis by causing death of these three populations of liver cells, while inhibitors of ECM production, which mainly affect HSC, might attenuate the disease. Alternatively, instead of M6P-HSA another ligand, characterised by a higher selectivity for HSC, can be coupled to the surface of liposomes. In this respect, human serum albumin modified with a cyclic peptide recognising either the PDGF receptor or collagen VI receptor on HSC might be an option.

More advanced quantitative methods for evaluating the uptake of M6P-HSA liposomes or other carriers systems such as modified HSA, by the different cell populations in diseased livers, are crucial for the further development of targeted drug carriers for the treatment of liver fibrosis. Immunohistochemical analysis provides only qualitative or semi-quantitative data which are not appropriate to estimate the concentrations of the carrier and/or drug in HSC and other major cell populations of the liver like Kupffer cells, endothelial cells and hepatocytes. As a result, the effects of an applied treatment using drug carriers are difficult to evaluate. Mechanisms underlying the in vivo interaction of drug carriers with cells in a disease as complex as liver fibrosis are not easy to elucidate but understanding these mechanisms is pivotal to the improvement and development of drug-carrier mediated therapies. Isolation and purification of the different cell types involved in this disease from the fibrotic liver is an option for quantitative determination of drug carrier uptake. However, because of excessive ECM deposition, the isolation of pure cell populations is difficult, and therefore established isolation methods must be adapted to the fibrotic condition of the livers.

In chapter 5, M6P-HSA liposomes were used to prepare targeted particles containing the Hemagglutinating Virus of Japan (HVJ) for the delivery of therapeutic genes or antisense oligonucleotides to HSC. HVJ vectors were shown to have high transfection efficiency in vitro and in vivo (16), yet uptake of these vectors in HSC is not possible without targeting. Fusion with the cell membrane is the mechanism by which HVJ infects cells. It was also demonstrated that HVJ are able to fuse with liposomes. We incorporated a plasmid containing a reporter gene into inactivated HVJ. Subsequently, these HVJ envelops were fused with M6P-HSA liposomes. The particles obtained in this fusion were characterised and their ability to target HSC in the fibrotic liver was determined. The data indicate that newly formed particles resulting from the fusion of HVJ envelops and M6P-HSA
liposomes are targeted to HSC in the fibrotic liver. However, the transfection efficiency of M6P-HSA-HVJ-liposomes in an in vitro test system was low. The reduced transfection efficiency might be due to the loss of plasmid DNA during the fusion process and/or to the redirection of the particle to the M6P/IGF II receptor. Additional experiments are required to elucidate the precise mechanism of transfection using the M6P-HSA-HVJ liposomes. Future studies along these lines might ultimately provide a suitable vehicle for the delivery of genes or antisense oligonucleotides to HSC using M6P-HSA-HVJ liposomes so as to inhibit the activation of these cells in the fibrotic liver and thus, reduce fibrosis.

In summary, in this thesis we demonstrated that liposomes can exert two functions in the therapy for liver fibrosis. Firstly, targeted liposomes can be used as carriers of antifibrotic compounds to HSC which are crucial cells in the development of fibrosis. Secondly, liposomes containing DLPC may modulate different processes such as inflammation and fibrogenesis in different cell types of the fibrotic liver. Thus, the dual functionality of liposomes as a drug carrier system may be successfully exploited in new approaches to treat liver fibrosis.

References


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Perspectives

Chronic liver disease, primarily caused by factors like hepatitis C or B infection, alcohol abuse or metabolic disorders often leads to fibrosis in this organ. Liver transplantation is the ultimate treatment of advanced fibrosis but because of lack of liver donors, high costs and complexity of this therapy, there is a strong urge to establish alternative treatments. Currently, various strategies are investigated for their efficacy in attenuating fibrosis, but so far none of them has been approved for use in humans. Low specificity of experimental treatments towards particular cells in the liver that are involved in the fibrotic process often hamper the antifibrotic effects in vivo and alter the results obtained with this compounds in vitro. Therefore, cell specific delivery of drugs to the fibrotic liver using carriers such as liposomes represents an attractive alternative for traditional treatments.

Liposomal drugs available on the market for cancer treatment prove their potential in the therapy of chronic disease. The practical use of liposomes as carriers in liver fibrosis would require separate toxicity and safety studies, but liposomes are in principle suitable for a long-term therapy.

According to current views, HSC producing extracellular matrix constituents are a major target for antifibrotic therapies. In order to deliver liposomes to HSC, selective targeting strategies need to be employed. On the basis of research presented in this thesis we conclude that targeting of liposomes to HSC is feasible. However, the partial uptake of the targeted liposomes by other cells than HSC complicates the applicability of these liposomes. Thus, highly selective liposomal carriers to HSC should be a priority for future research. In addition, methods for quantitative determination of the amount of liposomes and carried compound delivered to the HSC need to be developed.

Liver fibrosis is a complex disease, in which besides ECM deposition other processes like inflammation and oxidative stress occur simultaneously and contribute to the development of fibrosis. Therefore the role of other cell types, such as Kupffer cells and liver endothelial cells should not be ignored. To effectively cure liver fibrosis, the uptake of our carrier system by these cells may be quite relevant as a concerted action of two or more compounds interfering with different pathways in the disease process may be required for optimal effectivity. Targeted liposomal drug carriers have been shown to be able to target all major cell populations of the liver such as hepatocytes, Kupffer cells, liver endothelial cells and now also HSC. This opens the possibility to selectively modulate the action of different cells involved in the fibrotic process. However, future studies using liposomal drugs for treatment of liver fibrosis will have to demonstrate their practical applicability.
If $A$ equals success, then the formula is: $A = X + Y + Z$.
$X$ is work. $Y$ is play. $Z$ is keep your mouth shut.

Albert Einstein
chapter 7

samenvatting
Leverfibrose ontstaat als gevolg van schade aan het orgaan. Dit leidt tot de vorming van littekenweefsel, wat onder normale omstandigheden essentieel is voor het genezingsproces van de lever. Wanneer het proces van littekenvorming echter chronisch geactiveerd wordt, kan dat leiden tot blijvende schade. Deze activatie kan verschillende oorzaken hebben: chronische infecties, zoals door het hepatitis B of C virus, chronisch alcoholisme of langdurige blootstelling aan andere toxische stoffen, ernstige zwaarlijvigheid en overdadige consumptie van vetten (resulterend in niet-alcoholische steatohepatitis, NASH), erfelijke stofwisselingsziekten, zoals de ziekte van Wilson, of leverontsteking als gevolg van auto-immuunziekten.

Het littekenweefsel dat ontstaat na deze chronische beschadiging bestaat uit diverse extracellulaire matrix (ECM) componenten, met name collageen type I en III. Leverfibrose is een dynamisch proces, waarbij de excessieve productie van ECM samenvalt met een verminderde afbraak hiervan. Tevens verandert de samenstelling van ECM; er ontstaat een stugge structuur. Uiteindelijk kan fibrose zich ontwikkelen tot een potentieel dodelijke ziekte die bekend staat als levercirrose die door veel mensen geassocieerd wordt met alcoholisme. Het is echter ook mogelijk dat de ontwikkeling van fibrose stagneert, en dat de lever uiteindelijk zelfs goeddeels geneest en weer normaal gaat functioneren. De lever heeft een zeer goed herstellend vermogen, maar een chronische schade kan ernstige en blijvende pathologische veranderingen induceren.

Hoe levercirrose zich klinisch manifesteert hangt af van de ernst van de primaire oorzaak, maar ook van de mate waarin fibrose al is opgetreden. Dit heeft tot gevolg dat men een grote verscheidenheid aan ziektebeelden ziet; zowel volledige afwezigheid van symptomen als volledige leverfalen komen voor. Leverfibrose is dan ook ziekte die zich in een laat stadium manifesteert. Tot veertig procent van mensen met leverfibrose heeft nog geen duidelijke klachten en het kan langer dan tien jaar duren voordat die ontstaan. Daarom is het van groot belang dat de diagnostiek ontwikkeld wordt, vooral om op een betrouwbare wijze een indicatie te kunnen krijgen van de vroege fases van het fibrose proces. Op dat moment zal de lever in het algemeen nog in staat om te herstellen wanneer de oorzaak weggenomen wordt. Ook wanneer de ziekte zich verder ontwikkelt heeft, kan het fibrose proces nog vertraagd worden, en soms kan zelfs genezing optreden wanneer de oorzaak van de chronische beschadiging weggenomen wordt. Voor een adequate behandeling is dan echter therapeutische interventie nodig. Onderzoek naar medicijnen en therapieën voor de behandeling van leverfibrose vindt plaats in laboratoria en klinieken wereldwijd, maar op dit moment zijn er nog geen behandelingen die goedgekeurd zijn voor algemene klinische toepassing. Daarmee is levertransplantatie op dit moment de enige effectieve behandeling van ernstige leverfibrose en cirrose. Echter aan transplantaties kleven ernstige bezwaren zoals de risicovolle operatie, en het tekort aan donor levers.

Zowel in Europa als in de Verenigde Staten is levercirrose, na kanker, de meest voorkomende doodsoorzaak binnen de groep van lever- en maag-darmziekten. Daar komt bij dat uit epidemiologische prognoses blijkt dat leverfibrose in de nabije toekomst steeds vaker voor zal komen als gevolg van een toenemend voorkomen van infecties met het hepatitis C virus (zowel toename van het aantal infecties, als het steeds ouder worden van reeds geïnfecteerden). Ook zal NASH vaker voorkomen als gevolg van het toenemende probleem van obesitas.

In de vroege jaren negentig van de vorige eeuw identificeerde men de lever stellaatcel (hepatic stellate cell, HSC) ofwel stervormige levercel als het celtype dat cruciaal is in
de ontwikkeling van leverfibrose. In een gezonde lever hebben stellaatcellen een rustend fenotype en verzorgen de opslag van vitamine A. Bij leverschade komen groeifactoren en signaalstoffen vrij uit andere levercellen, zoals de hepatocyten, Kupffer cellen en de leverendotheelcellen (LEC), die de HSC activeren. In deze toestand verliezen de HSC het opgeslagen vitamine A en gaan grote hoeveelheden collageen produceren. De toename van de hoeveelheid collageen in de lever verstoort de lever architectuur en bemoeilijkt vervolgens de leverfunctie. Daarnaast gaan de geactiveerde HSC zich vermenigvuldigen en migreren ze in de richting van de beschadiging. Ze produceren dan ook zelf signaalstoffen, die cellen uit het immuunsysteem aantrekken en een ontstekingsproces in gang zetten. Het gevormde littekenweefsel en de HSC die zich kunnen samentrekken, veroorzaken een hoge weerstand in de lever waardoor er een bloeddrukverhoging ontstaat in de aanvoerende bloedvaten (de vena porta). Dit is een ernstig symptoom bij patiënten met levercirrose want het leidt tot veel klinische problemen. Op basis van deze gegevens is het aannemelijk dat een therapie die in staat is om de activering van HSC en de overmatige productie van collageen te stoppen een bijzonder waardevolle bijdrage kan leveren aan de behandeling van patiënten met leverfibrose.

Voor een dergelijke therapie is het noodzakelijk om medicijnen met een anti-fibrotisch effect met behulp van geneesmiddeldragers zo specifiek mogelijk in de HSC af te leveren (targeting). Op deze manier kan men hoge concentraties van het medicijn in deze celpopulatie bereiken, zonder dat het middel elders in het lichaam ophoopt en daar ongewenste toxische effecten heeft.

Liposomen zijn een voorbeeld van geneesmiddeldragers. Liposomen zijn vet bolletjes bestaande uit een dubbellaag van fosfolipiden die een waterige ruimte omsluiten waarin wateroplosbare medicijnen ingesloten kunnen worden. Vetoplosbare medicijnen daarentegen, kunnen worden opgeslagen in de lipide dubbellaag die het membraan van de liposoom vormt. In beide gevallen kan een hoge concentratie geneesmiddelen in het liposoom gebracht worden. Liposomen, toegepast voor het afleveren van medicijnen, worden vaak gemaakt van fosfolipiden die niet of negatief (anionisch) geladen zijn. Hieraan wordt cholesterol toegevoegd om de stabiliteit van de membraan te vergroten. Het membraan van liposomen lijkt sterk op dat van een gewone lichaamscel. Hierdoor zijn liposomen bio-compatibel, wat betekent dat ze afbreekbaar, niet immunogeen en niet giftig zijn.

In het onderzoek beschreven in dit proefschrift hebben we liposomen gebruikt om medicijnen selectief in HSC af te leveren en gekeken of het mogelijk is om op deze manier een antifibrotische therapie te ontwikkelen. Om ervoor te zorgen dat de liposomen na injectie ook daadwerkelijk naar de HSC gaan, hebben we een eiwit, aangeduid als M6P-HSA, gekoppeld aan het oppervlak van de liposomen. Dit eiwit, humaan serum albumine (HSA) gemodificeerd met mannose-6-fosaat (M6P), wordt herkend door geactiveerde HSC in een fibrotische lever, doordat het bindt aan een receptor (de mannose-6-fosaat/insuline-achtige groeifactor type II receptor, ofwel M6P/IGF II receptor) op het oppervlak van deze cellen.

In hoofdstuk 2 van dit proefschrift laten we zien dat M6P-HSA daadwerkelijk gekoppeld kan worden aan liposomen, waarbij deeltjes worden verkregen met een diameter van zo’n honderd nanometer d.w.z. klein genoeg om fenestraties in het leverendotheel te passeren en HSC te bereiken. Incubatie van deze liposomen met gekweekte HSC leidde ingerdaad tot een verhoogde celbinding en opname van de M6P-HSA liposomen. Het was opmerkelijk
dat de M6P-HSA liposomen ook sterk bonden aan niet geactiveerde HSC, alhoewel deze cellen niet de eerder genoemde M6P/IGF II receptoren tot expressie brengen. Uit deze en andere experimenten werd geconcludeerd dat er een tweede type receptor is die de M6P-HSA liposomen herkent. Omdat M6P groepen een negatieve lading geven aan het albumine, is het mogelijk dat M6P-HSA ook bindt aan de zogenaamde scavenger receptoren, waarvan bekend is dat zij ionische macromoleculen binden. Alhoewel er weinig bekend is over het voorkomen van scavenger receptoren op HSC, wijzen onze resultaten erop dat deze cellen scavenger receptoren tot expressie kunnen brengen. Na injectie van M6P-HSA liposomen in ratten met een geïnduceerde leverfibrose (BDL ratten), bleken deze liposomen daadwerkelijk te worden opgenomen door HSC in de fibrotische lever.

In hoofdstuk 3 werden M6P-HSA liposomen verder gekarakteriseerd in een rattenmodel voor leverfibrose. Het bleek dat liposomen met daaraan M6P-HSA gekoppeld snel uit de bloedsomloop van BDL ratten verdwenen en dan voornamelijk in de lever teruggewonden werden. Deze snelle verdwijning uit het bloed kon geremd worden door de pré-injectie met vrij M6P-HSA, terwijl vrij (ongemodificeerd) HSA geen effect had. Daaruit kan worden afgeleid dat de waargenomen kinetiek van de M6P-HSA liposomen verklaard kan worden door de aanwezigheid van M6P-HSA groepen in het liposoom. Behalve HSC bleken ook de Kupffer cellen en LEC M6P-HSA liposomen op te nemen. Aangezien van deze beide celtypen bekend is dat ze de scavenger receptoren hebben, was deze opname, met het oog op de in vitro resultaten, te verwachten. Wanneer ratten een pré-injectie met van polynosine zuur, een remmer van de scavenger receptor gemedieerde opname, kregen, bleek dat de M6P-HSA liposomen veel minder snel uit de bloedsomloop verdwenen en bleek ook de opname door Kupffer cellen en LEC sterk geremd. Verdere analyse van HSC toonde aan dat deze cellen scavenger receptor type A tot expressie brachten. De expressie van die receptor was echter sterk verminderd in geactiveerde HSC.

Samenvattend laten de resultaten uit deze hoofdstukken zien dat M6P-HSA liposomen snel door de lever worden opgenomen maar dat er meerdere receptoren betrokken zijn bij de herkenning van deze liposomen. Het gaat hierbij om de M6P/IGF II receptor en de scavenger receptor. De consequentie daarvan is dat de M6P-HSA liposomen niet alleen door HSC maar ook door andere celtypen in de lever zoals Kupffer- en endotheel cellen worden opgenomen. Bij de keuze van het medicijn dat ingesloten wordt in het liposoom moet hier terdege rekening mee worden gehouden. Een hoge opname in de doelcel kan worden verkregen, maar het middel mag niet toxisch zijn voor deze andere celtypen.

In hoofdstuk 4 worden de effecten beschreven van liposomen die het bioactieve vet dili-noeoleoylfosfatidylcholine (DLPC) bevatten. Dit effect is onderzocht op gekweekte HSC en in fibrotische BDL ratten. Eerder was al aangetoond dat DLPC de activatie van HSC kan remmen en dat het de progressie van leverfibrose kan verminderen. In onze aanpak werd DLPC geïntegreerd in het membraan van de liposomen. Dit biedt de mogelijkheid om ook nog medicijnen in het liposoem in te sluiten waardoor de DLPC liposomen een tweeledig anti-fibrotisch effect zouden kunnen hebben; naast een functie als geneesmiddeldrager heeft het liposoom dan ook een direct effect in de doelcel. In vitro studies lieten zien dat DLPC liposomen zowel met als zonder M6P-HSA gekoppeld aan het lipid membraan, een anti-fibrotisch effect hebben. De expressie van een aantal pro-fibrotische genen werd geremd. In vivo daarentegen, waren de effecten van DLPC liposomen op leverfibrose minder uitgesproken. Anti-fibrotische effecten werden alleen
waargenomen in dieren die behandeld werden met DLPC liposomen waaraan geen M6P-HSA gekoppeld was, terwijl liposomen met M6P-HSA nauwelijks een anti-fibrotisch effect in BDL ratten lieten zien. Om de oorzaak van de verschillen tussen de in vitro en in vivo situatie te achterhalen werden effecten van DLPC liposomen op gekweekte Kupffer cellen en LEC bestudeerd. Het bleek dat in deze cellen de expressie van diverse genen die ontstekingsprocessen regelen werd beïnvloed door de opgenomen DLPC liposomen. Hoewel het netto effect van DLPC liposomen op Kupffer cellen, LEC en HSC niet duidelijk is, lijkt er een duidelijke invloed van de DLPC liposomen op de cel reacties in de fibrotische lever. Opmerkelijk was dat de DLPC liposomen de opslag van glycogeen in hepatocyten bevorderden. Het verlies van hepatocyten en functieverlies van de overblijvende cellen zijn belangrijke gevolgen van leverfibrose. De hoeveelheid glycogeen die in de hepatocyten is opgeslagen is mogelijk een goede indicatie van de algehele conditie van hepatocyten. Over het algemeen resulteert leverfibrose in het verlies van cytoplasmatisch glycogeen. De toename van glycogeen in de hepatocyten na injectie met DLPC liposomen duidt derhalve op een beschermende werking van deze liposomen bij leverfibrose.

**Hoofdstuk 5** behandelt de toepassing van M6P-HSA liposomen in combinatie met het hemagluttinerend virus van Japan (HVJ) voor het afleveren van therapeutische genen of antisense oligonucleotiden in HSC. Voor HVJ is aangetoond dat het zowel in vivo als in vitro heel efficiënt cellen kan transfecteren, maar de opname in bepaalde doelcellen in vivo blijft een probleem. Op dezelfde manier waarmee het virus kan fusieren met het cellmembraan, kan het virus echter ook fuseren met liposomen. In deze studie werd een plasmide met luciferase als reporter gen, geïncorporeerd in geïnactiveerd HVJ, waarbij een zogenaamde HVJ envelop wordt gevormd. Deze HVJ enveloppen werden vervolgens gefuseerd met M6P-HSA liposomen. De verkregen partikels werden vervolgens gekarakteriseerd en hun efficiëntie om de HSC in de fibrotische lever te bereiken werd bepaald. De gevormde deeltjes werden inderdaad selectief door HSC opgenomen. In vitro was de transfectie efficiëntie van de M6P-HSA-HVJ liposomen echter laag. Dit is mogelijk een gevolg van een verlies aan plasmide DNA gedurende de fusie, of van een veranderde infectieroute van het virus via de M6P/IGF II receptor. Additionele experimenten zijn nodig om het transfectiemechanisme van M6P-HSA-HVJ liposomen te verduidelijken. Het uiteindelijke doel van deze studie is aan te tonen dat genen kunnen worden afgeleverd met behulp van M6P-HSA-HVJ liposomen in HSC teneinde het proces van leverfibrose te remmen. Hiervoor zijn echter meer vervolgstudies noodzakelijk.

Samenvattend kan gesteld worden dat het gebruik van selectief gestuurde of getargete liposomen als dragers van medicijnen naar de HSC in de leverfibrose een reële optie is om een therapie te ontwerpen voor deze ziekte. We hebben aangetoond dat de liposomen snel accumuleren in de lever en de doelcel bereiken, hoewel ook andere lever celtypen de gemodificeerde liposomen opnemen. De doelcellen zijn te beïnvloeden door de lipid samenstelling van het liposoom en ook een modulatie van genen met behulp van virusspartikels geïncorporeerd in de liposomen lijkt mogelijk. De kennis gegenereerd in dit project kan de aanzet zijn voor een nieuwe, rationele farmacotherapie gebaseerd op liposomen die selectief farmacologisch actieve stoffen afleveren in cellen betrokken bij leverfibrose waaronder de zo belangrijke HSC.
Stelling 12 in practice.
appendix I

acknowledgement
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contributing authors
abbreviations
Acknowledgement

I think the idea of studying abroad was born when I attended secondary school. I was fascinated by the fact of living abroad, getting to know new people and new culture. I thought this would be an exciting experience and definitely more challenging than studying in my own country. Although I had Germany or Great Britain in my mind as a destination, fate brought me to a place in between these countries, to the Netherlands. And now, years after my secondary school time, as I’m finishing Ph.D. studies, I can say that these four years indeed were exciting and challenging. I learnt how is to be a researcher and gained experience in doing laboratory work, had the privilege of living in the lovely town of Groningen and met a lot of people without whom I wouldn’t be here and completing this thesis wouldn’t have been possible. To all of you, being close and far I’m endlessly thankful. However, let me mention some people by name.

First of all, I would like to express my gratitude to Prof. Gerrit Scherphof. I came to Groningen because you obtained an Ubbo Emmius grant for me. I’m very grateful, not only for your guidance in scientific matters, our discussions and your comments on my manuscripts, but also for your friendliness and concern about things not related to experiments. You kept my spirits up while I was waiting endlessly for my visa to come in, arranged for me to participate in the Liposomal Workshop in Oberjoch even when I was not yet appointed as a Ph.D. student and helped me organise my visit to the laboratory of Prof. Kaneda at the Osaka University. For all of this and more, thank you!

Of course this thesis wouldn’t have come about without Prof. Klaas Poelstra and Dr. Jan Kamps, my supervisors, who shared my joy and frustration at the mundane research work on daily base. I always appreciate you enthusiasm and optimism, which provided a counterweight to my innate scepticism. I’m thankful for your support of my ideas and encouraging me to try “high risk” experiments. Klaas, I truly admire your ability to find and envision success. I always appreciated our discussions on manuscripts and I think nobody else can guide people through sections of stained tissue in such an interesting way. I’m also appreciative for your help in finalising this thesis as a first promoter.

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Finally, I would like to express my gratitude to Prof. Dirk Meijer also my promoter. I was always happy to see your interest in my project. The regular meetings were bringing valuable discussions which allow me to see different aspects of my research in the broader perspective.

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Because of my working in two different departments (the department of Pharmacokinetics and Drug Delivery and the section Medical Biology of the department of Pathology and Laboratory Medicine), situated in two different buildings, my Ph.D. time could be considered somewhat exceptional. Two offices and many labs meant running back and forth remembering where I left what all day and, often causing quite a bit of frustration.
Fortunately, there is always another side of the medal, since being in two groups meant twice the amount of birthday cakes and “Lab-days” twice a year.

From Department of Pharmacokinetics and Drug Delivery, I obviously wish to thank Catharina first of all, not only for the isolation of the cells, but also for your help with the synthesis of modified albumin, animal experiments, stainings and hundreds of other small things which I bothered you for during these four years. Annemiek, how would I perform animal experiments without you? I’m very grateful for your flexibility, sometimes even coming to the lab during the weekends. Alie, many thanks for the helpful hand whenever I needed it. It was always good to be around you, because of your smile and the positive attitude you radiate. Jai and Alie, I shared my office with you with pleasure. Jai, you were my company for work in the evenings and weekends. I enjoyed our discussions about science and many other subjects, as well as your stories about India – I hope I will soon visit this fascinating country. Teresa, Teresita my friend and my “sister-in-arms” during the last four years. Thank you for all your support, your care and your optimism inside and outside of the lab. Heni, my starting out in this department wouldn’t have been so easy without you. Teresa, Jai, Rick, Werner, Marja, Annemarie, Ester, Kai, Adriana, Janja, Anshar: it was fun to share the experience of being a Ph.D. student with you, not only within the walls of faculty building, but also on conferences, in pubs and at the bowling centre.

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When I started my Ph.D., the Liposome Research group belonged to the department of Cell Biology. I enjoyed my time on the 6th floor of the FMW building with Xuedong, Martin, Alida, Ewa and Ingrid a lot; thank you all for a warm and friendly working environment. I also wish to express my gratitude to Gerry Hoogenberg. Thanks to you, the pile of formalities which needed to be arranged after moving to this new country did not turn into nightmare. Dear Ph.D. students from the 10th floor: Kacper, Luc, Delphine and Zuzana, the chatting and gossiping, mostly somewhere in the corridors, always provided great amusement.

In December 2003, the Liposome Research group joined with the Endothelial Biomedicine and Vascular Drug Targeting group in the Medical Biology section. Within this context, I’m grateful to Prof. Ingrid Molema for her interest, our discussions and her support, especially during the final period of my Ph.D. Her inspiring enthusiasm and valuable comments are close to being famous. Henriëtte, hartstikke bedankt voor je hulp met de bereiding van liposomen, de isolatie van cellen en voor alle andere dingen die je voor mij hebt gedaan. Het delen van een werkbank met jou was erg plezierig. Maar ik wil je vooral bedanken voor het geduld waarmee je steeds Nederlands met mij wilde praten.
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I’m grateful beyond expression to the people I left when coming here: my family. My parents, Ewa and Benedykt, thank you for your continuous support, encouragement and faith in me. Especially when living abroad, it is important to know that you are always on my side. I think that now, at the end of my Ph.D., is a good moment to thank you as well for all the time and effort you put into my education. This Ph.D. is the result of all these years. Misio, my younger brother who knows the harsh reality of life better than I do, but still has great sense of humour. I was missing you quite a lot over here. I hope that, one day, we can live closer to each other. Many thanks for some great holidays in the Polish country side, and for all your care. Aunt Wisia and uncle Pawel, Kasia and Przemek your care and interest is overwhelming. Thank you for your warmth and help over the years. I was always happy to see you come to the station just to say good bye, even if it made leaving again even more difficult for me.

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Asia

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List of publications


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Aco-HSA</td>
<td>cis-aconitic anhydride modified human serum albumin</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>BDL</td>
<td>bile duct ligated</td>
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<tr>
<td>CCl₄</td>
<td>carbon tetrachloride</td>
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<td>³H-COE</td>
<td>[³H]cholesterol oleyl ether</td>
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<td>Chol</td>
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<td>DLPC</td>
<td>1,2-dilinoleoyl-sn-glycero-3-phosphocholine</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>GAPDH</td>
<td>glyceraldehydes-3-phosphate dehydrogenase</td>
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<td>human serum albumin</td>
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<td>HSC</td>
<td>hepatic stellate cells</td>
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<td>HVJ</td>
<td>Hemagglutinating virus of Japan</td>
</tr>
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<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>KC</td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>LEC</td>
<td>liver endothelial cells</td>
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<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<tr>
<td>M6P/IGF II</td>
<td>mannose 6-phosphate/insulin-like growth factor receptor</td>
</tr>
<tr>
<td>M6P-HSA</td>
<td>mannose 6-phosphate modified human serum albumin</td>
</tr>
<tr>
<td>MPB-PE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(maleimidophenyl)butyramide]</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>platelet derived growth factor</td>
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<td>POPC</td>
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<tr>
<td>poly I</td>
<td>polyinosinic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
</tbody>
</table>
Gate of Todai-ji (Great Buddha temple) in Nara.
appendix II

The adventure in Japan

Joanna E. Adrian
GUIDElines October 2005
Early morning: before the alarm clock rings, trucks coming to the small factory behind the house wake me up. The house is situated in a rather interesting neighbourhood, with a small paddock in front of the house, a large gambling palace and highway to the left, the aforementioned factory behind it and a quaint apartment building to the right. There’s a small river running through this scene, in which a couple of turtles live. After the night on the futon, my muscles and bones are slightly sore. The bed is thin and rather cool, but this is not all that unpleasant given the warm nights here in Osaka. This is one of Japan’s largest ports, and the heart of a metropolitan area second only to the megalopolis of Tokyo several hundred kilometres to the north. Not that my small apartment in the far north of the city really gives the impression of being located in a buzzing and relevant city. It’s mainly the trucks that do the buzzing here.

After a quick shower and some breakfast, it is time leave for the lab. It is sunny and rather hot already this morning. In the middle of the day, it will be 30 degrees. The Suita campus of Osaka University, my destination now, is 25 min by foot from my house. I walk uphill along a smelly highway, where the newest branch of the Osaka City Monorail is under construction. Its concrete tracks with magnetic propulsion system tower high above the road. Land is very expensive here, so it was cheapest to just build this futuristic rollercoaster above the road that the city already owned. On the ground, Osaka is a town designed for cars and not for bikes and pedestrians. The first have broad highways, spanning two levels or more where necessary. The last have to share narrow and bumpy pavements. Entering Suita campus itself brings relieve though, it is quiet here. Only cars with special (and expensive) permission are allowed to enter the campus, and the institutes are surrendered by half wild ponds, patches of bamboo forest and, naturally, cherry trees. It is amazing how many of these there are around here. They grow everywhere, but you really notice them during the cherry blossom time. It lasts only around a week, and Japanese people celebrate it by having mandatory parties on the grass below the cherry blossoms.

The institutes on the Suita campus are all related to the beta sciences and engineering. It is here, also, that one finds the Osaka University Hospital, the largest hospital of Osaka, and its Graduate School of Medicine, reputedly the best medical school in Japan. Within the rather monumental looking building of the Graduate School, I take the elevator to the tenth floor, the left wing of which houses the Division of Gene Therapy Science of Professor Yasufumi Kaneda.

The research of this group focuses on using the Hemagglutinating virus of Japan (HVJ,
or Sendai virus) as vectors for gene therapy. The major advantages of HVJ are its high transfection activity in various types of tissues and its being non-infectious for humans, which makes HVJ vectors potentially usable for medical purposes. HVJ enters the cell by fusing with the cell membrane, a process which is mediated by the virus’ membrane proteins. Reports from the laboratory of Professor Kaneda showed that HVJ can also fuse with liposomes (lipid vehicles) containing plasmid DNA. As a result of this fusion, viral proteins are introduced into the membrane of liposomes, significantly increasing transfection activity of these liposomes.

For my PhD project, under the supervision of Klaas Poelstra and Jan Kamps, I’m working in two departments: the Department of Pharmacokinetics and Drug Delivery, and the Medical Biology section. The aim of this project is the development of liposomes targeted to the hepatic stellate cells in the fibrotic liver. In a diseased organ, these cells produce large amounts of collagen, making them attractive targets for anti-fibrotic therapy. One of the approaches to the treatment of fibrotic livers would be gene therapy. In November 2004, I wrote a mail to Professor Kaneda, proposing a research project which combines the high transfection efficiency of HVJ with the targeting properties of our liposomes. Professor Kaneda accepted my proposal, and invited me to perform experiments in his laboratory. Since HVJ vectors lack cell specificity, the aim of the study was to investigate whether fusion of HVJ with our targeted liposomes will form HVJ liposomes characterised by both specific interaction with certain cell types and good transfection activity.

Every morning, when I enter the lab, I’m greeted by a cheerful ohaio gozaimas! (good morning!) from Okuno-san, one of the technicians. In this group, there are between 30 and 40 people, but only two technicians. Here, everybody is doing all their experiments by themselves. In spite of the grandeur of the building on the outside, the laboratories themselves are small and filled to the absolute limit with people, laboratory animals, equipment and chemicals. It all looks rather chaotic and there isn’t much free space, like most of Osaka. But it’s the people that matter more than things, and the people here are very friendly. Their hospitality is great and they are always willing to help. Fortunately, they have a lot of opportunities to help me. It is not just that it is a new lab for me – it is a
Japanese lab to boot! This means manuals, protocols and software are most often written in unreadable Japanese, just like catalogues, forms, ordering systems, group seminars and many other large and small things (like labels on waste containers). It was sure to make working here a lot of fun, of the confused and adventurous sort. Unfortunately, not all people in the group speak much English either. On the other hand, working here can be fast and efficient, mainly because of the very short delivery times of ordered items (often only one or two days) and the free access to all kinds of equipment.

Lunch time is around one o’clock in the afternoon, when I buy a sandwich in the shop and join some colleagues that are eating in the seminar room. The typical Japanese lunch consist of rice or noodles with some fish or sea weeds or other kind of sea animal and a bit of vegetable. To drink, green tea is most popular. It can be bought everywhere, even from vending machines, and several kinds are usually available. After lunch, I have a meeting with Professor Kaneda. Although he is very busy person, he has time for me whenever I need it, and provides me with all the help he is able to give. Our discussion is short, but contrite and interesting as usual. He has been educated as a medical doctor, and his opinions are therefore often coming from different point of view as mine, or those of my supervisors in Groningen. I learnt a lot from him.

I work until six o’clock, when I walk to the large cafeteria at the centre of the campus for dinner. I can try Japanese dishes here, and it is actually cheaper then cooking by myself. Luckily, the menu is exhibited on the wall and each dish is described by names in both Japanese and Latin writing, and accompanied by a picture. This time, I take *tamagodon* (a bowl of rice, with scrambled eggs and vegetable on top) and *miso suru* (a kind of soup, based on fermented soybeans). After dinner, I go back to the lab. Early in the evening, it is still hot and almost dark. The night comes very fast here, even in the middle of summer. Of course, the laboratory is still full of people. The atmosphere is good here, everybody seems to enjoy being at work and nobody is in a hurry to go back home. I chat a bit with a colleague about the lab party which took place yesterday in the luxurious apartment of one of the associate professors. She, like some other people in the lab, is a medical doctor and she works in the hospital as well. Sometimes she needs to recuperate during the long day at work, and then she sleeps on her desk for about half an hour. She’s always a bit worried that the professor will see her sleeping like that, because it is important, in the hierarchy of the lab, for your superiors to have a favourable impression of you. Of course, the professor knows, but he is understanding enough to pretend never to notice. Around ten o’clock it’s rush hour on the campus and people are going back home.
On my way home, I pass a supermarket to buy some breakfast for tomorrow. As usual, I walk through the big section of sea food, which is like a free visit to the aquarium full of weird sea monsters. A bit frozen perhaps, but still quite interesting. The shops here have long opening hours, and if you forget something there are plenty of convenient stores that are open all night. However, this convenience is not quite extended to financial matters. For such an advanced economy, Japan is remarkably cash-oriented and one shouldn’t expect to be able to pay with plastic anywhere. And unlike the stores, ATM’s are open strictly during the day and one should be careful not to be too late for them.

Before I go to sleep, I talk with my mother using Skype. In Poland, it is now four in the afternoon. Tomorrow will be a Saturday, and I’m planning to visit some temples in Kyoto. The former capital of Japan is only thirty minutes by train from Osaka, but its character is totally different. It is a historic and cultural city, with some two thousand temples. On Sunday, I’m going to relax in an onsen, a traditional Japanese bath, and have a dinner in the neighbourhood restaurant where they serve the Osaka speciality of okonomiyaki, a small pancake of Japanese potatoes with oyster sauce, cabbage and bonito flakes. There’s always something interesting to do here.

If I had to make the decision once again, I would again decide to go for a short research project to Japan. This experience belongs to those that happen only once in your life.

March-July 2005
1. To improve upon existing drug delivery systems, it is necessary to enlarge our understanding of the mechanism by which drug carriers interact with cells in vivo. \[this\ \text{thesis}\]

2. Our knowledge of cells in diseased tissue is often more extensive than that of the same cells in a healthy condition.

3. Current experimental antifibrotic therapies tend to focus on targeting hepatic stellate cells selectively. However, given the involvement of all types of liver cells and the wide spectrum of factors causing liver fibrosis, multiple cell targeting is relevant and should receive more attention. \[this\ \text{thesis}\]

4. The field of liposomal drug delivery systems is in dire need of a breakthrough comparable to the invention of sterically stabilised liposomes. \[London\ 2005\ \text{ILS conference}\]

5. When living abroad, one tends to learn about one’s own country in particular.

6. Laziness and stupidity are unlimited sources of easy money.

7. One knows that a country is bound for agony when its government starts reducing the budget for higher education.

8. In democracies, it is an unfortunate fact that the winner tends to be the loudest politician, rather than the wisest one.

9. The world is presented the bill for current global political stability in the form of regional conflicts.

10. Parents would do well to realise that the tolerance level of other passengers for their children’s behaviour in a train is probably much lower than theirs.

11. It is natural for universities to adapt to changing circumstances, but the present demand for valoration of knowledge should not lead to a situation where research groups simply embrace corporate methods. This tends to stimulate research on small scale variations of known themes, so as to heighten the chances of short term results and financing, at the expense of new perspectives.

12. Horse riding would be a valuable addition to the normal school curriculum, as it teaches one how to be consequent.

Joanna E. Adrian
6 december 2006