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Summary

Liver cirrhosis is a chronic disease, which may be elicited by viruses (hepatitis B, C, and D), chronic alcoholism, prolonged obstruction of the bile flow, chronic exposure to hepatotoxic agents (e.g. drugs or environmental toxins), or inherited diseases such as hemochromatosis, Wilson's disease, glycogen storage diseases, and α_1-antitrysin deficiency. The disease is associated with a high mortality, it is the seventh leading cause of death by disease. The hall-mark of liver cirrhosis is the excessive deposition of extracellular matrix proteins in the liver. This process of scarring is called fibrogenesis. The scar tissue that forms in cirrhosis harms the normal structure of the liver and blocks the flow of blood through the organ. The loss of functional liver tissue slows the processing of nutrients, hormones, drugs, and toxins by the liver. Also, the hepatic synthesis of many proteins and other substances is impaired. The hepatic stellate cells are predominantly responsible for the enhanced production of extracellular matrix proteins in the cirrhotic livers.

In this thesis, we used the bile duct ligation (BDL) model in rats as the experimental model for cirrhosis. This rat model resembles the pathology of cirrhosis in patients, which is caused by a blockade of the bile flow, such as primary biliary cirrhosis. Other reasons determining our choice for the BDL model were the quick development of the fibrotic process (within 3 weeks after ligation), the low mortality of the rats, and the high reproducibility of the pathological events. In the BDL model, liver injury starts at the portal areas in contrast to other models of fibrosis, such as CCl_4 intoxication, which is associated with centrilobular lesions. No model perfectly mimics the human disease. However, a final common pathway of fibrogenesis in most experimental

models is the activation of hepatic stellate cells, which is an important feature for our studies.

To date, no effective antifibrotic drugs are available to reverse the process of liver cirrhosis. The treatment of patients aim at a limitation of further damage to the liver or at an attenuation of the complications such as kidney failure, ascites, or hepatoencephalopathy. In the past decades, a liver transplantation has become a relevant option for many patients, but the hospital costs and the availability of donor livers hamper the application of such an operation in general practice. A pharmacologic intervention would be a better option for antifibrotic therapy, but as outlined before no adequate antifibrotic drugs are available. To improve the pharmacological effects of antifibrotic drugs, we aim at a specific delivery of these drugs to the different cell types in the liver. Therefore, we examined the cellular specificity of modified albumins in cirrhotic livers. Furthermore, we developed new carriers that specifically bind to or are taken up into hepatic stellate cells, the cells that play a key role in the pathogenesis of liver cirrhosis.

Pathology.

Liver cirrhosis is a complicated disease, since all resident hepatic cell types are involved and each cell type plays its own specific role during the progression of the disease. Initial liver damage usually affects the hepatocytes. As a consequence, these cells release mediators that activate the Kupffer and sinusoidal endothelial cells. In addition, the release of chemokines and the upregulation of adhesion molecules on endothelial cells will induce the intrahepatic infiltration of inflammatory cells. Kupffer and endothelial cells are involved in the inflammatory response that sustains throughout the fibrogenic process. The release of a broad array of cytokines, growth factors, reactive oxygen species (ROS), and other mediators subsequently activate hepatic stellate cells. After activation, stellate cells proliferate and migrate into the injured areas and transdifferentiate into myofibroblast-like cells. These cells are to a major extent responsible for the enhanced production of the extracellular matrix proteins (mainly collagen type I and III) that are deposited in the liver tissue.
Insight in the pathogenesis of the disease is necessary to rationally identify the targets for the carrier and the coupled drugs, and to identify (early) markers that reflect the severity of the disease. These markers can be applied to evaluate the effects of therapeutic interventions. Several tissue and plasma markers in different stages of the disease may serve as early markers. Examples of early

markers for the fibrotic process are described in **chapter 3**, in which we studied the generation of reactive oxygen species (ROS) and nitric oxide radicals (NO) after induction of fibrosis. ROS (consisting of H_2O_2 and superoxide anions) were produced at all stages of the disease, but peak levels were observed at 1 to 2 weeks after ligation. ROS were mainly detected in the portal areas, the sites where most inflammatory cells were present, but they were also found in the parenchymal and necrotic areas of fibrotic livers. In addition to this ROS staining, we were also able to detect lipid peroxidation products (i.e. 4-hydroxynonenal adducts) in these fibrotic livers at sites where ROS were produced.

Furthermore, we showed that an increased production of NO took place in fibrotic livers due to an upregulation of the enzyme inducible nitric oxide synthase (iNOS). This enzyme was upregulated immediately after induction of fibrosis and its expression was present in the portal areas mainly in the inflammatory cells and in groups of adjacent hepatocytes in the parenchymal area. In addition, iNOS expression was found in both of these cells types around necrotic areas. iNOS expression was enhanced early after induction of fibrosis, that is at four days after ligation. The total hepatic iNOS expression disappeared at two weeks after ligation.

Since a correlation between NO production and collagen formation has been suggested, we studied the development of the fibrogenesis after administration of the iNOS-selective inhibitor S-methylisothiourea in vivo. An inhibition of NO production did not cause significant differences in the total collagen content of the liver. In the portal areas where collagen deposition was most prominent in bile duct ligated rats, no changes were observed in the staining for collagen type I and III. Only around necrotic areas enhanced staining for collagen type I and III was found in rats treated with S-methylisothiourea. Therefore, the results obtained in this study indicate a local and transient anti-fibrotic action of NO produced by iNOS in fibrotic livers.

The early induction of iNOS and the production of ROS in the fibrotic rat livers renders these parameters as (early) markers for hepatocyte damage, which may allow the early detection of therapeutic effects of antifibrotic agents.

Targeting to hepatocytes, endothelial and Kupffer cells.

In the past, modified albumin carriers have been developed that selectively accumulate in hepatocytes, Kupffer cells, or endothelial cells of the liver. This cell selectivity was obtained by sugar and charge modification of albumin.

Lactosylation, mannosylation, and succinylation are standard modification procedures for the targeting to the above mentioned hepatic cells. The cellular selectivity of these modified albumins was predominantly examined in healthy animals. Therefore, we started our project by evaluating the organ and intrahepatic distribution of these modified albumins in rats with fully developed liver fibrosis, that is, three weeks after ligation of the bile duct. The distribution characteristics and kinetic parameters were nearly identical in healthy and diseased rats which demonstrates the applicability of these macromolecules for the targeting of drugs to the different cells in the cirrhotic livers (**Chapter 4**).

Targeting to hepatic stellate cells.

Hepatic stellate cells are the key cells involved in the formation of the extracellular matrix proteins. Moreover, these (activated) cells produce an array of mediators that enhance and sustain the fibrotic process independently from the activity of Kupffer, endothelial, and inflammatory cells. This central role for hepatic stellate cells prompts the development of agents that interfere with the activation of this cell type. Targeting of antifibrotic agents using albumin carriers is an option to obtain a selective drug accumulation in activated hepatic stellate cells. Until recently, no carrier molecule was available that distributed to stellate cells. Therefore, we developed carriers that interacted with membrane receptors on stellate cells, which are upregulated after activation of this cell type. Since a strong upregulation of the mannose 6-phosphate/insulin-like growth factor II receptor was reported on the cell membrane of activated stellate cells, we modified albumin with mannose 6-phosphate groups ($M6P_x$-HSA). An increase in the degree of mannose 6-phosphate substitution on the core protein albumin induced a parallel increase in the hepatic stellate cell-specific accumulation *in vivo* in rats with liver fibrosis. $M6P_x$-HSA with a low degree of sugar loading ($x<10$) remained in the plasma (>75% of the administrated dose), whereas an increase in the molar ratio of M6P:HSA to 28:1 caused an preferential accumulation in the liver (59±9% of the administrated i.v. dose at t=10 min). Studies on the *in vivo* intrahepatic distribution of $M6P_x$-HSA revealed that 70±11% of the intrahepatic staining for $M6P_{28}$-HSA was found in hepatic stellate cells (**Chapter 5**). *In vitro* studies confirmed the binding of $M6P_{28}$-HSA to rat hepatic stellate cells, in particular to primary cultures of activated hepatic stellate cells. Results demonstrated that $M6P_{28}$-HSA binds to specific receptors on the target-cells. Furthermore, rapid internalization of $M6P_{28}$-HSA was demonstrated that occurred via the endosomal/lysosomal

pathway. Similar results regarding binding and uptake were obtained in studies with slices prepared from human livers. Sinusoidal endothelial cells were responsible for the hepatic uptake of $M6P_{28}$-HSA in normal human livers, whereas hepatic stellate cells contributed primarily to the uptake of this neoglycoprotein in cirrhotic human livers (**Chapter 6**).

New carriers to hepatic stellate cells were designed based on the use of cyclic peptides that recognize specific receptors on these target-cells. We selected the collagen type VI and platelet derived growth factor (PDGF) receptors, since the expression of these receptors is upregulated on stellate cell membranes during fibrosis. Cyclic peptides were prepared that contained the amino acid sequence recognizing the receptors of collagen type VI and PDGF-BB. The use of peptides as homing devices to gain access to target cells is a new concept described in this thesis and a patent application on this subject has recently been filed by us (**Appendix**).

In **chapter 7**, the results obtained with the carrier pCVI-HSA are described. Albumin substituted with RGD containing cyclic peptides displayed a specific interaction with hepatic stellate cells *in vitro* and *in vivo*. pCVI-HSA was taken up in activated hepatic stellate cells, although at a slower rate and to a lesser extent as $M6P_{28}$-HSA. *In vivo* results showed preferential accumulation of pCVI-HSA in fibrotic and normal rat livers, respectively 62±6% and 75±16% of the dose was found in this organ 10 min after administration. Subsequent analysis of these livers demonstrated that in fibrotic livers 73±14% of the hepatic cells that bound the carrier could be identified as hepatic stellate cells, whereas endothelial cells contributed mostly to the total liver uptake of pCVI-HSA in normal rats.

The peptide modified albumin pPB-HSA was studied for its applicability as a stellate cell-specific carrier via binding to the PDGF receptor (**Chapter 8**). Specific binding of pPB-HSA to PDGF receptors was demonstrated by performing competition studies in fibroblast cultures with native PDGF-BB (^{125}I labelled) and increasing amounts of pPB-HSA. *In vivo* experiments established the liver specific distribution of pPB-HSA in normal rats and in rats with liver fibrosis. Moreover, hepatic stellate cells largely contributed to this hepatic uptake in fibrotic livers after intravenous injection. *In vitro* studies confirmed the binding of pPB-HSA to primary cultures of rat hepatic stellate cells, in particular to the activated cells.

Conclusions.

The studies presented in this thesis demonstrate the applicability of modified abumins as drug carriers to cells in cirrhotic livers. Now all hepatic cell types, each of them playing a role in the development of liver fibrosis, can be specifically targeted using modified albumins. Derivatization of albumin with lactose, mannose, or succinic acid resulted in specific distribution to respectively hepatocytes, Kupffer cells, or endothelial cells in fibrotic livers. The lack of cell-selective drug carriers to hepatic stellate cells at the beginning of our project is now compensated by the availability of three different types of carriers. Whether neoglycoproteins or peptide-modified albumins represent the most optimal carrier remains to be established. The carriers $M6P_{28}$-HSA, pCVI-HSA, and pPB-HSA all allow cell-specific delivery of antifibrotic agents to hepatic stellate cells. Since the mannose 6-phosphate/insulin-like growth factor II receptor and the amino acid sequences recognizing the collagen type VI and PDGF receptors are similar in rats and in humans, these carriers may be applicable in patients as well. Further research is warranted to explore the possibilities of drug targeting in cirrhotic patients.