Angiogenesis in liver fibrosis
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GENERAL INTRODUCTION
1. Liver fibrosis

Liver is known as the most regenerative organ which has the capability to renew and repair itself after injury. However, when the injury is sustained, chronic inflammation and accumulation of extracellular matrix (ECM) persist and lead to a progressive substitution of liver parenchyma by scar tissue, resulting in liver fibrosis. The inflammatory response by macrophages is one of the first events that occur following liver injury due to viral infections, alcohol consumption, or hepatotoxic substances. They respond among others with an increased production of cytokines, such as TGF-β, TNF-α, IL-1, IL-6, and IL-10 that are predominantly secreted by the resident macrophages, known as Kupffer cells (KC). These cytokines are involved in the activation of hepatic stellate cells (HSC), which results in excessive ECM deposition, containing mainly collagen. In the normal liver, quiescent HSC have a role in storing vitamin A, but upon activation they lose their vitamin A droplets, transdifferentiate into myofibroblast-like cells, proliferate, contract and release pro-inflammatory, profibrogenic and promitogenic cytokines. Another important feature of liver fibrosis is vascular changes which involve capillarization of hepatic sinusoids and angiogenesis that lead to an abnormal angioarchitecture of the liver.

The complexity of the processes involved in liver fibrosis makes it difficult to understand the disease, which complicates the development of effective treatment for this disease. Over the past few years, numerous compounds have been developed as antifibrotic, and their efficacies have been tested either in in vitro models using human and animal HSC or in vivo animal models of liver fibrosis, or both. However, none of these compounds have passed clinical trials and reached the market as therapy for fibrosis. Several different strategies were employed in the effort to develop a pharmacotherapy for fibrosis. A first strategy was to reduce the initial injury with hepatoprotectants, for instance by using antioxidants that can interfere with reactive oxygen species (ROS) formation, as an elevated intracellular ROS concentration was shown to be linked to the increased production of profibrogenic mediators by KC and recruitment of circulating inflammatory cells. Silymarin is an antioxidant that was shown to have antifibrotic properties in a rat model of biliary cirrhosis. However, it failed to show significant effects in patients with primary biliary cirrhosis. A second strategy was directed to the activated myofibroblasts and involved interference with fibrosis-relevant pathways in activated myofibroblasts such as kinase inhibitors or siRNA, stimulation of matrix degradation, for example by inhibiting the activity of collagen cross-linking enzyme lysil oxidase like 2 (LOXL2), induction of reversion of activated HSC, or induction of cell death in the activated HSC, for instance by utilizing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This latter approach resulted in elimination of activated HSC but this approach raised a concern of risking the resident hepatocytes...
as well ⁴, ²⁶. Attempts to target the antifibrotic molecules to the activated myofibroblasts by coupling them to ligands that are recognized by specific receptors on the surface of these cells were successful in animal studies, but this approach has not been tested in man yet ¹³, ¹⁴, ²⁷-²⁹. As a third strategy, therapies directed to the inhibition of angiogenesis have been investigated in the recent years ²¹, ³⁰, ³¹.  

2. Angiogenesis and its role in liver fibrosis  

Angiogenesis, defined as the formation of new blood vessels from pre-existing ones, is one of the features in liver fibrosis and cirrhosis that was first introduced by Moschowitz in 1948 in a histological study in patients with Laennec cirrhosis ³². A few years later, angiogenesis was shown to be accompanied by increased portal cellularity, fibroblast proliferation, bile duct proliferation and altered liver structures in patients with cirrhosis ³³. However, it lasted until 1984 until the first anti-angiogenic approach in experimental liver fibrosis was published ³⁴, more than 10 years after the first published finding on the role of angiogenesis in tumor progression ³⁵. Since then, the role of angiogenesis in liver fibrogenesis has been studied extensively.  

Structural and anatomical changes, and particularly the increased collagen disposition, in the liver during fibrogenesis have been proposed to affect liver perfusion and compromise oxygen supply ¹⁰, ³⁶. In addition, new formation of blood vessel takes place in the fibrotic liver, as was also observed in cancer, where neovascularization is initiated to compensate for the shortage of oxygen supply ¹⁰, ³⁷, ³⁸. Whether this angiogenesis in liver fibrosis is a consequence of the compromised oxygen supply still remains to be confirmed. Moreover, the activated HSC, endothelial dysfunction and the inflammatory cells were hypothesized to be involved in the production of pro-angiogenic factors and the creation of a supporting milieu for neovascularization in fibrogenesis ¹, ¹⁰, ³⁶.  

Although it has been shown that angiogenesis is positively correlated with liver fibrogenesis, the role of angiogenesis in liver fibrosis is hitherto not elucidated. There is still a debate on whether this angiogenesis is a defense mechanism in response to the fibrosis and helps to protect the hepatocytes against oxygen deprivation, or whether it supports and even aggravates the fibrotic condition. Several anti-angiogenic compounds that were tested both in vitro using HSC and in vivo using mouse or rat models of liver fibrosis, such as brivanib, TNP-470, sunitinib and sorafenib, have shown antifibrotic effects in these models ²¹, ²², ³⁰, ³¹. However, these compounds are multi-target kinase inhibitors, which imply that the antifibrotic effect cannot unequivocally be ascribed to their anti-angiogenic properties. For example, an anti-angiogenic compound that inhibits activation of vascular endothelial growth factor receptor 2 (VEGFR2), also inhibits the activation of platelet derived growth factor receptor β (PDGFRβ) ²². Since PDGFRβ is an important receptor
involved in activation and proliferation of HSC, the antifibrotic effect of sunitinib could also be ascribed to its direct effect on HSC rather than to its anti-angiogenic effect. Moreover, there are conflicting data showing that angiogenesis inhibition eventually can aggravate liver fibrosis and decelerate fibrosis resolution. For example, cilengitide, a specific inhibitor of integrin αvβ3 and integrin αvβ5, has been shown to have anti-angiogenic effect in patients with recurrent glioblastoma in phase II clinical trial and antifibrotic activity on human HSC in vitro. Although it was shown that cilengitide inhibits angiogenesis in rat models of secondary biliary fibrosis and panlobular fibrosis, this compound aggravated the fibrosis condition in both models in spite of its antifibrotic effect. Another example is the recent discovery of the effect of genetic ablation of vascular endothelial growth factor (VEGF) in scar-infiltrating myeloid cells in a fibrosis model of bile duct ligation (BDL) and CCl4-exposed mice. The effect of genetic ablation was evaluated during fibrogenesis and during recovery after the fibrosis induction was terminated. The specific genetic targeting prevented the sinusoidal angiogenesis during fibrogenesis in CCl4-exposed mice but not in the BDL mouse model. However, no effect was observed on the degree of liver fibrosis during fibrogenesis, but the specific genetic ablation of VEGF prevented the resolution of fibrosis in both models during the recovery phase.

Angiogenesis is tightly controlled by the balance of pro- and anti-angiogenic factors. These factors can influence one or more of the different steps in the angiogenesis process. Most angiogenic factors have been characterized in the pathogenesis of tumor growth and some have been investigated for their involvement in liver fibrosis, i.e. VEGF and angiopoietin-1. These factors are either produced by the endothelial cells or by other cells present in the liver. For example, VEGF-A (one of the VEGF subtypes which is known as a potent angiogenic activator) is involved in the increased permeability of the vessel wall, in endothelial cell proliferation and migration and in mesenchymal proliferation and migration. VEGF-A is one of the most potent angiogenic activators and
an increased VEGF-A expression in the fibrotic liver has been shown in some studies in human, mouse and rat. In contrast, recent studies showed that VEGF-A is decreased in fibrogenesis and re-augmented during fibrosis resolution in mouse models of CCl₄ and BDL induced liver fibrosis. There have been suggestions for the explanation of this discrepancy, such as the use of different fibrosis models or different methods to quantify VEGF-A expression, but the most feasible explanation for the finding in the fibrosis model of CCl₄-exposed mice was the different time point for measuring the VEGF-A level. Kantari-Mimoun et al. showed that an increased VEGF-A expression at 6 weeks of CCl₄ exposure was followed by a decreased level after 12 weeks of exposure, indicating a transient peak after liver injury with subsequent decrease in prolonged CCl₄ exposure. In contrast, Nakamura et al. found a decreased VEGF-A expression even at an earlier time point (4 weeks of CCl₄ exposure). No specific explanation of this phenomenon has been found yet.

**Fig. 1** Mechanism of angiogenesis. Blood vessel formation is a multifactorial process. The first step is destabilization through Ang-2 (1) and hyperpermeability (2) of the vessel wall. Followed by EC proliferation and migration (3). Cell-to-cell contact is established through VE-cadherin and integrins (4). Tube formation is completed via TNF-α, FGF and PDGF (5), followed by mesenchymal proliferation and migration (6) and pericyte differentiation (7). Vessel formation is completed after stabilization (8). Some of these steps can be inhibited by endostatin, angiotatin and thrombospondin. Ang, angiopoietin; EC: endothelial cell; EGF, epithelial growth factor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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Liver fibrogenesis is mainly sustained by hepatic myofibroblasts (MF) which represent a heterogeneous population of cells. It is clear that HSC are the leading actors in liver fibrogenesis because hepatic MF are mainly originated from activated HSC. However, the role of hepatocytes in liver fibrogenesis has not been completely understood. Hepatocytes, which comprise ~80% of liver mass, are often regarded as “victims” and “bystanders” in liver fibrosis and have been proposed to contribute in liver fibrosis through a process of epithelial to mesenchymal transition, but this issue remains controversial. Nevertheless, hepatocytes express important angiogenic factors, i.e. VEGF-A, and thrombospondin-1 (THBS-1). The contribution of hepatocyte to angiogenesis in fibrosis has not been investigated yet and is one of the aims of the studies that are discussed in this thesis. We aimed to investigate if targeting of interferon alpha (IFNα) to the hepatocytes to inhibit the expression of angiogenic factors by the hepatocytes could result in the reduction of fibrosis. The effect of IFNα on angiogenesis aside from its known antiviral activity was discovered by Brouty-Boyè and Zetter in Folkman’s laboratory and published in 1980. IFNα as angiogenic inhibitor has been used in hemangioma and cancer therapy since 1988. However, IFNα receptors are expressed in most of the tissues in the body which makes the strategy of utilizing IFNα in this study inefficient. Therefore, a modified IFNα was designed by coupling galactose moieties to IFNα, which will be recognized by the asialoglycoprotein receptors that are expressed abundantly on hepatocytes.

3. Evaluation of Angiogenesis in the Liver

One of the problems in studying the role of angiogenesis in liver fibrosis is the absence of rapid and specific assays to evaluate angiogenesis and the efficacy of potential therapies, and to investigate possible targets within the angiogenic process and its effect on liver fibrosis. A number of in vitro, ex vivo and in vivo assays have been developed based on the different steps involved in the mechanism of angiogenesis (Fig. 1).

- Endothelial cells cultured in vitro, testing proliferation, migration, and differentiation

Discovery of the method to isolate and culture endothelial cells derived from human umbilical veins was reported in the annual meeting of the Federation of American Societies for Experimental Biology in 1973 by Folkman and Jaffe. Since then, freshly isolated or commercially available human umbilical vein endothelial cells (HUVEC) have been one of the mostly used models in angiogenesis study in vitro. In vivo, in the process of formation of new blood vessels, endothelial cells from existing blood vessels migrate towards an angiogenic stimulus, such as pro-angiogenic factors produced by tumor cells, and behind this migrating front, endothelial cells proliferate to provide the necessary cells that can form the new vessel. Subsequently, the new outgrowth of endothelial cells will reorganize into a three-dimensional
tubular structure. The HUVEC model is used to characterize this proliferation, migration and differentiation process. During 2D culture, the HUVEC proliferate and migrate and form blood vessel-like structures. Based on this behavior, three assays are commonly applied to study the mechanism of and the effect of mediators on angiogenesis, i.e. a proliferation assay, a migration assay and a differentiation assay. In the proliferation assay, the effect of angiogenic or anti-angiogenic compounds is tested by assessing the net cell number with a cell counting device or with other common cell proliferation assays, such as the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, the incorporation of [³H] Thymidine or of BrdU. This method was used to study the anti-angiogenic effect of sorafenib and sunitinib on the proliferation of HUVEC and prostate tumor derived endothelial cells (PTEC). The results showed that both compounds inhibited the proliferation of HUVEC but only sunitinib inhibited the proliferation of PTECs. Although the different effect was unexpected because both compounds share a similar target, i.e. the vascular endothelial growth factor receptor 2 (VEGFR2), it was suggested that it might be due to the different level of activation in normal (HUVEC) and tumor (PTEC) endothelial cells of the intracellular pathways that are the target of sunitinib. This study highlighted the importance of carefully selecting the most relevant cell type to investigate the effect of anti-angiogenic compounds on endothelial cells. Moreover, two different assays for the evaluation of the effect on the endothelial cells proliferation were used. The combination of two or more different methods is strongly recommended to achieve the most reliable conclusions in the proliferation assay. The MTT assay or direct cell counting is often combined with a measure of DNA synthesis such as BrdU or [³H] Thymidine incorporation assay. Although proliferation assays provide simple and rapid methods, each assay has technical problems. For example, cellular autofluorescence is one of the problems in the evaluation of BrdU content of the cells with fluorescence activated cell sorters (FACS). The most frequently used migration assay evaluates chemotaxis as driving force for migration using the Boyden chamber, in which the endothelial cells are cultured on the upper layer of a cell-permeable filter and allowed to migrate towards an angiogenic stimulus added to the medium below the filter. The Boyden chamber was used for the evaluation of oxytocin effect on angiogenesis using HUVEC. The result showed that oxytocin induced HUVEC migration in a concentration-dependent manner, but a decreased number of migrated cells was observed at the highest concentration. It was suggested that the bell-shaped response curve is typical for chemotactic substances, but this might also occur because of the loss of the concentration gradient. The problem with maintaining the gradient concentration between the upper and lower chambers in the Boyden chamber assay is one of the technical difficulties in
this assay, especially during prolonged incubation time, because of the equilibration between the chambers over time due to diffusion of the effector molecule.\textsuperscript{64}

In the tube formation assay, the differentiation of endothelial cells into a tubular structure is measured as a model for the formation of new blood vessels. This assay is mostly used to test novel angiogenic stimuli or inhibitors as a first screen before animal testing.\textsuperscript{67} The endothelial cells are plated onto or into a layer of gelled basement matrix (Matrigel is the commonly used matrix). After the cells are attached to the matrix, they migrate towards each other, align and form tubular structures within 1h and completely form tubes within 12h.\textsuperscript{64,67} The required time for the cells to form tubules in this assay is variable and depends among others on the type of matrix, the total protein concentration of the matrix, the source and type of endothelial cells. Therefore, it is important to optimize these factors before testing a compound using this assay.\textsuperscript{67}

Using this method, the influence of the matrix type on the differentiation rate was shown: rat capillary endothelial cells cultured on collagen I and III showed minimal tubule formation, while culturing on collagen IV and V showed extensive tube formation.\textsuperscript{64,68}

\textbf{Organ culture}

Since an \textit{in vitro} assay of endothelial cells in monoculture does not include the surrounding cells (fibroblasts, macrophages, and pericytes) which also have important role in angiogenesis, there was an urge to develop an \textit{ex vivo} assay that bridges the gap between the \textit{in vitro} and \textit{in vivo} results. The rat aortic ring assay was introduced as the first \textit{ex vivo} angiogenesis assay by Nicosia and Ottinetti, and was later extended to the use of mouse aorta showing similar responses to those of rat aortic rings.\textsuperscript{69-71} In this essay, the thoracic aorta, dissected from rat or mouse, is cut into rings and these rings are placed individually into drops of matrix (collagen, Matrigel or fibrin) in a well plate. The medium is added when the gel has solidified and the embedded ring is further incubated for one to three weeks. The endothelial microvessel outgrowth from the ring is used as the parameter to determine angiogenesis. The number and the length of the outgrowths can be measured and used to evaluate angiogenesis quantitatively.\textsuperscript{69-71} This assay represents the \textit{in vivo} situation better than other \textit{in vitro} assays because the system includes pericytes as supporting cells in neovascularization.\textsuperscript{64} However, the use of aorta is far from ideal because angiogenesis is a microvascular process.\textsuperscript{64}

\textbf{Chick chorioallantoic membrane (CAM)}

This assay is categorized as \textit{in vivo} assay because it uses the intact chick embryo in the egg. The CAM is a highly vascularized extraembryonic membrane which has important roles during embryonic development, among others, gas exchange.\textsuperscript{72} In this assay, the CAM inside the egg is exposed by cutting a window in the shell or by transferring the entire egg without shell to a
plastic culture dish which allows better imaging of the blood vessels. Test substances can be added directly into the CAM by placing it on the filter disc and the angiogenic effect can be measured by counting the blood vessels around the filter disc. One of the advantages in this assay is its ability to support the growth of inoculated xenogenic tumor cells, in which the primary tumor is rapidly formed due to the highly vascularized environment in the CAM. Thus, besides testing the anti-angiogenic activity of the substances, this assay allows us to test the antitumor activity in the same system. Since CAM itself is well vascularized, one of the technical difficulties in this assay is to distinguish new capillaries from the existing ones. Moreover, it is important to keep in mind that this assay is run on chicken cells, which may limit the clinical translation of the assay.

There are several other assays that have been developed for the study of angiogenesis after the discovery of tumor-associated angiogenesis, such as the corneal angiogenesis assay and the Matrigel plug assays. But the aforementioned three assays are the most frequently used ones, because they are well validated, technically easy, relatively low cost, and can be quantitatively interpreted. However, these assays have some drawbacks that should be taken into consideration when interpreting the test results. For example, species difference might be a problem in clinically translating the results from the mouse or rat aortic ring assay and the chick CAM assay to the human situation. Although human endothelial cells such as HUVEC are used successfully in in vitro assays, it only portrays the response of one cell type and ignores the fact that mural cells are also involved in the new formation of blood vessels. This issue was tackled by co-culturing mural cells with endothelial cells. Several methods have been published on how to co-culture both cell types, but up to now this needs further optimization.

For the application of these assays for studying angiogenesis in liver fibrosis, it is important to take into account that they were developed to study angiogenesis in carcinogenesis and some specific limitations hamper their applicability in liver fibrosis-associated angiogenesis studies. The main limitation is that the distinctive features of hepatic angiogenesis, which are substantially different from homologues processes in other organs or tissue, are not well reflected in these models. One of the unique features of the liver is the presence of two different microvascular structures, i.e. the sinusoids lined by fenestrated endothelium and the large vessels lined by a non-fenestrated endothelium. Therefore, hepatic angiogenesis cannot be simply evaluated with an assay using human umbilical vein endothelial cells (HUVEC) for instance, because HUVEC represent the macrovasculature only, whereas there are strong indications that also the sinusoidal endothelial cells are involved in hepatic angiogenesis. The use of liver sinusoidal endothelial cells (LSEC)
has been reported in some in vitro studies, but the same issue on lack of cell-cell interaction in in vitro assay using HUVEC also applies here as in fibrosis-associated hepatic angiogenesis, the HSC and other liver MF are key players. These cells produce several growth factors, such as VEGF and PDGF, that also stimulate angiogenesis, and they also express the receptors of these growth factors, which mean that the assay using only LSECs might ignore the possibility of the competition of the cells for the angiogenic stimulus or inhibitor.

Precision-cut liver slices (PCLS) from rat, mouse and human liver have been used as model to study fibrosis since the introduction of the model by van de Bovenkamp et al. in 2006. The PCLS serves as a mini-model of the liver because in this model, all liver cells are present in their natural environment with intact cell-cell and cell-matrix interactions during the incubation. Liver slices (~200 µm of thickness) can be prepared from different species, including rat, mouse and even human. Both normal and cirrhotic livers can be used in this model. Human liver tissue was obtained from patients following partial hepatectomy in metastatic colorectal cancer or from organ donors, remaining as surgical surplus after reduced-size liver transplantation. An incubation of PCLS for two days has been shown to increase fibrosis markers at mRNA and protein levels, including collagen 1, alpha smooth muscle actin and HSP47. A recently published study on rat PCLS incubated for five days showed a high increase of collagen disposition with maintained viability, morphology and function. Although it has been well validated as fibrosis model, this model has never been formally used to investigate fibrosis-associated angiogenesis. If signs of angiogenesis would be found in cultured slices, it would pave the way to develop a novel liver-specific approach in unraveling the role of fibrosis-associated angiogenesis.

4. Aim and scope of the thesis

The studies described in this thesis are focused on the role of angiogenesis in liver fibrosis. Different approaches were used to elucidate the controversial role of fibrosis-associated angiogenesis. The first strategy as described in chapter 2 and 3 was to use angiogenesis inhibitor to study the role of hepatocytes in fibrosis-associated angiogenesis. In this study, IFNα was used as angiogenesis inhibitor to interfere with the balance of angiogenesis factors produced by hepatocytes, i.e. VEGF-A and thrombospondin-1 during liver fibrogenesis. Therefore, we aimed to target IFNα to the hepatocytes. This was done by coupling IFNα to galactose, resulting in a galactosylated protein, which is specifically recognized by the asialoglycoprotein receptors expressed abundantly on hepatocytes. Moreover, this allowed us to unravel the role of hepatocytes in liver fibrogenesis. In chapter 2, the synthesis of the galactosylated IFNα and its characterization is described, and the biological activity of the construct was tested in vitro using
HepG2 and HUVEC cells. In chapter 3, this construct was tested in an in vivo fibrosis model of carbon tetrachloride (CCL₄)-exposed mice.

In chapter 4, the effect of the pro-angiogenic factor VEGF-A in liver fibrosis was investigated in order to elucidate the controversial in vivo discoveries on the exact role of VEGF-A in liver fibrogenesis. Besides studying the expression in vivo of VEGF-A and related receptors in livers of CCL₄-exposed mice with low and high degree of fibrosis, the direct effect of VEGF-A on liver fibrosis was tested in mouse and human PCLS.

In chapter 5, three different assays were developed using PCLS in order to generate a liver-specific model to study angiogenesis. These assays differ in the readout, the degree of fibrosis, and the employed method. In the first assay, rat PCLS were incubated with and without VEGF-A and sunitinib for two days, and the mRNA expression levels of fibrosis and angiogenesis markers were measured. In the second assay, rat PCLS were incubated for five days in two different media, i.e. Williams’ E Medium (WME) and RegeneMed®. Blood vessel formation was subsequently studied using immunofluorescence. The third assay was adapted from the aortic ring assay, by embedding PCLS in collagen matrix for five days and the microvessel outgrowth was evaluated.

Lastly, in chapter 6 the results are summarized and discussed and perspectives on future directions towards a better comprehension on the role of angiogenesis in liver fibrosis are presented.

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