The role of decorin in liver fibrogenesis and hepatocarcinogenesis

Ph.D. Thesis

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INTRODUCTION

Chronic liver diseases cause severe healthcare problems worldwide. Hepatitis infections, alcoholism, metabolic disorders and other environmental factors are to be mentioned as major factors inducing the impairment of liver. These etiological factors enhance connective tissue deposition resulting in the development of liver fibrosis. The long-lasting process leading to cirrhosis is accompanied with structural rearrangement of the liver with the appearance of pseudolobules. Cirrhosis is thought to be irreversible despite a few reports of its regression occasionally. The only therapy available so far is the liver transplantation. Liver cirrhosis greatly increases the risk of liver cancer, especially hepatocellular carcinoma (HCC). However this malignant tumor is not always associated with cirrhosis, may develop in non-cirrhotic surrounding.

During the fibrogenesis, the deleterious factors activate hepatic stellate cells (HSC) enhancing their extracellular matrix (ECM) production, leading to connective tissue accumulation in the Disse space. In parallel, fenestrae of endothel cells and microvili of hepatocytes are lost, basal membrane closed capillaries take the place of sinusoids. These

events remarkably reduce the communication between the blood and hepatocytes.

The actual amount of ECM is the result of synthetic and catabolic processes. These events are precisely regulated for maintaining the optimal structure essential for differentiated hepatocyte functions. The main stimulating factor of ECM production is the transforming growth factor-β1 (TGFβ1), whereas in matrix degradation matrix metalloproteinases (MMPs) play central roles.

In the majority of liver cancers, chronic inflammation induced fibrosis or cirrhosis precedes the development of tumor, although this is not essential for tumor formation. At the same time, the process resulting in the overproduction of extracellular matrix favors cancer development by at least by two ways; by

- inducing accelerated hepatocyte regeneration leads to insufficient DNA damage repair,
- the presentation of signals coming from the environment is impaired in the pathological matrix.

The extracellular matrix of the connective tissue is a complex mixture of macromolecules interacting with each other and with cells of the connective tissue. As a result, matrix macromolecules provide structural integrity, influence cell growth regulation, migration and differentiation. The

matrix consists of collagens, non-collagen glycoproteins, elastin and proteoglycans (PGs). Proteoglycans are able to influence the effects of growth factors and cytokines, moreover, a growing number of evidences show that PGs are directly involved in signal transduction through different matrix receptors.

Decorin is a small leucine-rich proteoglycan containing one chondroitin sulfate or dermatan sulfate side chain. It is present in a small quantity in normal healthy liver. During fibrogenesis the amount of decorin significantly increases together with other ECM proteins. Previous studies showed that the core protein of decorin is able to bind the TGFβ1, directly blocking the bioactivity of the growth factor, thus, it has a protective effect against fibrosis. Decorin is an effective growth inhibitor. It was proved to bind different receptor tyrosine kinases (EGFR, IGFR, Met), resulting in inhibition of cell proliferation. This blockage of receptors on the surface renders tumor cells into growth arrest and hinders the tumor growth by keeping tumor cells in quiescence. The functional $p21^{Waf1/Cip1}$ protein that causes G1 phase arrest is indispensable for the tumor suppressor action of decorin. The elevated concentration of decorin around tumor cells may be a form of paracrine defensive mechanism by stromal cells counteracting

the growth of malignant cells on the invasive front of solid tumors.

OBJECTIVES

The main goal of the recent work was to answer if decorin has a role in liver fibrogenesis and in the development of liver cancer. For this purpose the following investigations were carried out:

1. For *in vivo* studies, generation of homozygous decorin knockout mouse strain from heterozygous animals, their morphological examinations with a special focus on the liver.
2. Induction of liver fibrogenesis, monitoring the progression and regression of the disease in decorin knockout and wild type mice. Examinations of connective tissue production and degradation at mRNA and protein levels.
3. Examination of fibrosis-activated signaling pathways, in the absence of decorin with a special interest on the effect of TGFβ1.
4. Induction of hepatocarcinogenesis in cirrhotic and non-cirrhotic livers of decorin knockout and wild type mice. Morphological analysis of the evolved liver tumors.

5. Identification of active signaling pathways of hepatocarcinogenesis at mRNA and protein levels in wild type and decorin knockout mice.

MATERIALS AND METHODS

For generation of decorin-null (Dcn-/-) mouse strain, the genotype of individual mice was determined by conventional PCR technique.

For induction of hepatic fibrosis, animals were subjected to thioacetamide (TA) treatment for 4 months (TA4) followed by an additional 4 months (recovery phase, TA4+4) in which the drug was withdrawn. Three animals were sacrificed at each time point at monthly intervals.

The amounts of different collagen proteins were determined by morphometrical analysis of picrosirius-stained sections, immunostaining, dot blot and hydroxiproline determination, their mRNA levels were examined by qRT-PCR. Decorin was analyzed by immunostaining and dot blot. Activity of two matrix metalloproteinases (MMP-9 and MMP-2) was determined by gelatinase assay, the gene expression of MMP inhibitors TIMP-1 and PAI-1 were analyzed by qRT-PCR. To examine the effect of TGFβ1, mRNA levels of the growth factor and the TGFβ1-induced early response gene

(TIEG) were studied by qRT-PCR, in addition, we examined the phospho-Smad2, phospho-Smad3, Erk1/2 and phospho-Erk1/2 protein levels all known members of the TGFβ1 signaling pathways (Western blot). For i*n vitro* studies, decorin gene was silenced by specific siRNA in LX-2 human hepatic stellate cell line, then cells were treated with TGFβ1. Alpha-smooth muscle actin (αSMA) and collagen-1 mRNA and protein determinations served as markers of stellate cell activation (immunostaining, dot blot, Western blot, qRT-PCR). The amount of decorin and the efficiency of silencing were studied by dot blot and qRT-PCR. P-Smad2 and P-Smad3 protein levels were analyzed by Western blot.

For hepatocarcinogenesis studies, Dcn-/- and wild type mice were treated with TA for 7 months (tumors develop in cirrhotic surrounding), or injected with a single dose of diethyl nitrosamine (DEN) and sacrificed after 9 months of the shot (tumors develop in non-cirrhotic surrounding). We determined the turmor prevalence in each group and the average tumor number per liver. The tumor morphology was studied on hematoxilin-eosin-stained sections. For analysis of active signaling pathways oligoarray hybridization was applied (Cancer Pathway Finder OligoArray, SABiosciences). Evaluation of array results was carried out by R programming language and heat map illustration after densitometry of the

membranes. For validation of array results signaling molecules of interest were studied at mRNA and protein levels by qRT-PCR, Western blot and immunostaining.

RESULTS

I. Generation of decorin-null mouse strain and its morphological characterization

We succeeded to generate homozygous Dcn-/- mouse strain from heterozygous animals by careful selection for breeding based on the genotype of the offspring determined by DNA isolation and PCR analysis.

Dcn-/- mice are characterized by large hairless areas appearing mostly on their back. In these sites the epidermis becomes thinner; hairs are missing or fracture in the follicle. The diameter of collagen fibers is extremely diverse; from giant laterally fused fibers to tiny ones can be observed in contrast to the uniform fibers seen in wild type mice. The Dcn-/- livers showed no difference compared to the wild type animals.

II. Results of fibrogenesis and regeneration studies

For our i*n vivo* experiments we induced liver fibrosis in decorin-null and wild type mice by administering

thioacetamide (TA). Dcn-/- mice accumulated more connective tissue in their livers as compared to the wild-type mice in response to chronic liver injury. Four months after the cessation of TA treatment (TA4+4), the majority of connective tissue fibrils positive for PS were resolved in the liver of wild type animals. In contrast, Dcn-/- livers still contained considerable amounts of fibrous tissue.

Next, using antibodies against collagen I, III and IV a significant increase of these proteins was observed in the livers of Dcn-/- mice compared to wild type ones both in fibrosis (TA4) and at the end of the recovery phase (TA4+4). By immunostaining, as expected, decorin was detectable only in wild-type animals where it was deposited in periportal connective tissue and around the central veins. Notably, during fibrogenesis decorin levels increased in the wild-type animals and accumulated along fibrotic septa. After 4 months of regression the amount decreased to a level practically identical to that measured in control animals.

Next, we determined the mRNA levels of collagens type I, III, and IV using qRT-PCR analysis. Notably, despite their elevation we found no statistically significant changes in mRNA levels of any collagens tested after 4 months of liver injury. However, at the end of the regression period (TA4+4) the steady-state levels of all the three types of collagens were

significantly elevated in Dcn-/- animals vs. wild-type mice, suggesting that rather impaired degradation than overproduction may be responsible for the elevated protein levels.

Indeed, in the wild-type fibrotic livers, activity of both MMP-2 and MMP-9 significantly increased and to a greater extent than that of decorin-null animals. These changes regressed by the end of the recovery period showing only subtle differences between the two genotypes. Fibrogenesis in Dcn-/- livers was characterized not only by low activity of MMP-2 and MMP-9 but also by enhanced expressions of TIMP-1 and PAI-1, two well-known MMP inhibitors, especially at the end of the recovery period.

Collectively, these results suggest that decorin is directly involved in regulating the fine balance of synthesis and degradation during hepatic fibrosis and healing.

To establish whether TGFβ1 would exert a stronger effect in animals lacking functional decorin, we determined the expression of TGFβ1 and TIEG as a marker for the efficacy and activity of the growth factor. Quantitative RT-PCR revealed approximately 6-fold increase in TIEG mRNA level in Dcn-/- liver samples compared with wild-type during both fibrogenesis and regression, whereas TGFβ1 mRNA levels increased by 3-fold even in the Dcn-/- control group. After 4

months of TA treatment, there was a 20-fold increase of TGFβ1 in Dcn-/- samples in contrast to only a threefold increase in wild-type. After the regression period, TGFβ1 expression decreased to barely detectable levels, even lower than the wild-type controls.

To investigate what molecular pathways could be operational in our experimental animal model, we determined the amounts of Erk1/2 and their phosphorylated forms (P-Erk1/2), as well as phospho-Smad2 (P-Smad2) and phospho-Smad3 (P-Smad3), all known downstream effectors of the TGFβ1 signaling pathways. In addition, we determined the levels of α-smooth muscle actin (αSMA), a known marker of fibrogenic cells such as the hepatic stellate cells. In fibrotic livers (TA4), regardless of their genetic background, the amounts of Erk1/2 and P-Smad2 significantly increased as determined by Western blot. However, at the end of regression period, the amounts of these proteins did not statistically differ from controls. Concurrently, the levels αSMA were significantly elevated after 4 months of treatment and after the recovery period in the decorin-null animals compared with wild type. However, although Erk1/2 were not phosphorylated in the wild type fibrotic liver, the level of activation increased to 183% in decorin-null samples. At the end of the recovery phase, lower amounts of active Erk1/2 were detected vs.

untreated samples, and Dcn-/- lysates contained significantly higher amounts of Erk1/2 as compared with wild type. The level of P-Smad3 protein increased during fibrogenesis showing a 2.3-fold increase in wild-type and 8.37-fold increase in decorin-null livers after 4 month of TA treatment. At the end of the recovery period (TA4+4) similar amounts of P-Smad3 were detected as in control samples. These results suggest that the lack of decorin leads to enhanced accumulation of αSMA-positive cells, and to increased phosphorylation of Erk1/2 and Smad3 proteins without significantly affecting the levels of P-Smad2.

To determine whether hepatic stellate cells, known as the main fibrogenic cell type in the liver, could be involved in the action of decorin, we investigated the effects of TGFβ1 in the presence or absence of endogenous decorin. To this end, we used human hepatic stellate LX2 cell line. Decorin was silenced by specific siRNA (siDcn), whereas control cells were transfected with scrambled (scr) siRNA. The siRNA-mediated silencing reduced the decorin mRNA level to ~38%. After TGFβ1 exposure for 48 h, siDcn cells exhibited significantly higher collagen I and αSMA mRNA levels in contrast to cells transfected with scrambled siRNA. At the protein level, decorin silencing caused a greater increase in collagen type 1 in the media of cells exposed to TGFβ1, and

only a modest elevation without exposure to the growth factor compared to scr cells. In agreement with the *in vivo* data, TGFβ1 caused enhanced decorin production in LX2 stellate cells. In full support of the results presented above, immunofluorescence staining for αSMA of hepatic tissues from various animals showed increased immunopositive cells in the decorin-null livers, especially at 4 months. Similarly, enhanced αSMA actin immunostaining was observed when endogenous expression of decorin was decreased in LX2 hepatic stellate cells, and these effects were more enhanced by TGFβ1 treatment. Moreover siDcn cells exhibited a significant increase in the amount of αSMA compared to scr cells determined by Western blot. These findings support and corroborate the *in vivo* data that showed elevated amounts of αSMA in Dcn-/- liver samples both at 4 months of TA treatment and after the recovery period. Collectively, our findings indicated that endogenous hepatic decorin has a key role in the regulation of stellate cell activation insofar as genetic ablation of decorin or RNA interference strategies culminate in enhanced activation of stellate cells and a pronounced profibrotic phenotype.

We have previously shown that the *in vivo* phosphorylated form of Smad2 and Smad3 proteins was elevated due to TA treatment but only P-Smad3 was significantly higher in Dcn-/-

samples. The results of our cell culture model system correlated well with those obtained with the animal experiments because after 48 h of TGFβ1 exposure the extent of increase in P-Smad2 was comparable in cells transfected with either scrambled or decorin-specific siRNAs. In contrast, after TGFβ1 treatment, P-Smad3 levels were significantly higher in siDcn cells than those transfected with scrambled siRNA. Thus, decorin deficiency potentiates TGFβ1/Smad3 axis suggesting that decorin *in vivo* might regulate the fine balance of connective tissue remodeling.

III. Results of hepatocarcinogenesis studies

Liver cancer was induced by either thioacetamide (TA) or diethyl nitrosamine (DEN) to model tumors developing in cirrhotic and non-cirrhotic livers respectively in both wild type and decorin-null mice. The tumors evoked by the two drugs exhibited highly different phenotypes. After 7 months of TA-treatment, tumor cells were rich in cytoplasm which showed strong eosinophil staining. These tumors were surrounded by connective tissue capsule, whereas DEN-induced tumor cells had narrow cytoplasm with basophil staining, and they often invaded the veins. Connective tissue deposition around the tumor was rarely seen. After TA exposure Dcn-/- animals developed higher number of tumors with cirrhotic surrounding

with a higher prevalence compared to wild type ones. However, no significant difference could be revealed between the two genotypes after DEN exposure despite the similar tendency seen case of TA.

To uncover what molecular pathways are responsible for the phenotypic differences of tumors with different origin, and the possible mechanisms operate behind the enhanced susceptibility for tumor formation of decorin null mice, we carried out gene expression analyses. To this end, a membrane hybridization technique was applied containing specific probes for genes of cell cycle regulation, DNA repair, apoptosis, aging, adhesion, angiogenesis, invasion, metastasis, several transcription factors and signaling molecules. The results obtained from array experiments suggested that several members of the cell cycle regulation and MAPK signaling pathway changed in our experimental set up. Furthermore, they revealed differences between the two genotypes. These pathways were chosen for further analysis. From the members of MAPK signaling pathway expression of the EGF, IGF growth factors, the EGFR, c-met receptors, the Grb2, Raf1, MEK1 changed, displaying higher expression in Dcn-/- samples. In addition, alterations in expressions of genes responsible for controlling the restriction point at G1 phase of the cell cycle G1 phase were observed such as $p21^{Waf1/Cip1}$,

$p27^{Kip1}$, $p16^{INK4A}$ tumor suppressors, retinoblastoma (Rb), Cdk4/6 that phosphorylates Rb at the Ser780 and forms a complex with cyclin D1.

Changes in $p21^{Waf1/Cip1}$ mRNA levels were validated by qRT-PCR. After 7 month of TA exposure 108-fold expression was seen in wild type samples vs. control ones, in contrast to the 62-fold observed in Dcn-/- livers. The DEN treatment had less effect on the $p21^{Waf1/Cip1}$ mRNA level, but still wild type livers exerted a 21-fold, while Dcn-/- ones only a 2-fold increase.

At protein level determined by Western blot, in both TA and DEN-induced tumors the lack of decorin caused elevated phosphorylation of Rb at Ser780, and that went parallel with the decreased $p21^{Waf1/Cip1}$ levels. These observations indicate the enhanced activity of CDK4 allowing the cell to pass the restriction point.

As previously seen, the array analysis revealed gene expression changes in several members of the MAPK pathway, showing elevated mRNA levels in Dcn-/- samples compared to wild type. The final effectors of this pathway are the Erk1/2 (p44/42) proteins. No difference was seen in the amount of inactive unphosphorylated Erk1/2 either between tumors with different origin (TA, DEN) or between genotypes (wild type, Dcn-/-). Non-parenchymal cells of connective

tissue septa in cirrhotic areas showed strong positivity. Most of the tumor cells in both types of cancer showed intensive immunopositivity as well. The active phosphorylated form was present in a higher amount in Dcn-/- samples regardless of the tumor origin. These findings denote that the lack of decorin leads to enhanced proliferation and survival of tumor cells.

NEW OBSERVATIONS

1. The lack of decorin favors the development of fibrosis, inhibits the healing process of the liver by interfering the degradation of the connective tissue.
2. When no decorin is present in the liver, the connective tissue production induced by TGFβ1 is enhanced as a consequence of stimulated P-Erk1/2 and P-Smad3 signaling pathways leading to stellate cell activation.
3. The phenotype of TA and DEN-induced liver cancers is remarkably different.
4. The lack of decorin promotes primary liver cancer development in cirrhotic livers, characterized by enhanced Erk1/2 activation.

5. The increased MAPK action provoked by the lack of decorin triggers the cell cycle progression and the formation of liver cancer.

LIST OF PUBLICATIONS

Publications in the topic of the thesis:

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