Role of decorin in hepatocarcinogenesis

PhD Theses

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1. Introduction

The epidemiology of liver cancer is complex, and it is difficult to distinguish between primary liver tumors and the large number of secondary tumors. Hepatocellular carcinoma (HCC) represents the most common primary liver cancer, but the much rarer angiosarcoma and hepatoblastoma are also of primary origin.

Because of its poor prognosis, HCC is ranked as the second deadliest cancer worldwide with a mortality/incidence rate of 0,95. In 2012, 745 000 deaths were caused by hepatocellular carcinoma (9.1% of all cancer deaths).

Although HCC is a rapidly progressing, fatal disease of which therapies are still unsatisfactory, many information is available to promote its prevention. Sustained HBV or HCV hepatitis infection is responsible for three quarters of the liver cancer worldwide, so preventing these virus infections is a possible way of reducing the number of cases. The infection causes chronic hepatitis, which induces increased regeneration and promotes fibrotic and then cirrhotic processes in the liver tissue. This results in formation of dysplastic cell groups (focuses, nodules) from which later carcinoma can develop, but HCC can emerge directly from the cirrhotic background, without focus formation. In cases without viral infection, chronic damage of liver tissue by alcohol abuse, or direct genotoxic agents such as aflatoxin B1 can provoke development of HCC. Furthermore, literature reports the involvement of certain lifestyles and/or inherited factors such as smoking, obesity, diabetes, or contraceptives, which may also contribute to the formation of liver cancer.

HCC is highly polymorphic at molecular level. This complexity is partly explained by its etiology, and the diverse pathogenic processes behind its development. During the progression of HCC, malfunctions of several signaling pathways and mediator molecules were observed (such as WNT/β-catenin, PI3K/AKT/mTOR, RAS/MAPK, HGF/MET,

EGFR, IGF, VEGF and PDGF), however the role of connective tissue in HCC progression is still poorly understood.

All tissues and organs, including the liver tissue contain a well-organized network of non-cellular components, called extracellular matrix (ECM). The ECM does not only provide a structural integrity in which parenchymal cells can be embedded, but it also controls a variety of cellular processes such as growth, migration, differentiation, survival, homeostasis and morphogenesis. The ECM is made up of a variety of macromolecules, and its composition is different and specific for each tissue. The main components of the ECM are fibrillar proteins such as collagens, elastin, fibronectin, laminins, as well as glycoproteins, proteoglycans and glucosaminoglycans. All of them are strongly acidic and hydrated molecules, which linked to each other can form large fibrillar structures.

The ECM is not only important in physiological processes, but it also has a crucial role in malignant transformation and in regulation of tumor cell behavior. During malignant transformation, the ECM surrounding tumor cells undergoes quantitative and qualitative changes which impair the regulation of dynamic organization of the ECM and ultimately lead to pathological activity supporting tumor development.

Primary hepatocellular carcinoma often emerges from cirrhosis or chronic inflammatory fibrosis, although this is not an indispensable condition for tumor development. In case of chronic inflammation, the accelerated hepatocyte regeneration leads to insufficient DNA repair and increased mutation rate in liver cells. Furthermore, abnormal transformation of the extracellular matrix also modifies the amount, localization and role of the signaling factors present in the tissue environment, thus destroys the normal, original function.

Decorin is a small, leucine-rich proteoglycan (SLRP) of the ECM consisting of a small protein core and an O-

glycosylated GAG side chain. Both the core protein and the GAG chain can bind to many molecules that play important role in various signaling pathways. Accordingly, decorin can regulate the physiological and tumorous microenvironment and typically have an anti-tumorigenic effect. In 2012, decorin was referred as the "guardian from the matrix" in analogy with the p53 protein, which is the "guardian of the genome".

The lack of decorin from tumor stroma correlates with lower survival rate in invasive breast cancer patients. In addition, the amount of decorin is significantly reduced in the stroma of various tumors, including bladder cancer, where the decorin is present in large amounts in normal conditions. Decorin and p53 double mutant mice (DCN-/-; TP53-/-) dies much earlier in aggressive T-cell lymphoma than those carrying only p53 mutation. However, systemic delivery of soluble decorin to the body (for example by adenovirus vector) significantly reduces neoangiogenesis in several solid tumors.

Further experimental observations have demonstrated that decorin is able to bind to cell surface tyrosine kinase receptors such as EGFR (epidermal growth factor receptor), Met receptor, or IGFR, and thus it is antagonist of several growth factors involved in the regulation of cell cycle and proliferation. Ectopic decorin expression inhibited the proliferation of several neoplastic cell lines with different tissue origin. In such stably transfected cells, inhibition of the cell cycle was typically accomplished via the $p21^{WAF1/CIP1}$ pathway. As a confirmation, decorin failed to block cell proliferation in a cell line (HCT116 human colon carcinoma) without functional p21.

In addition, decorin can bind and block TGFβ, thereby inhibiting the proliferation of certain cells sensitive to the growth factor.

These observations have demonstrated the tumor suppressor role of decorin and that could be considered as a potential therapeutic factor inhibiting tumor progression and metastasis alone or in combination with chemotherapeutic agents respectively.

Our group has previously studied the role of decorin in the development and regeneration of liver fibrosis and its relationship with TGFβ in fibrosis and liver cirrhosis. However, the effect of decorin on the development of hepatocellular carcinoma has not been unraveled yet. Therefore, on the one hand we performed *in intro* studies on HepG2, Hep3B, HuH7 and HLE hepatoma cell lines in which cells were exposed to human recombinant decorin and on the other hand we induced primary liver tumors in wild-type and decorin knock-out ($Dcn^{-/-}$) mice *in vivo* to investigate the role of decorin in hepatocarcinogenesis.

2. Aims

1. Although it has been previously demonstrated that decorin may have anti-proliferative effects on some hepatoma cells, our goal was to investigate whether administration of human recombinant decorin can inhibit the proliferation of multiple hepatocellular carcinoma cell lines, and to decide whether the growth inhibitory effect of decorin is universal among hepatoma cell lines.
2. To complement and support our *in vitro* observations with *in vivo* results, we planned to investigate the role of decorin in experimental hepatocarcinogenesis by comparing wild type and decorin knock-out ($Dcn^{-/-}$) mice.
3. Our further aim was to explore and map signaling pathways responsible for the observed effects, to detect the quantitative and qualitative changes in the participating molecules, and to identify additional possible target molecules.
4. Furthermore, based on the evaluation of our studies our goal was to draw a comprehensive picture of the role of decorin in hepatocarcinogenesis.

3. Methods

In vitro assays: Hepatoma cell lines (HepG2, Hep3B, HuH7 and HLE) were used. Cells were treated with human recombinant decorin produced by our group. Cell proliferations were tested by MTT assay and experimental samples were generated for further assays.

In vivo studies: C57B1/6 and decorin knock-out mice were treated with thioacetamide (TA) and diethylnitrosamine (DEN) hepatotoxic agents, to induce liver cancer.

Samples from tissue cultures and animal experiments were used in molecular assays.

mRNA expression assays: RNA was isolated from frozen liver tissue and cell cultures by TRI reagent, and after reverse transcription reaction the following target genes were tested on mice: CDKN1A, AP4, GS, AFP and on human cell lines: WEE1, CDC25A. The results were expressed in CT values, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Protein activity, localization and expression assays: Measuring the activity of tyrosine kinase receptors relative receptor phosphorylation was investigated by RTK Proteome Profiler Array. To determine the localization of each target molecule ***ICC and IHC*** methods were applied with appropriate antibodies. ***Western blot*** technique was used for quantitative comparative assays for proteins. The tested target molecules were: *decorin, c-Myc, phospho-c-Myc (Thr58), p44/42 MAP kinase, phospho-p44/42 MAP kinase (Thr202/204), β-Catenin, phospho-β-Catenin (Ser33/34/Thr41),* $p21^{WAF1/CIP1}$*,* $p27^{KIP1}$*, Pan-Akt, phospho-Akt (Thr308), GSK3β, phospho-GSK3α/β (Ser21/9), phospho-Rb (Ser780), phospho-Rb (Thr821), phospho-CDK1 (Tyr9), PDGFRα, PDGF AB, p-Y.*

To detect TGF-β1 secretion, the level of growth factor was measured from cell culture media using the Solid Phase Sandwich ***ELISA*** kit of R&D systems.

The ***statistical analyses*** were performed by at least three independent measurements, in which mean and standard deviations were calculated. Each calculation was done by using GraphPad Prism 4.03 software. The normal distribution of data was tested by the D'Agostino and Pearson test. The significance of the differences was determined by the non-parametric Mann-Whitney test or Student's t-

test, depending on the distribution of the data. The differences of tumor incidence in wild type and Dcn knock-out groups and their significance was evaluated by χ square test. Repeated experiments were carried out independently, comparison of the results proved the reproducibility of the experiments. Only reproducible, significant differences were considered to be significant. Significance was declared at $p < 0.05$ level as used in general practice.

4. Results

Results of in vitro experiments

HepG2: Decorin reduced cell proliferation over 72-hour experiment. Out of the cell surface RTKs examined, only EGFR was active. Phospho-EGFR levels were significantly reduced after 48 h of decorin treatment in a concentration-dependent manner, while in parallel, the dephosphorylation of ERK1 and ERK2 was observed. The amount of $p21^{WAF1/CIP1}$ protein increased with 4.8-fold and 3.7-fold in DCN50 and DCN100 treated groups compared to the control, whereas $p27^{KIP1}$ increased 1.74 and 1.5-fold. Furthermore, decorin resulted in decreased GSK3α/β phosphorylation and elevated levels of phospho-β-catenin in HepG2 cells. In addition, β-catenin disappeared from the nucleus of HepG2 cells and translocated to the cell membrane in immunocytochemical assays. Decorin significantly reduced the secretion of TGF-β1 as well.

Hep3B: Proliferation of Hep3B significantly decreased upon decorin treatment. Changes in EGFR, InsR and IGF-IR phosphorylation were detected by the tyrosine kinase array. Similarly to HepG2, a significant decrease in EGFR phospho-protein level was observed, however we detected increased activation of InsR and IGF-IR receptors. Parallel to the activation of IGF and insulin receptors, significant elevation was observed in the phosphorylation of Akt on the Thr308 residue. Additionally, while ERK1 phosphorylation showed a 37% increase in both treated groups, no significant change in pERK2 levels was detected. Although no $p21^{WAF1/CIP1}$ was observed in controls o decorin-exposed Hep3B cells, the amount of $p27^{KIP1}$ protein showed a significant increase after 48 hours of decorin treatment. There was a slight increase in phospho-GSK3β level but no significant change in the amount of phospho-c-Myc and phospho-β-catenin was detected in Hep3B cells after treatment. In further studies, we showed that decorin is able to induce CDK1 phosphorylation in Hep3B together with the increase of WEE1 mRNA expression. In addition, decorin reduces cellular secretion of TGF-β1.

HuH7: In case of HuH7 cells, the inhibitory effect of decorin on proliferation was observed only after 48 hours. Similarly to Hep3B, cells have activated EGFR, InsR and IGF-IR receptors under normal conditions. Phosphorylation of EGFR was significantly reduced in a dose-dependent manner upon decorin treatment. In addition, changes in phosphorylation of InsR and IGF-IR receptors were detected in DCN100 group; the phosphorylation rate decreased by 34% and 28%, respectively. Consequently, the amount of phosphorylated Akt decreased as well. Interestingly, significant ERK activation was provoked by decorin; a 2.80-fold and 3.93-fold increase of phospho-ERK1 and a 12.74-fold and 19.83-fold increase of phospho-ERK2 was observed in the DCN50 and DCN100 groups compared to the control. No $p21^{WAF1/CIP1}$ CDK inhibitor was detected in either control or decorin treated HuH7 cells and only minor, non-significant change was observed in $p27^{KIP1}$ levels. Although GSK3β phosphorylation was elevated by 32% in the DCN50 group, it was spectacularly reduced by 62% in the DCN100 group, similarly to phospho-GSK3α. In addition, significant c-Myc phosphorylation was detected after a 48-hour decorin treatment but phospho-β-catenin could not be detected in HuH7 cells. No significant change in secreted TGF-β1 concentration was observed upon treatment.

HLE: In the fibroblast-like HLE cells, only a minute, non-significant decrease in proliferation was detected upon decorin exposure, and no active phosphorylated tyrosine kinase receptor was observed. Nevertheless, a significant reduction in phospho-Akt level was measured by 66% and 64% in DCN50 and DCN100 groups compared to control cells. No significant change was seen in ERK1/2 phosphorylation. While $p27^{KIP1}$ did not change in the presence of decorin, the amount of $p21^{WAF1/CIP1}$ elevated significantly. Only a slight decrease was observed in phosphorylation of GSK3 proteins, but treatment with the higher decorin concentration resulted in a 37% elevation of phospho-c-Myc level. In addition, significant β-catenin phosphorylation was detected; a 2.76-fold increase in the DCN50 group and a 3.07-fold increase in DCN100, verified by immunocytochemistry. The greatest reduction of TGF-β1 concentration provoked by decorin was seen in case of HLE cells compared to the other hepatoma cell lines.

Results of in vivo experiments

Phenotypical changes

As expected, different hepatocarcinogenesis models resulted in tumors with different phenotypes. The metabolism of thioacetamide (TA) occurs through cytochrome p450 in hepatocytes, which first leads to fibrosis and then cirrhosis. Accordingly, treatment with TA causes hepatocyte hyper-regeneration, which acts as an initiator of hepatocarcinogenesis in the cirrhotic liver. TA-induced tumor cells typically had large cytoplasm, with strong eosinophilic staining, and tumors were usually surrounded by connective tissue capsules. In contrast, high dose of diethylnitrosamine (DEN) causes direct DNA damage without fibrotic changes. These tumor cells had a small basophil cytoplasm and often penetrated the blood vessels.

93% of decorin deficient mice developed a macroscopic liver tumor by TA administration, whereas it was only 22% in wild-type animals. Similarly to TA, DEN exposure caused tumor formation in 44% of $Dcn^{-/-}$ animals, while only 27% of wild-type animals had liver cancer, however the difference did not reach statistical significance. Simultaneously, significantly higher tumor volume was measured in decorin knock-out animals than that of wild-type mice.

Molecular changes

Four tyrosine kinase receptors, namely PDGFRα, EGFR, MSPR (also called RON) and IGF-IR have been identified to have significantly higher phosporylated level in the absence of decorin compared to wild-type during experimental hepatocarcinogenesis induced by both TA and DEN.

Since the the most significant changes were seen in the amount of pPDGFRα, this receptor has been subjected to our further studies. We have shown that PDGFRα is mainly localized in non-parenchyma-type cells in healthy liver such as fibroblasts or myofibroblasts. However, it

can appear on the surface of tumor cells as well. Furthermore, by double immunostaining we found that decorin and PDGFRα did not colocalize, therefore we examined whether decorin could directly bind the PDGF ligand. To this end, immobilized human recombinant decorin was incubated with PDGF AB and immunoreaction with PDGF AB specific antibody was performed. As a result, the PDGF specific signal appeared on the membrane points containing the decorin leading to the conclusion that decorin is able to directly bind to the PDGF.

Since decorin generally exerts its tumor suppressor activity via $p21^{WAF1/CIP1}$, its changes were analyzed in our experimental system. In control, untreated animals, low levels of p21 were detected by immunohistochemistry in the livers of both wild-type and decorin-deficient mice. TA exposure caused a significant increase in the amount of p21 in wild-type animals; hepatocytes, connective tissue cells, as well as tumor cells exhibited intense immunostaining. In contrast, the accumulation of p21 was not observed in decorin knock-out mice, and was almost completely missing from the nucleus of tumor cells. DEN also provoked an increase in p21 level, but the lack of decorin had less influence on the process. To gain further insights into the regulation of G1/S transition in our experimental model, the status of different phosphorylation sites of the retinoblastoma (Rb) protein was investigated using phospho-specific antibodies (Ser780 and Thr821). Intensive Rb phosphorylation was observed at Ser780 regulated by CDK4 in TA-induced liver tumors which was more pronounced in $Dcn^{-/-}$ samples than in wild-type (2.65-fold and 1.77-fold increase). DEN exposure resulted in 40% higher Rb phosphorylation at the Ser780 phosphorylation site in decorin-deficient animals than in wild-type counterparts. CDK2 is responsible for the phosphorylation of the Thr821 residue. Although the TA and DEN hepatotoxins both increased the phosphorylation of Thr821 of retinoblastoma in all cases, we could not detect differences between wild type and decorin knock-out mice.

In our further studies, strong cytoplasmic accumulation of phospho-c-Myc was observed in tumorous and non-tumor cells of wild-type animals regardless to the type of carcinogen applied. In contrast, decorin-deficient tumor cells contained less phospho-c-Myc than the surrounding tissue.

Thioacetamide increased the β-catenin amount; strong immunostaining was observed in the cirrhotic areas, especially in the proliferating cholangiocytes. However, apart from a few exceptions where it showed cytoplasmic or weak nuclear localization, β-catenin retained its membranous localization in most hepatocytes and tumor cells. In contrast, in DEN-induced liver tumors the protein showed significant translocation from the membrane to the nucleus, and significantly less inactivated phospho-β-catenin was detected in decorin deficient livers than that of wild type ones.

In control samples, phospho-Akt was practically undetectable, while its amount dramatically increased after TA and DEN exposure. Since significantly higher phospho-Akt level was detected in decorin-deficient tumors, we assumed that the Akt pathway becomes activated in the absence of decorin during experimental hepatocarcinogenesis.

The amount inactive forms of GSK proteins, the phospho-GSK3α and phospho-GSK3β increased upon TA administration. The same effect was seen when DEN was applied, but interestingly no difference in the amount of p-GSK3β between the genotypes either in untreated control or treated groups.

Activation of ERK1/2 proteins showed the most remarkable change among signaling mediator molecules. The phosphorylation level of ERK1 increased 2.3-fold and 3.6-fold by TA exposure in wild type and $Dcn^{-/-}$ samples. In case of p-ERK2 TA induced a 4.7-fold increase in decorin-deficient animals, while it was only a 1.24-fold elevation in wild-type mice. In DEN induced tumors, decorin deficiency led to ERK1/2 activation, 2.9-fold and 5.3-fold increase in p-ERK1 and p-ERK2 levels compared to wild type samples. Accordingly, the ERK1/2 pathway is continuously activated in the absence of decorin.

5. Conclusions

We have verified the tumor inhibitory effect of decorin on hepatoma cell lines *in vitro*. Each cell line reacted with decreased proliferation to decorin treatment, although only minimal effect was seen in HLE cell line. Accordingly, decorin treatment resulted in molecular changes, which however differed in each cell line. While HepG2 cells showed well-known, canonical pathway activations upon treatment, such as inhibition of EGFR activation and increased expression of $p21^{WAF1/CIP1}$, the effects were mediated via insulin and IGF-IR receptors in Hep3B and HuH7 cells. G2 / M phase block was detected in Hep3B cells provoked by decorin, an effect not described previously. Based on the decreased TGF-β1 secretion of HLE, it is assumed that decorin effects rather on the migration in these fibroblast-like cells, than the proliferation.

Decorin was more effective during TA provoked carcinogenesis, as more pronounced tumor development was observed than in DEN-induced liver tumors where no cirrhosis occurs *in vivo*. In the cirrhotic liver, decorin accumulates along the fibrotic septa and in the connective tissues around the tumors, while much less decorin can be found in DEN-exposed livers without cirrhosis. It should be noted, that enhanced degradation of the extracellular matrix by MMPs is a common process both during fibrogenesis and carcinogenesis. In light of this, we assume that as a consequence of MMP activity, decorin can be released from the collagen-bound state which causes local accumulation of the soluble proteoglycan in the invasive front of the tumors. In this case, the amount of decorin is higher when TA is applied than that of DEN due to connective tissue accumulation. Therefore, the tissue concentration of available soluble decorin is much higher around the HCCs with cirrhotic background than around the cirrhosis-free liver tumors. This may cause the more potent tumor inhibitory activity of decorin in TA-induced liver cancer.

Decorin deficiency elevated the basic activity of PDGFRα, EGFR, RON and IGF-IR tyrosine kinase receptors, and consequently increased cell growth supporting and survival promoting signals in tumors. The decorin-free tissue environment and thus RTK activation signals via Ras/MEK/ERK pathway permanently. In our experiments, this pathway proved to be the major signal transduction pathway. Furthermore, Akt activation may also has an important role, together

with the inhibition of c-Myc and β-catenin degradation. In the nucleus, c-Myc induces the AP4 transcription factor, a known transcriptional repressor of $p21^{WAF1/CIP1}$ tumor suppressor protein. Consequently, the reduced level of $p21^{WAF1/CIP1}$ will no longer be sufficient to inhibit the CDK4/Cyclin4 complex that causes E2F release through retinoblastoma phosphorylation, resulting in the cell passing through the restriction point in G1 phase.

In addition, we have shown that decorin inhibits the PDGF receptor during hepatocarcinogenesis, probably not by direct blocking of the receptor, but by binding its PDGF AB ligand.

New findings regarding the relationship between decorin and hepatocellular carcinoma:

1. Decorin exerts its tumor suppressor activity via different signaling pathways among different hepatoma cell lines.
   a. although decorin typically causes block G1 in the cell cycle through the induction of $p21^{WAF1/CIP1}$,
   b. as seen in case of Hep3B cells, it may also cause a G2 phase block if conditions are suitable
   c. decorin may also be a direct target of insulin and IGF-IR receptors, as decorin exposure was able to increase or reduce their phosphorylation experimentally.
2. Lack of decorin supports the tumor progression in hepatocarcinogenesis. In decorin deficient animals, both types of liver tumors were increased in numbers and volume compared to the wild type and this difference was more pronounced in TA treated animals.
3. The presence or absence of decorin influenced mainly the RTK / Ras / MAPK pathway in TA treated animals, while the role of β-catenin was more pronounced in DEN treated mice.
4. Lack of decorin results in constitutively active tyrosine kinase receptors, of which the MSPR/RON receptor has not been identified before as a possible target of decorin.

5. PDRFRα receptor activation is probably not directly inhibited by decorin, but by binding its ligand, the proteoglycan prevents PDGF from linking to its receptor.

6. Publications

Publications in context of the thesis

1. Baghy, K., **Horvath, Z**., Regos, E., Kiss, K., Schaff, Z., Iozzo, R. V., & Kovalszky, I. Decorin interferes with platelet-derived growth factor receptor signaling in experimental hepatocarcinogenesis. The FEBS journal. 2013;280(10):2150-64.

2. **Horvath, Z**., Kovalszky, I., Fullar, A., Kiss, K., Schaff, Z., Iozzo, R. V., & Baghy, K. Decorin deficiency promotes hepatic carcinogenesis. Matrix Biol. 2014;35:194-205.

3. **Horvath, Z**., Reszegi A, Szilak L, Kovalszky I, Baghy K., Tumorspecific inhibitory action of decorin on different hepatoma cell lines. BMC Cancer, 2018. Submitted.

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