The Role of the Syndecan-1 in the Fibrogenesis of the Liver

PhD thesis

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I. INTRODUCTION

The liver cirrhosis is a serious illness that can be characterized by pathological changes of liver structure and by accumulation of connective tissue elements, which often means worldwide life-threatening complications and serious medical problems up to this day. Virus infections (hepatitis B and C viruses), pathological alcohol consumption, several metabolic diseases (Wilson disease, haemochromatosis), autoimmune diseases (autoimmune hepatitis, primer biliaris cirrhosis) or consumption of foods polluted with alpha toxin may cause pathological connective tissue accumulation, liver fibrosis. Despite the warning data an efficient, conservative therapy does not stand for either to turn back the liver cirrhosis, only the liver transplantation offers substantive recovery from the illness. Looking at the low number of the donor livers, selection of the suitable recipient is one of the most difficult task of the specialists dealing with the transplantation. Different score systems provide making objective decisions during the selection of the recipients. The MELD score (Model for End-Stage Liver Disease) takes into account only objective laboratory values, as the values of serum bilirubin and creatinine, as well as INR (International Ratio). The MELD-score is a good prognostic marker of the short-term, three months mortality, but it is necessary to take into consideration its limits beside the usefulness of the point system. For example the score systems takes into account only the INR during the bleeding disturbance being attached to the liver diseases, but the other reasons of bleeding disturbance (e.g. thrombocyte disfunction) is left out of consideration. According to the above

mentioned facts the development of the liver cirrhosis and better recognition of its molecular mechanisms may enable the development of newer therapeutic targets and diagnostic means.

In the course of the liver fibrosis we talk about increased connective tissue accumulation, which evolves because of the balance decomposition of connective tissue synthesis and degradation. The lymphocyte infiltration and the activation of the Kupffer cells can be observed due to the liver damaging agents. The lesion of the hepatocytes and the inflammatory process activate the HSC cells into myofibroblasts, which plays an important role in the accumulation of the connective tissue. The myofibroblasts produce extracellular matrix components, matrix metalloproteases, their inhibitors and TGFβ, one of the most important mediator cytokin of fibrosis.

According to the classic idea that the extracellular matrix (ECM) serves as the frame of the tissues, it was found in the course of latter decades, that the ECM proteins take part in the regulation of growth, cell migration and differentiation of the cells. We examined the role of the syndecan-1 (CD138) - which is one of the proteoglycans in ECM complex network – in the fibrogenesis of the liver.

Sulfatated polysaccharid chains (glucosaminoglicans, GAG) attach to the protein frame of the proteoglycans through O-glycoside bounds. GAG side-chains can be heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) and keratan sulfate (KS). The syndecan-1 is the member of the four-member syndecan family belonging to the transmembrane proteoglycans. Its name cames from the Greek syndein (to join) word. Heparan sulfate and chondoroitin

sulfate chains attach to its axis protein. While the molecule's cytoplasmatic domain is preserved and among the single species is identical, its extracellular part is different, it shows a specific sequence for each species. For the members of the syndecan family is typical the so-called shedding mechanism. An enzymatic cleavage occurs in the region of the axis protein close to membrane in the course of the shedding. The fragment formed on this way acts as a parakrin and autokrin regulatory factor and it appears in the blood plasma. The ripping enzymes are called shedases, from which the most famous are MMP-2, -9, -7, MT1-MMP (MMP-14) and MT3-MMP. Syndecan-1 can be found in the matured, adult tissues significantly in the epitheliatic tissues and on the surface of the plasma cells and pre-B cells.

Our workteam had previously examined the change of syndecan-1 and other proteoglycans in different liver diseases. In a healthy liver syndecan-1 can be found mainly on the basolateral surface of the hepatocytes, it's expression on the rest of the cells is significantly less. The quantity of syndecan-1 grows during the liver fibrosis, primarily on the surface of the hepatocytes and cholangiocytes, stromal reaction cannot be observed. Examining hepatocellular carcinomas (HCC), being fibrotic or without fibrosis and their not tumourous environment, we can see, that the quantity of syndecan-1 rises more in the cirrhotical cases.

II. OBJECTIVES

On the basis of the earlier results we wished to examine the role of syndecan-1 in the liver fibrogenesis. Answering the question in the first step our target was to form a mouse strain that steadily produces human syndecan-1 in their liver. We generated experimental liver fibrosis in these mouse strains and used suitable control animals to examine the progression of the liver fibrosis during four months. We wished to examine the effect of TGFβ1 in vitro cellular model system with co-culture model systems of myofibroblasts of syndecan-1-overproducing and of control hepatoma cells. In the above mentioned model systems we examined the production of the connective tissue proteins on protein and mRNA level, and the activity of the proteases that demolish the connective tissue proteins. Furthermore we measured the activation of the signal transmission routes that play an important role in the liver fibrosis. Finally we examined the change of the syndecan-1 quantity in patient samples, and corralated that with clinical data.

III. MATERIALS AND METHODS

The transgene mice were prepared according to our order in the Central Medical Research Institute with the leadership of Gábor Szabó. In the transgenic construction, which was made by László Szilák, the sequence of human syndecan-1 cDNS was cloned (mAlb/hSynd1) after an albumin promoter. Due to the albumin promoter the human syndecan-1 is produced in a constitutive manner only on the surface of the hepatocytes.

We induced liver fibrosis with thioacetamide (TA) in the wild and human syndecan-1 transgene (hSDC1 +/+) animals. We diluted the thioacetamid in concentration of 300 mg/l in the drinking water of the animals, the animals received the treatment from their 4 week old age. We made the treatment through 4 months. We used not only the liver of the animals, but we took their blood off, which we centrifugated and stored the plasma at -80 °C. We fixed the half of every liver in 10% buffered formalin solution and embedded the samples in paraffin according to the standard method applied in the pathology department. We prepared hematoxilin-eosin and picrosirius-red painted sections.

We used in our in vitro experiments direct and indirect co-culture model of human LX-2 immortalised HSC cell line, and syndecan-1 transfected Hep3B and control Hep3B (hepatocellular carcinoma) cell lines. We induced the cellular models to transform the LX2 cells to myofibroblast with TGFβ1.

We examined in the in vivo experiment accumulated quantity of the connective tissue with the analysis of picrosirius-red morfometry. We studied the percentual proportion of the

accumulated connective tissue, we tracked the change of the collagen-1 quantity through fluorescent immunhistochemical examination, with RT-PCR and with Dot-blot in an in vitro model system in the medium of the cell cultures. We investigated in the in vivo and in vitro models with fluorescent immunhistochemisry and with Westerd-blot the change of SMA, which is the marker of the activated myofibroblasts. We tracked the activity of signal transmission routes - which are important in the liver fibrogenesis - with Western-blot and RT-PCR. During the examination of the signal transmission routes we found decreased TGFβ-1 effect in the presence of syndecan-1, so we examined the direct connection between syndecan-1 and TGFβ-1. We incubated the recombinant TGFβ-1 attached to membrane with the medium of syndecan-1 transfected Hep3B cells. We performed syndecan-1 immunreaction on them, besides appropriate positive and negative control.

We investigated the concentration of the human syndecan-1 and the mice TGFβ-1 serum with ELISA method. We made Western blot analysis to examine the change of the quantity of human, mice and total syndecan-1 in the proteoglycan samples from the animal livers.

We prepared tissue multi block (tissue microarray –TMA) from the different etiology formalin fixed, paraffin-embedded human cirrhotic liver samples, picking up two-two block representative cores. The liver samples were originated from the archive of the 1st Department of Pathology and Experimental Cancer Research. 13 cases originated from alcoholic liver damages, 4 cases from hepatitis B and 13 cases on the ground of hepatitis C virus infection of liver cirrhosis. We made syndecan-1 immune reaction from the TMA and

measured the strength of the immune reactions with the DensitoQuant module of the QuantCenter software, that belongs to the Panoramic Viewer program.

IV. RESULTS

Without liver fibrosis collagen can be only found around the vessels in the wild and in the transgenic mice. The quantity of the accumulated connective tissue increased in both groups due to the treatment. We made morfometry analysis to define quantitatively the accumulated connective tissue. Based on the results the accumulated connective tissue was significantly higher in the second and third month of the treatment in wild group compared to the transgenic group, while the difference levels off between the two groups at the fourth month.

We saw a similar picture to the pirosirius red sections during the examination of collagen-1 in fluorescent immunhistochemical experiments. Examining the change of the collagen-1 with RT-PCR on mRNA level it can be found, that the quantity of collagen-1 mRNA is significantly less in the transgenic mice, compared to the wild group during the thioacetamid treatment, while the difference between the two groups equalizes in the fourth month of the treatment.

We tracked the activation of myofibroblasts with the change of the quantity of SMA. We observed SMA positivity around the vessels in the liver of the untreated animals, while the SMA reaction we experienced during the thioacetamid treatment well correlated with the observations of the collagen-1 examination. The SMA quantity measured from the liver lysates never exceeded in the transgene mice the values we got in the wild types during the examined months.

Based on the results we had made until now we may draw the conclusion, that in case of excessive syndecan-1 presence the decreased production of the connective tissue can be the explaination to the reduced accumulation.

TGFβ1 is one of the most important cytokins in the liver fibrosis, which plays an important role in the activation of the HSC cells, so we examined the activation of TGFβ1 signal transmission route. Studying the expression of TGFβ1 and its early response gene, TIEG, we experienced the activation of both genes during the thioacetamid treatment, but the quantity of mRNA proved to be significantly lower in the transgenic mice compared to the wild group. The pSMAD2 and pSMAD3 proteins are important members of the canonised signal transmission route of TGFβ1. During the investigation of their quantity we found a similar difference, in the second month of the TA treatment they were present in significantly higher quantity in the liver isolates of the wild groups compared to the syndecan-1 transgenic mice.

Beside the TGFβ1 we examined other activity of signal transmission routes (e.g.: ERK, Akt, GSK, NFKB and FAK) known to play important role in the liver fibrosis. Each examined route was activated due to the thioacetamid treatment compared to the untreated specimen. From the above-mentioned signal transmission routes we can highlight the ERK route, which is the non-canonized route of the TGFβ1. We experienced a similar difference during the ERK investigation to the previous ones. They were present in significantly higher quantity in the first and second months of the treatment in the wild group compared to the transgenic ones.

In the next step we wished to examine the effect of TGFβ1 in an in vitro model, in which we used syndecan-1 transfected and control Hep3B and LX2 immortalized HSC cells. We applied TGFβ1 treatment in 2 ng/ml concentration. In the co-culture models, where a considerable amount of human syndecan-1 is present in the medium, the TGFβ1 effect was not able to prevail, and the expression level of SMA and TIEG proved to be significantly lower compared to the cultures that were made with non-transfected hepatomes.

According to the above-mentioned results we can prove, that the syndecan-1 inhibits the TGFβ1 activated signal transmission routes, so we examined, what kind of connection can be proved between the syndecan-1 and the TGFβ1 cytokin.

We immobilized recombinant TGFβ1 onto PVDF membrane, which we incubated with human syndecan-1 overproducing hepatoma cell medium. After suitable washing steps we made an immune reaction with antibody reacting with extracellular syndecan-1. We watched a specific reaction on the areas, that suit to the immobilized TGFβ1, so we proved bounding between syndecan-1 and TGFβ1.

During the next step we examined whether the syndecan-1 and the TGFβ1 complex could appear in the plasma of mic, so we applied ELISA method. The quantity of human syndecan-1 shows a significant increase in case of thioacetamid treatment, compared to the untreated specimen. The serum concentration of the TGFβ1 in the mice during the thioacetamid treatment showed a decreasing tendency in the wild samples, while we experienced a stable level in

the hSDC1 +/+ sera, although the difference between the two groups did not proved to be significant.

According to the ELISA results we proved, that in the presence of human syndecan-1 the TGFβ1 of the mice appears in the peripheral blood, presumably being bound in complex based on the previous results.

We examined the changes of the human, the mice and the total syndecan-1 quantity in our in vivo samples. Without treatment the human syndecan-1 appeared on the basolateral surface of the hepatocytes. The quantity of the human syndecan-1 shows continuous decrease due to the thioacetamid treatment. To determine the quantity of mice syndecan-1 we made mice syndecan-1 Western-blot from the total proteoglikan isolatum.

Due to the thioacetamid treatment the quantity of the mice syndecan-1 grew progressively in both groups. The endogenous mice syndecan-1 shows a slighter uprise in the human syndecan-1 transgenic mice. The quantity of the total syndecan-1 grows in both groups, but the quantity of syndecan-1 was higher in the strains of transgenic mice in every examined month because of the overproduction of syndecan-1. The quantity of the heparan sulfate being attached to the side chains of the proteoglycnas partly followed what we experienced in the human syndecan-1 immune reactions in the transgenic mice. After the second month of the treatment the quantity of the heparan sulfate shows a progressive decrease till the completion of the experiment. The quantity of the heparan-sulfat increases in the liver of the wild specimen in the second month, while it decreases to the initial level at the end of the treatment.

We examined the quantity of MMP-2 and MMP-9 enzymes with zymography. The quantity of the MMP-2 and -9 increases in both groups due to the effect of the thioacetamid treatment, but we couldn't observe the presence of the active form of the enzymes.

We examined the quantity of the MMP-14 metalloprotease enzyme that is one of the enzymes that takes part in the syndecan-1 shedding. We studied the MMP-14 activity in the Hep3B/LX2 and Hep3B SCD1/LX2 co-cultures, too. The MMP-14 appeared in both cell model media, although its quantity was less compared to the syndecan-1 overproducing samples in case of the Hep3B/LX2 cell co-culture. While we can observe mainly the inactive form in the Hep3B/LX2 model, in case of the Hep3B SDC1/LX2 samples the active form can be found, furthermore the presence of syndecan-1 increases the quantity of MMP-14. We investigated the appearance and change of the enzyme in the mice samples with MMP-14 immune reaction during the treatment. The quantity of the MMP-14 increased in the second month of the thioacetamid treatment in the livers which produce human syndecan-1, in contrast to the other groups it can be observed in the liver of hSDC1 +/+ animals not only on the non-parenchymal cells being in the sinusoids, but in the plasma membrane of the hepatocytes in the second month of the treatment, too. Based on these results the syndecan-1 increases its own shedding via production of MMP.

Although we had already known from the previous results of our working group, that the quantity of syndecan-1 increases due to cirrhosis, we examined, whether it can be found relevant, from the viewpoint of diagnostic and therapy, useful difference in the clinical concern of the liver diseases. We didn't find significant difference

according to the three most common etiology factors amongst the several groups. Increase of MELD-score value showed a positive tendency with the syndecan-1 level, the difference between the lowest and the highest score group the difference proved to be significant.

V. CONSCLUSIONS

The principal statements of the thesis are follows:

- Production of human syndecan-1 protects against the accumulation of connective tissue in the early phase of the fibrogenesis.
- The reduced myofibroblast activity and the lower collagen-1 production stand in the background of the reduced connective tissue accumulation.
- The syndecan-1 binds TGFβ1.
- Concentrations of syndecan-1 and TGFβ1 are increasing parallel in the serum of the mice, so we can assume, that they are present in the serum at least partly in the form bound to each other.
- The signal transmission routes depending on TGFβ1 show a reduced activity in the liver of transgenic mice compared to the wild group, that can be explained with the relative TGFβ1 deficiency in the connective tissue.
- The syndecan-1 increases its own „shedding" in an autokrin manner through the MMP-14 activity.
- The quantity of syndecan-1 didn't show correlatation in the examined human cirrhotic samples with the etiology of the cirrhosis.
- We proved significantly higher quantity of syndecan-1 in the human cirrhotic samples with higher MELD-score values.

VI. PUBLICATIONS

VI.1. PUBLICATIONS RELATED TO THE THESIS

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VI.2. PUBLICATIONS UNRELATED TO THE THESIS

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