The relationship between fibrogenesis and ductular reaction in human cirrhotic livers and experimental models

PhD thesis

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Index

I. Introduction

Liver cirrhosis is the end-stage of several different chronic liver diseases. It is a diffuse process, which is – according to the WHO-definition – characterised by increased fibrogenesis (fibrosis) and the nodular rearrangement of the liver parenchyma. The most common etiological factors are alcoholic liver disease (ALD), hepatitis B (HBV) and C (HCV) virus infection, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), Wilson's disease, haemochromatosis and different storage diseases.

I.1. Characterisation of the newly formed connective tissue of cirrhotic liver

The cirrhotic liver consists of pseudolobules, formed by hepatocytes and connective tissue septa containing bile ducts, inflammatory cells and vascular structures.

The most characteristic feature of cirrhotic liver both macro- and microscopically is the increased amount of connective tissue (fibrosis). Connective tissue bundles dividing the parenchyma are called septa. According to the distribution of the connective tissue deposition, liver fibrosis has different forms – we can speak about perisinusoidal, periportal or pericentral fibrosis. Established cirrhosis is characterised by „bridging" fibrosis, where the newly formed connective tissue septa connect two vascular structures (porto-portal or porto-central septa are formed). Parenchymal extinction is a special form of fibrosis, where large fields of scar tissue replace the extinct parenchyme. There can be great differences in the morphology of liver fibrosis according to the causative factors.

Different semiquantitative scoring systems are used for the histological evaluation of liver cirrhosis (e. g. Scheuer-, Ishak-, Batts-Ludwig-, METAVIR-score), in which necroinflammatory activity (grade) and the extent of fibrosis (stage) are determined. The highest stage marks cirrhosis in each of these systems. The most important problems of these are the pronounced intra- and interobserver variability between pathologists. Besides, there can be great differences in the extent of fibrosis in a given stage, since the score is determined not only by the exact amount of fibrosis, but

also the structural alterations of the liver (localisation and extension of septa, spot of biopsy).

Nowadays we need histological parameters beside the semiquantitative scoring systems which can be objectively and reproducibly measured to accurately determine the extent of fibrosis and to monitor the progression or the possible regression of cirrhosis. Some authors advocate the use of digital morphometry beside the scoring systems to assess the amount of hepatic fibrosis. In this method the amount of collagen deposition is measured as a proportion of the total biopsy area (collagen proportionate area – CPA). Thus fibrosis is characterised by a continuous variable, which can be correctly used in statistical comparisons with different clinical or histological parameters. Many studies have shown that CPA values determined by digital morphometry correlate well with the fibrosis stage of the different scoring systems.

I.2. Ductular reaction in experimental models and in the human liver

Beside fibrosis the other most characteristic feature of cirrhotic liver is the so called ductular reaction; this term is used collectively for the bile duct-like structures appearing in the fibrotic septa. Ductular reaction contains cells with progenitor/stem cell properties, and it is theoretised to have a regenerative role. If it would be possible to recognise the exact role that liver progenitor cells play in regeneration, it would be possible to influence the course of cirrhosis either by enhancing the regenerative processes or by inhibiting the harmful processes (fibrosis, carcinogenesis) that accompany progenitor cell activation.

Liver has excellent regenerative capabilities. If 2/3 of the liver is surgically removed from a mouse or rat, liver weight is restored in 5-7 days by the proliferation of hepatocytes. If the proliferative capacity of hepatocytes is compromised by different liver injuries, liver progenitor cells become activated. According to the most accepted view liver progenitor cells derive from the stem cells residing in the canals of Hering.

The classic model of liver regeneration by progenitor cells is the AAF/PH (2-acetylaminofluorene/partial hepatectomy) model in rats. In this model it is proven that the progenitor cells recovering the liver parenchyme are of bile duct origin.

Nowadays liver stem cell research is mainly done in mice, where different models are used. In contrast to rat in mouse liver the origin and possible regenerative function of progenitor cells are debated.

Similarly to experimental models in human liver it is also a longstanding question if the ductular reaction observed in different acute and chronic injuries contain progenitor cells and whether it has any role in regeneration. In massive hepatic necrosis there are strong evidences for the regenerative role of ductular proliferations. In most chronic liver diseases ductular reaction can be observed as well, but its biological function is contorversial. Similarly to animal models and massive hepatic necrosis in human there are substantial arguments for its regenerative role in cirrhosis. In the last decades it has become a popular theory that in chronic liver diseases ductular reaction starts to have regenerative capability when the majority of hepatocytes have already became senescent (meaning they are incapable to proliferate anymore).

I.3. Animal models used for the study of cirrhosis

The most often used agents for the experimental study of liver cirrhosis are carbon tetrachloride (CCl_4) and thioacetamide (TAA).

CCl_4 is one of the longest used hepatotoxic agents in experimental settings. It is metabolised by the cytochrome p450 2E1 (CYP2E1) enzyme which is expressed by the pericentral and midzonal hepatocytes. During metabolization a thricloromethyl derivative is produced, causing centrilobular necrosis. By induction of the CYP2E1 enzyme the harmful effect of CCl_4 can be enhanced, and this way the development of fibrosis/cirrhosis can be speeded up. One of the inducing agents is phenobarbital (PhB), which – dissolved in drinking water – has been used in experimental models for a long time in combination with CCl_4.

During the metabolization of TAA thioacetamide-s-oxide is made by the flavin containing monooxigenase and cytochrome p450 enzymes. The free radicals that are produced during the reaction are responsible for the cell damaging effect. TAA is given intraperitoneally or dissolved in the drinking water to induce cirrhosis. It produces acute centrilobular necrosis but it damages the periportal hepatocytes as well.

I.4. The use of targeted therapeutic drugs in experimental models of cirrhosis

Some of the receptors that play a role in fibrogenesis and in the formation of ductular reaction are the receptor tyrosine kinases (e. g. EGFR, PDGFR, c-met), so it is not surprising that many tyrosine kinase inhibitors were examined in different experimental models of hepatic fibrosis and liver regeneration.

The first tyrosine kinase applied in clinical practice was the c-kit and PDGFR inhibitor imatinib (Glivec). The central role of PDGFR in fibrogenesis is the reason why the antifibrotic properties of imatinib were investigated experimentally in many different organs. Its antifibrotic effects were described in lung, heart, kidney and skin, but the most studied organ was liver, where it had effective antifibrotic properties in different experimental settings.

The EGFR inhibitor erlotinib was also used succesfully as an antfibrotic agent in different models.

These tyrosine kinases receptors play an important role in the regulation of ductular reaction as well, but most studies didn't describe the effects of the tyrosine kinase inhibitors on ductular reaction. Besides, most observations were only made in one experimental time point, so little is known about the dynamics of the antifibrotic effects of these drugs.

I.5. Regression of cirrhosis

Until recently cirrhosis was thought to be an irreversible end-stage of different chronic liver diseases. Today the regression of cirrhosis can be a realistic alternative, hence the approach towards the disease is changing. Nowadays instead of an illness with only one possible outcome, regardless of the etiology, cirrhosis is considered to be a heterogenic, dynamically changing disease, where even regression is possible in some etiologies (e. g. viral hepatitis, ALD). Along with this change the flaws of the scoring systems used for the evaluation of fibrosis also came to the fore. Since these systems view cirrhosis as a uniform end-stage, the given score doesn't contain information about the possibility of regression. Today the classification of cirrhosis is necessary to

distinguish between patients with different prognosis. Objectively, reproducibly measurable parameters are needed that could support classification and could show which cases would react well to therapy and which cases are irreversible. The etiological factors are becoming more important because of the possible regression, so finding parameters that vary according to the etiology would be of great significance.

II. Aims

I. To study the relationship of different morphological parameters (the area occupied by fibrosis, ductular reaction and activated myofibroblasts, the thickness of fibrotic septa, the proliferative activity of hepatocytes and ductular cells) in human cirrhotic liver samples.

II. To search for correlations between the morphological parameters of the human liver samples and the etiological factors, the laboratory values and the presence of cirrhotic complications in order to classify liver cirrhosis.

III. To study the dynamics of the progression of fibrosis and ductular reaction, and the proliferative activity of hepatocytes and ductular cells in mouse liver fibrosis models.

IV. To study the effects of the tyrosine kinase inhibitors imatinib and erlotinib on the progression of fibrosis and ductular reaction in mouse liver fibrosis models.

III. Methods

III.1. Human samples

We examined 56 explanted livers removed during transplantation at the Department of Transplantation and Surgery of Semmelweis University between 2010 and 2012. The etiological factors were HCV (30 cases), HBV (4), ALD (11), AIH (5), PSC (4), PBC (1), cryptogenic (1). In 13 livers (10 HCV, 1-1-1 ALD, AIH, PBC) HCC could be identified as well. We used three normal livers as controls. From every liver we made a section with at least 1 cm^2 area from the VII. segment, where usual biopsies are taken from. Our study was approved by the Regional and Institutional Comittee of Science and Research Ethics (decision no. 125/2010).

We collected the last laboratory values before transplantation in each case. The values gathered were the parameters of liver and kidney function (serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, serum total protein (TP), gamma-glutamyltransferase (GGT), international normalised ratio (INR), creatinine) and qualitative blood test results (hemoglobin level (Hb), white blood cell count (WBC), thrombocyte numbers (Thr)). From the serum bilirubin, creatinine and INR values we calculated the MELD-score. We also checked if oesophageal varices, variceal bleeding, ascites or hepatic encephalopaty was present in the history of the patients.

III.2. Experimental models

Our experiments were performed in the Animal Facility of our Institute on 8 week old male mice, kept under standard circumstances. The experiments were conducted by the protocols of the National Institutes of Health (USA), also keeping in line with the guidelines of Semmelweis University's Institutional Animal Care and Use Committee.

We have formed ten experimental groups:

Control models:

I. Wild type C57Bl/6 mice were treated with a combination of CCl_4 (0,2 ml/kg through oral gavage, dissolved in sunflower oil) and phenobarbital (PhB) (0,5 g/l, dissolved in drinking water) (I. WT+CCl_4, n=49).

II. Wild type C57Bl/6 mice were treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) (II. WT+TAA, n=50).

III. Mice expressing active TGFβ in their liver were treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) (III. TGFβ+TAA, n=61). These animals were transfected with porcine TGFβ1 cDNS bound to an albumin promoter, so their hepatocytes constantly produce TGFβ from the transgene. The blood levels of TGFβ is several times higher in these animals compared to wild type mice.

Drug treated models:

IV. Wild type C57Bl/6 mice treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) were given a daily dose of imatinib (Glivec, Novartis, Basel; 25 mg/kg dissolved in water, per os) (IV. WT+TAA+imatinib, n=54).

V. TGFβ transgenic mice treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) were given a daily dose of imatinib (25 mg/kg dissolved in water, per os) (V. TGFβ+TAA+imatinib, n=38).

VI. Wild type C57Bl/6 mice treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) were given a daily dose of erlotinib (Tarceva, Roche, Basel; dissolved first in dimethyl sulfoxide then water) (VI. WT+TAA+erlotinib, n=48).

VII. TGFβ transgenic mice treated with thioacetamide (TAA; 300 mg/l, dissolved in drinking water) were given a daily dose of erlotinib (dissolved first in dimethyl sulfoxide then water) (VII. TGFβ+TAA+erlotinib, n=38).

In models I-VII. treatment was continued for 18 weeks. We terminated 5-16 animals on the 3., 6., 9., 12., 15. and 18. week of the experiment. The data of ’0’ timepoint was measured from the liver of 5-5 healthy wild type and transgenic mice.

Therapeutic models:

Beside the models above, where the drugs were given to the animals from the same timepoint as the fibrosis inducing agent, the effects of imatinib and erlotinib were also studied on established cirrhosis. For this reason three models were formed:

VIII. Wild type C57Bl/6 mice were treated with thioacetamide for 27 weeks (TAA; 300 mg/l, dissolved in drinking water) (VIII. Ther. control, n=18).

IX. Wild type C57Bl/6 mice were treated with thioacetamide for 27 weeks (TAA; 300 mg/l, dissolved in drinking water). From the 19th week the animals recevied daily imatinib treatment as well (in the way described above) (IX. Ther. imatinib, n=18).

X. Wild type C57Bl/6 mice were treated with thioacetamide for 27 weeks (TAA; 300 mg/l, dissolved in drinking water) From the 19th week the animals recevied daily erlotinib treatment as well (in the way described above) (X. Ther. erlotinib, n=17).

We terminated 5-7 animals on the 21., 24. and 27. week of the experiment (3., 6.. and 9. week from the start of the drug treatment).

Every animal received 500 mg/kg bromodeoxyuridine (BrdU) intraperitoneally 20, 2, and 1 hour before termination.

III.3. Histological processing, morphometric studies

Formalin fixed, paraffin embedded blocks were made from both the human and mouse liver samples through routine histological processing. Parts of the mouse livers were frozen in isopentane cooled with liquid nitrogen and were stored on -80ºC. The paraffin embedded blocks were rehydrated with xylene and descending alcohol grades, then hematoxylin eosin (HE) staining, different special stains and immunohistochemical reactions were applied on sections made from each block. Immunohistochemical reactions were made with a Leica Bond immunostainer, using diaminobenzidine (DAB) as chromogen.

We analysed the extent of fibrosis on Picro Sirius red stained, paraffin embedded sections both in the human and mouse liver samples. Besides, in the human samples we

measured the width of the three thickest septa and drew an avarege to give the septum thickness value for each sample.

In human samples the area occupied by activated myofibroblasts and ductular reaction was measured on slides labeled with anti-SMA (cat. no.: M0851; Dako), and anti-CK7 (cat. no.: MU-255-UC; Biogenex) antibodies, respectively. In the mouse liver samples for the study of ductular reaction we performed CK19 (cat. no.: TROMA-III; Developmental Studies Hybridoma Bank) labeling on frozen sections.

In order to characterise the proliferative activity of hepatocytes and ductular cells in the human liver samples we counted 5000 hepatocytes and 500 ductular cells after performing Ki-67 immunohistochemical reaction (cat. no.: M7240; Dako). For the same reason the mice recieved BrdU. On the sections made from mouse livers we made an anti-BrdU immunohistochemical reaction (cat. no.: 347580; BD Pharmingen) and counted 2500 hepatocytes and 500 ductular cells.

III.4. Statistical methods

The statistical analysis was performed with Statistica 8.0 software (StatSoft Inc, Tulsa, OK). Deviation from Gaussian distribution of variables was tested with Kolmogorov-Smirnov and Lillefor's method. Correlation between the morphometric parameters of human cirrhotic livers and the laboratory values was examined with Spearman rank correlation test. The data divided into different groups (viral/nonviral, HCC/ non HCC) were compared with Mann-Whitney U test, since not every variable was of normal distribution.

In the experimental studies we used two way ANOVA (analysis of variance; every variable was of normal distribution, the two variables were the experimental group and the timepoint). Correlation between the variables within an experimental group was tested with Spearman rank correlation test. Results were considered significant at a p value less than or equal to 0,05.

IV. Results

IV.1. Correlation studies between the clinical data of the patients and the morphometric data of the cirrhotic liver samples

Both on the HE and Picro Sirius stained slides pseudolobules completely surrounded by fibrotic septa were present in the human liver samples. The pseudolobules were highlighted by the SMA immunohistochemical reaction, due to the septal localisation of the activated myofibroblasts. CK7 reaction was strongly positive in the normal bile ducts and in the epithelial cells of the ductular reaction as well.

The area occupied by fibrosis showed significant correlation with the area occupied by activated myofibroblasts (SMA), with septum thickness and with the ALP value as well. The SMA value correlated in a significant way with septum thickness and the extent of ductular reaction (CK7). The CK7 value also showed significant correlation with the AST value. In most samples we detected a very low (under 1%) Ki-67 index in both the hepatocytes and ductular cells. There was significant, negative correlation between the proliferative activity of hepatocytes and septum thickness. There was a significant correlation between the proliferative activity of hepatocytes and ductular cells as well, but – contrary to expectations – the correlation was positive. We didn't find any significant correlations between the presence of clinical complications (ascites, oesophageal varices, variceal beleeding, hepatic encephalopathy) and the morphometric parameters.

When comparing the samples with viral and non-viral etiology there were significantly higher ALP values and larger extent of fibrosis in the non-viral group. Besides, the patients with HCV/HBV induced cirrhosis had significantly higher hemoglobin levels and lower MELD-score.

IV.2. Results of the animal studies

IV.2.1. Control models

By the end of the experiment completely encircled pseudolobules could be observed in the liver of both the CCl_4, and TAA treated animals, but the dynamic of fibrosis development was different according to the injuring agent. In the WT+TAA

group the amount of fibrosis was initially low, then increased suddenly, while CCl_4 induced larger amounts of fibrosis already in the early timepoints. In the WT+CCl_4 group septa connecting the central veins could be observed on the 3rd week of the experiment, the same structures could be seen in the WT+TAA group on the 6th week. On the 6th week the extent of fibrosis was significantly higher in the WT+CCl_4 group compared to the WT+TAA group. By the 9th week the amount of fibrosis became similar in the two groups, then it remained similar throughout the experiment. In the TGFβ+TAA group the extent of fibrosis was larger in every timepoint than in the WT+TAA group.

Interestingly, on the 3rd week the area occupied by ductular reaction was comparable in the WT+CCl_4 and WT+TAA groups, despite the different amounts of fibrosis. However, in the later timepoints TAA induced much more pronounced ductular reaction, the difference was significant from the 9th week. Similarly to the extent of fibrosis, TAA treatment resulted in more extensive ductular reaction in the TGFβ transgenic mice compared to the wild type animals.

The two injuring agent effected the proliferative activity of hepatocytes and ductular cells differently as well. CCl_4 treatment resulted in very low proliferative activity, which remained constant throughout the experiment. In contrast, in the WT+TAA group after an initial rise a gradual decline could be observed in the proliferative activity, especially in the ductular cells. Increased TGFβ expression had no significant effects on proliferative activity in the TAA treated animals.

IV.2.2. The effects of imatinib and erlotinib on TAA induced cirrhosis

Imatinib treatment resulted in a striking decrease in the amount of fibrosis and ductular reaction in the TAA treated wild type animals. The extent of fibrosis was significantly lower on the 9th, 12th, 15th week, while the extent of ductular reaction was significantly lower on the 9th and 12th week compared to the WT+TAA group. A similar tendency was present in the erlotinib treated wild type group, but the difference was not significant in any of the timepoints compared to the WT+TAA model. The effects of both drugs were only temporarily. By the 18th week the amount of fibrosis and ductular reaction was similar in the drug treated and in the control wild type mice.

In the transgenic animals neither drug could influence the progression of fibrosis and ductular rection.

Both drugs could only effect the proliferative activity of hepatocytes and ductular cells in one timepoint each in wild type mice. In the imatinib treated group the proliferation of ductular cells was significantly lower on the 6th week, while in the erlotinib treated group the proliferation of hepatocytes was significantly lower in the 12th week compared to the control wild type animals. In the transgenic mice neither drug could effect the proliferative activity of hepatocytes and ductular cells significantly.

IV.2.3. The effects of imatinib and erlotinib on established TAA induced cirrhosis (therapeutic models)

We also studied the effects of imatinib and erlotinib on established, TAA induced cirrhosis in wild type mice. Neither drug could prevent the progression of fibrosis and ductular reaction. Erlotinib treatment didn't cause any significant differences in the morphometric parameters. In contrast, after 3 weeks of imatinib treatment (on the 21st week of TAA treatment) we noticed significantly elevated levels of fibrosis, ductular reaction and ductular cell proliferation. This effect was only temporary, in the later timepoints there were no significant differences in the studied parameters.

IV.2.4. Correlation analysis of the parameters measured in the cirrhotic models

We studied the correlations between the parameters measured in each group with Spearman's rank correlation. During the analysis we used all data from each group, regardless of the timepoint. The extent of fibrosis and ductular reaction showed strong, significant correlation in all experimental models. There was significant positive correlation between the area occupied by fibrosis and the proliferative activity of hepatocytes in two models (WT+CCl_4, WVT+TAA+erlotinib). In the same two models the amount of ductular reaction showed significant, positive correlatetion with the proliferative activity of hepatocytes. In contrast, there was negative, significant correlation between the extent of fibrosis and ductular cell proliferative activity in three

models (WT+TAA, TGFβ+TAA, TGFβ+TAA+imatinib) beside positive, significant correlation in one model (WT+TAA+imatinib). There was significant correlation between the area occupied by ductular reaction and the proliferative activity of ductular cells in two models; one with positive (WT+TAA+imatinib) and one with negative (TGFβ+TAA) direction. We didn't find significant, negative correlation between the proliferative activity of hepatocytes and ductular cells in any of the models, on the other hand, there was significant, positive correlation in two models (WT+TAA+imatinib, Ther. imatinib).

V. Conclusions

I. In human cirrhotic liver samples the extent of fibrosis showed significant correlation with septum thickness and the area occupied by activated myofibroblasts, while the area occupied by activated myofibroblasts showed significant correlation with septum thickness and the extent of ductular reaction. There was positive correlation between the proliferative activity of hepatocytes and ductular cells. According to our results ductular cells can play a regenerative role focally, in the late phase of cirrhosis.

II. In human cirrhotic livers there was significant correlation between the extent of fibrosis and ALP value, also between the extent of ductular reaction and AST value. We couldn't find significant correlation between the morphological parameters and the presence of cirrhotic complications.

III. The extent of fibrosis and ductular reaction showed strong, significant correlation in all of our mouse models, regardless of the injuring agent. We didn't find an inverse correlation between the proliferative activity of hepatocytes and ductular cells. According to our results the ductular cells didn't contribute to liver regeneration in the experimental models.

IV. Both imatinib and erlotinib decreased the extent of fibrosis and ductular reaction in thioacetamide treated wild type mice, but this effect was only temporary. Neither drug could effect the progression of fibrosis and ductular reaction in TGFβ transgenic mice.

VI. List of publications

Publications in the theme of the thesis:

1. **Rókusz A**, Nagy E, Gerlei Zs, Veres D, Dezső K, Paku S, Szücs A, Hajósi-Kalcakosz Sz, Pávai Z, Görög D, Kóbori L, Fehérvari I, Nemes B, Nagy P. (2016) Quantitative morphometric and immunohistochemical analysis and their correlates in cirrhosis - A study on explant livers. Scand J Gastroenterol 51:86-94. IF: 2,526

2. **Rókusz A**, Bugyik E, Szabó V, Szücs A, Paku S, Nagy P, Dezső K. (2016) Imatinib accelerates progenitor cell-mediated liver regeneration in choline-deficient ethionine-supplemented diet-fed mice. Int J Exp Pathol 97:389-396. IF: 1,780

3. Dezső K, **Rókusz A**, Bugyik E, Szücs A, Szuák A, Dorogi B, Kiss M, Nemeskéri Á, Nagy P, Paku S. (2017) Human liver regeneration in advanced cirrhosis is organised by the portal tree. J Hepatol, 67:430-436. IF: 15,040

4. **Rókusz A**, Veres D, Szücs A, Bugyik E, Mózes M, Paku S, Nagy P, Dezső K (2017) Ductular reaction correlates with fibrogenesis but does not contribute to liver regeneration in experimental liver fibrosis models. PLoS One, 12:e0176518. IF: 2,766

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1. Papp V, **Rókusz A**, Dezső K, Bugyik E, Szabó V, Pávai Z, Paku S, Nagy P. (2014) Expansion of Hepatic Stem Cell Compartment Boosts Liver Regeneration. Stem Cells Dev23:56-65. IF: 3,727

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4. Bugyik E, Szabó V, Dezső K, **Rókusz A**, Szücs A, Nagy P, Tóvári J, László V, Döme B, Paku S. (2018) Role of (myo)fibroblasts in the development of vascular and connective tissue structure of the C38 colorectal cancer in mice. Cancer Commun (Lond), 38:46. IF: -