Mini Review

Portal tract fibrogenesis in the liver

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The portal area is the ‘main entrance’ and one of the two main exits of the liver lobule. Through the main entrance portal and arterial blood reach the liver sinusoids. Through the exit the bile flows towards the duodenum. The three main structures, portal vein and artery with their own wall (and vascular smooth muscle cells) and bile duct with its basal membrane, are surrounded by loose myofibroblasts and by the first layer of hepatocytes and non-parenchymal cells. Chronic diseases of the liver can lead to development of liver cirrhosis, characterized by formation of fibrotic septa which can be portal–portal in the case of the chronic biliary damage or portal–central in the case of the chronic viral hepatitis. Central–central septa can also be observed under other pathological conditions. When damaging noxae are introduced to the liver, inflammatory cells are first recruited to the portal field, the first layer of hepatocytes may be destroyed (enlargement of the portal field) and portal (myo)fibroblasts become activated. A similar reaction may take place when the target of inflammation is the bile duct with consecutive reduction of the bile flow, activation of the portal (myo)fibroblasts, proliferation of bile ducts and destruction of the hepatocytes around the portal field. Increased matrix deposition may be the consequence. During the past years several publications dealt with the pathomechanisms of portal fibrogenesis as well as with its resolution. One of the most intriguing observations was that it is not hepatic stellate cells of the hepatic sinusoid, but portal (myo)fibroblasts which rapidly acquire the phenotype of ‘activated’ (myo)fibroblasts in the early stages of cholestatic fibrosis. These may also become the main mesenchymal cells of the porto-portal or porto-central fibrotic septa. This article reviews the similarities as well as differences between the mesenchymal cells of the portal tract and of the fibrotic septa vs ‘activated’ stellate cells of the hepatic sinusoids, and discusses the debate over their relative contributions to liver fibrogenesis.

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Liver damage and repair

The portal field of the liver contains three vessels, the portal vein, the portal artery and the bile duct. Whereas the portal vein as well as the portal artery both have their own wall with smooth muscle cells (SMC), the bile duct with its basal membrane is surrounded directly by periductular fibroblasts. Loosely placed around the portal vein and artery as well as in the adjacent connective portal tissue, an additional cell type, portal myofibroblasts, can be identified. This cell type is thought to be the main source of laminin, tenascin, collagen type IV, elastin and fibrillin in the connective tissue of the portal

area.^{1,2} In the case of infectious liver diseases such as viral hepatitis, the initial histological changes leading to portal fibrogenesis are characterized by accumulation of inflammatory cells and matrix deposition around the portal vein. On the other hand, biliary fibrosis occurring secondary to bile duct obstruction is morphologically characterized by a quick activation of the fibroblasts, a progressive enlargement of the portal fields caused by edema and extensive bile duct proliferation, and massive deposition of collagens in portal tracts and the periportal areas with only modest signs of inflammation.^{3,4}

Liver fibrosis, remodelling, cirrhosis

It is well known that the liver possesses the ability of a complete *restitutio ad integrum* following a single damage when the damaging noxae are eliminated. Restitution can also be observed following

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resolution of mechanical obstruction by decompression or surgical anastomosis in cholestatic liver diseases. In all cases, apoptosis of inflammatory cells and activated mesenchymal cells and enhanced matrix degradation seem to play important roles.^{5–7} In the case of ongoing liver damage, however, the imbalance between matrix synthesis and matrix degradation together with hepatocellular necrosis lead to formation of porto-portal and of porto-central fibrotic septa.

Although first reports suggesting a certain reversibility of fibrotic changes in the liver appeared in literature in the late 1970s and early 1980s,⁸ the prevailing opinion was that the fibrotic process in the liver was self-perpetuating.⁹ However, the molecular similarities to the wound-healing process^{10,11} and the observation that matrix-producing cells undergo apoptosis^{5,12,13} strongly suggested that some matrix deposits might disappear when the damaging noxae are eliminated or inflammation is suppressed as by corticosteroids^{2,14–17} (Figure 1).

Mesenchymal cells of the portal field and identification markers

During the last 40 years, research focused on the cellular origin of enhanced hepatic matrix synthesis within the damaged liver tissue. Whereas this could be shown to a certain extent for hepatocytes as well as for bile duct epithelial cells, it was soon clear that the main contributors to matrix production were mesenchymal cells.¹⁸ In addition to periductular fibroblasts, portal myofibroblasts, smooth muscle cells of the wall of the portal vessels, and second layer fibroblasts of the central vein may possess the capacity of synthesizing connective tissue proteins. The hepatic stellate cells (HSC, formerly known as Ito cells¹⁹ or lipocytes²⁰) are located in the space of Disse between the hepatocytes and the sinusoidal endothelial cells and are also of mesenchymal origin. Studies in the early 1970s suggested that HSC could undergo an activation process, during which they lose their lipid droplets, acquire a fibroblastic phenotype and express the smooth muscle alpha actin (SMA)-gene. After collagen deposition was demonstrated next to activated

HSC, their major role in liver fibrosis was suggested.²¹

Taking advantage of the abundant fat content of the HSC, an isolation method for HSC from the rat liver was developed, and, interestingly, in culture this cell type showed the changes to a myofibroblastic appearance similar to those observed *in vivo*, now called a myofibroblast-like cell.²² The finding that activated HSC can proliferate and produce much more collagen, when compared to hepatocytes and sinusoidal endothelial cells, strengthened the suggestion of HSC being the principal matrix-producing cell type of the liver,^{23–25} a dogma which still holds. The collagen produced by hepatocyte cultures was attributed to contaminating ‘HSCs’.²⁶ However, accurate analysis of the cell numbers in the parenchyma failed to show a proliferation of activated HSC.^{27,28} Increased desmin positivity and BrdU- or ³H-thymidine incorporation in desmin-positive cells observed in animal experiments were interpreted as proliferation of HSCs.^{29,30} Without wanting to reduce the importance of the HSC, two errors might have been made: first, not taking the other mesenchymal cell types into consideration as potential source of connective tissue proteins; and second, a too high degree of confidence in the purity of the HSC cultures by claiming at the same time that collagen production in hepatocyte cultures was due to contaminating mesenchymal cells.^{26,31} Now, *in vitro* data strongly suggest that activated HSC do not proliferate but become polyploid by endoreplication and die by apoptosis when survival factors are missing.^{5,13,32} These data also strongly suggest that observed proliferation of cultured mesenchymal cells is due to contaminating myofibroblasts.³³ Probably because of the appointment of the HSC as major matrix producer in the liver, additional focus on the other mesenchymal cells of the liver has been neglected, although early on, Leo *et al*³⁴ described a method for isolation of myofibroblasts from the rat liver.

Repeatedly during the last 20 years, there have been single publications suggesting that portal tract (myo)fibroblasts, and fibroblasts, including the second layer cells of the central veins and the fibroblasts of the liver capsule, are potentially fibrogenic. This was established in particular by

Figure 1 Example of regression of liver cirrhosis. Goldner staining of human liver cirrhosis due to viral hepatitis B. (a) Before treatment was initiated. (b) After treatment with interferon- α and lamivudine. Note that the disappearance of the inflammatory infiltrate is accompanied by a strong reduction of matrix protein depositions and by recovery of the hepatocytes.

the studies of Bhunchet and Wake in 1992 (model of serum-induced liver fibrosis) and of Tuchweber *et al*⁴ in 1996 (model of bile duct ligation), demonstrating that activated portal fibroblasts rapidly express the SMA gene and synthesize connective tissue proteins.³⁵ Following these observations, Desmouliere *et al*³⁶ showed that deposition of extracellular matrix proteins in the portal area preceded enhanced SMA expression in activated periductular fibroblasts.

However, these published data, as well as the observation that SMA- and desmin positivity is already present in the normal liver in the periportal and pericentral areas, did not displace the suggested central role of activated HSC. On the contrary, SMA- and/or desmin positivity in mesenchymal cell isolations was used to declare isolated mesenchymal cells as activated HSC even though studies in the early 1990s cast doubt on the specificity of these markers.^{37,38}

Although ultrastructural detection of 'dense plaques' was a useful marker for myofibroblasts³⁸ as distinct from HSC, it was not useful for large-scale investigation of the purity of HSC cultures. The first step in the latter direction was the observation of a different life cycle of the two mesenchymal cell types in culture. Whereas portal myofibroblasts could be subcultured several times, activated HSC from rat liver died by apoptosis.^{5,33} This crucial difference allowed us to discover differences in the gene-expression profiles. Whereas both cell types showed similar expression patterns of I-CAM, V-CAM, desmin, vimentin, collagen IV, fibronectin and SMA, several differences could be observed. Cultured portal myofibroblasts were positive for fibulin-2 and IL-6 mRNA, whereas CD95L, α 2-macroglobulin, P100 and reelin were exclusively produced by activated HSC^{2,33,39–42} (Table 1). The use of these markers for *in vivo* identification of activated HSC, portal (myo)fibroblasts and septal myofibroblasts led to the conclusion that myofibroblasts of the septum strongly resemble the (myo) fibroblasts of the portal field. This observation was supported by an extensive study of Cassiman *et al*⁴¹ which was performed by adding additional genes such as synaptophysin, neurotrophin, neural growth factor (NGF), α -B-crystallin (ABCRY) and tyrosine kinases to the list of distinguishing markers.⁴³

These data suggest that myofibroblasts may migrate from the portal tract into the developing septa. The other possibility, namely that HSC of the sinusoid migrate toward the portal tract⁴⁴ becomes less plausible. Further support for the hypothesis comes from the study of Lorena *et al*⁴⁵ in this issue. In a model of cholestatic liver injury they observed in the enlarged portal fields a patchy elastin deposition in areas with SMA-positive myofibroblastic cells and also in the vessel walls. Elastin was also found in cirrhotic livers within the fibrous septa but not around SMA-positive cells (which may represent activated HSC) in the parenchyma.

Table 1 Comparison of expression profiles of mesenchymal subpopulations in the liver

Marker	Portal myo-fibroblasts	Portal fibroblasts	Portal smooth muscle cells	Activated HSC
Lipid droplets ^a	inducible	inducible	?	+++
Collagen synthesis	+	+	?	+++
α -SMA	+	—	+	+
Desmin	+ or —	+	+	+
Fibulin-2	+	?	?	—
IL-6	+	?	?	—
GFAP	—	—	—	+/-
Synaptophysin	—	?	?	+
Elastin	+	+	—	—
TGF- β_1	?	?	?	+ ^c
responsiveness ^b				
NTPDase1	—	—	+	—
NTPDase2	+	+	—	—

^aPresence of lipid droplets in the cytoplasm is a very unspecific marker.

^bWhen a wide range of connective tissue genes (collagen I, III, IV, laminin, fibronectin, entactin, tenascin, undulin, proteoglycans) is studied.

^cRat.

NTPDase = ecto-nucleoside triphosphate diphosphohydrolase (CD39L1).

In spite of this recent progress, additional questions arise. First, do non-HSC populations of (myo)fibroblasts, although similar in producing extracellular matrix proteins, belong to a homogeneous population? Expression profiles of myofibroblasts vs activated HSC suggest at least three different fibroblastic subpopulations in addition to activated HSC:³¹ portal myofibroblasts; septal myofibroblasts (with identical expression profiles) and the interface myofibroblasts (myofibroblast-like cells at the septal/parenchymal interface) with an expression profile intermediate to the other two.⁴⁶ Our FACS analysis of freshly isolated rat liver myofibroblast suggests the presence of several populations of cells with different sizes (unpublished data). Second, do myofibroblasts and HSC originally derive from the same cell type and if yes, what are the circumstances leading to a different phenotype? Third, do myofibroblasts derive from fibroblasts? Uchio *et al*⁴⁷ showed that portal fibroblasts acquire CRBP-1-expression-capacity and convert into myofibroblasts. On the other hand, HSC lose their retinol stores but maintain their high expression of CRBP-1 during activation. These data suggest that activated fibroblasts and activated HSC may converge to a phenotypically and functionally similar but not identical myofibroblastic cell type of different origin.

Similar comparisons in humans are not possible. In fact, data published so far have been obtained using mesenchymal cells from liver biopsies without the possibility of identifying the site of origin. Unfortunately, the isolation and study of the HSC of the human liver have not been pursued systematically so far.⁴⁸

Connective tissue proteins and mediators

During liver damage, expression of a large group of cytokines is enhanced, among which TGF- β_1 , TNF- α , PDGF and IGF-I are thought to be of special importance for liver fibrogenesis. Transforming growth factor β_1 quickly became 'the' fibrogenic mediator *par excellence* after TGF- β_1 gene expression was found to be increased in the inflamed damaged liver. TGF- β_1 was considered to be the main fibrogenic component present in the supernatants of inflammatory macrophages isolated from damaged livers.⁴⁹ However, it was also observed that TGF- β_1 transcripts were expressed in desmin-positive perisinusoidal cells described as HSC and myofibroblasts, as well as in portal fibroblasts, during the fibrotic process induced by CCl₄ administration or bile duct ligation.⁵⁰ As the procollagen $\alpha 1(I)$, $\alpha 1(III)$ and $\alpha 1(IV)$ genes also were increased and detectable in the same cell types it was suggested that TGF- β has an important role in the fibrogenesis as by a paracrine effect on mesenchymal cells.⁵¹

In vitro studies on rat liver HSC demonstrated that TGF- β_1 stimulated expression of collagen types I and III as well as other matrix proteins.^{52,53} Together with data suggesting that TGF- β_1 potentiates the effect of mitogens like PDGF on HSC,⁵⁴ TGF- β_1 was referenced as the main fibrogenic mediator according to its effects on the presumed main matrix producer in liver fibrogenesis.⁵⁵ This central role of TGF- β_1 has been increasingly challenged as its capacity of upregulating connective tissue genes decreases considerably when applied to myofibroblasts ('transdifferentiated HSC').⁵⁶ Again, the very probable involvement of the portal (myo)fibroblasts, the periductal fibroblasts and eventually the smooth muscle cells of the vessel walls and the role of several other mediators will need to be the topic of future research.

Of additional importance in the study of liver fibrogenesis are the cell-matrix interactions. The extracellular matrix (ECM) is regarded as a complex architectural network, with precisely organized molecules that determine the structure of tissues. The ECM provides the framework for cellular regulation of polarization, migration, proliferation, differentiation, cell survival and even cell death. Signalling from the ECM occurs via different ways. ECM components can transmit signals via transmembrane receptors, such as through the integrins or transmembrane proteoglycans of HSC and myofibroblasts.⁵⁷ The ECM also serves as a binding reservoir for several key cytokines such as TGF- β , TNF- α , PDGF or IL-6. The ECM therefore plays an important role in the control of matrix synthesis as well as its degradation by protecting these fibrogenic factors from proteolysis and by modulating their bioactivity and availability as a function of matrix binding capacity.^{58,59}

A further example of the dynamic ECM is fibrillin-1 monomers, which polymerize into ordered aggregates

to form the microfibrils. The microfibrils themselves are filamentous structures found in the extracellular space of most organs and are responsible for the biomechanical properties of the tissues. Microfibrils without associated elastin are found in the ECM of nonelastic tissues. Fibrillin and elastin form elastic fibers. In the normal human liver, fibrillin-1 and elastin are found in the portal tracts (vessel walls and connective tissue) as well as in the wall of centrilobular veins. In the perisinusoidal areas and at the interface of the portal tract to the hepatocytes, however, only fibrillin-1 is detectable.² In normal rat liver, fibrillin-1 is not found in the sinusoids. During cholestasis, fibrillin-1 expression increases drastically in the enlarged portal areas. In the case of toxic liver injury (CCl₄-model) fibrillin-1 colocalizes to centrilobular SMA-positive areas. In cirrhotic livers, fibrillin-1 is found extensively in SMA-positive regions, but elastin is found only within the septa.⁴⁶ Notably, fibrillin-1-positive activated HSC of the liver parenchyma are elastin negative.⁴⁵

Although *in vitro* data on elastin expression (which could become a true marker for activated myofibroblasts) are missing in this issue's article by Lorena *et al*⁴⁵ and the authors used a so-called HSC line (not well delimited from myofibroblasts by markers mentioned above), the *in vivo* data of the paper support the following concept: activated HSC acquire SMA expression and deposit fibrillin-1-positive but elastin-negative ECM, whereas non-HSC myofibroblasts deposit ECM that contains both fibrillin-1 and elastin. This then permits proposal of the following pathophysiological mechanism of portal fibrogenesis: Liver damage first induces myofibroblasts and then fibroblasts of the portal tracts to activate and deposit matrix. The resultant changed environment enables 'proliferation' and 'migration' of activated mesenchymal cells into the parenchyma along the sinusoids as the beginning of septum formation. Here the disturbed ecosystem is followed by activation of HSC, themselves contributing to the enhanced deposition of extracellular matrix at the interface between the border of the septum and the parenchyma. Owing to the inability of activated HSC to produce elastin, only nonelastic fibers are formed in sinusoids, potentially serving as defense line against a further expansion of the forming septa (Figure 2).

Concluding remarks

From studies on animal as well as on human material, we have learned that the portal tract mesenchymal cells may be of crucial importance in development of liver fibrosis and/or cirrhosis. The mesenchymal cells of the hepatic sinusoid HSC have been studied mostly in the rat system and are almost the only fibrogenic cell studied *in vitro*. If we consider their capacity to synthesize connective

Figure 2 Hypothesis of septa development in case of portal fibrogenesis. Whereas viral hepatitis leads to formation of porto-central septa featuring activation of HSC, cholestatic liver diseases lead to portal–portal septa laid down by portal-tract-derived mesenchymal cells. The pink fields represent areas of enhanced matrix production.

tissue proteins and to react to TGF- β_1 treatment, they are indeed ideal candidates for ‘the’ fibrogenic cells. On the other hand, when we consider their life cycle they resemble more terminally differentiated cells. It is probable that the activated HSCs play a major role in the perihepatocellular fibrosis as observed in the case of alcoholic fibrosis or of hemochromatosis. However, thorough consideration must now be given to the role of non-HSC mesenchymal cells to hepatic fibrogenesis—both in the rodent and in the human.

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