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TGF-beta signaling in alcohol induced hepatic injury

Christoph Meyer, Nadja M Meindl-Beinker, Steven Dooley

Molecular Hepatology, Alcohol dependent Diseases, II. Medical Clinic, Faculty of Medicine Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

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1. ABSTRACT

Chronic alcohol consumption is a risk factor for the development of chronic liver disease. Ethanol exerts its detrimental effects by various means: Directly via toxic metabolites, and indirectly by affecting the gut barrier leading to elevated levels of endotoxins in the blood challenging the liver. These factors, together with the resulting inflammatory and profibrogenic cytokine production, drive the organ's response, characterized by activation of hepatic stellate cells. Recent evidence argues for other cell types besides hepatic stellate cells, including hepatocytes, as additional sources of fibroblasts producing extracellular matrix and to be responsible for scar formation. Besides mediating hepatocyte apoptosis, TGF-beta additionally induces fibroblastoid transdifferentiation. This process is accompanied with loss of epithelial marker proteins and upregulation of fibrosis related proteins. These findings challenge the current view of the passive role of hepatocytes in liver fibrosis. In line, hepatocyte-specific inhibition of the TGF-beta pathway prevents CCl_4 induced liver injury. Hence, this review focuses on the interplay of TGF-beta and alcohol in chronic liver disease with special emphasis on the potential contribution of hepatocytes.

2. INTRODUCTION

Chronic liver diseases belong to the most common causes of death worldwide. The etiology is multifactorial and spans from viral hepatitis over autoimmune disorders to toxic injury, including chronic alcohol abuse (1, 2). The development of alcohol induced liver cirrhosis proceeds in stages reflecting different degrees of damage in the liver. The first alteration occurring is the fatty liver (steatosis) accompanied by changes in lipid metabolism and elevated fat droplet deposition in the liver parenchyma (3, 4). This reversible state can proceed to steatohepatitis and fibrosis when alcohol consumption is not stopped (5, 6). Both feature inflammatory processes such as infiltration of leukocytes, and cytokine production, e.g. TGF-beta, TNF-alpha and PDGF, and extracellular matrix (ECM) remodelling comprising expression of metalloproteinases, CTGF and type I collagen (1, 7, 8). Finally, advanced fibrosis can lead to liver cirrhosis bearing the risk of liver failure due to extensive scar formation, loss of liver parenchyma, nodule formation and alterations in blood flow with potential portal hypertension (2, 9, 10). This review focuses on ethanol induced liver injury with special emphasis on the

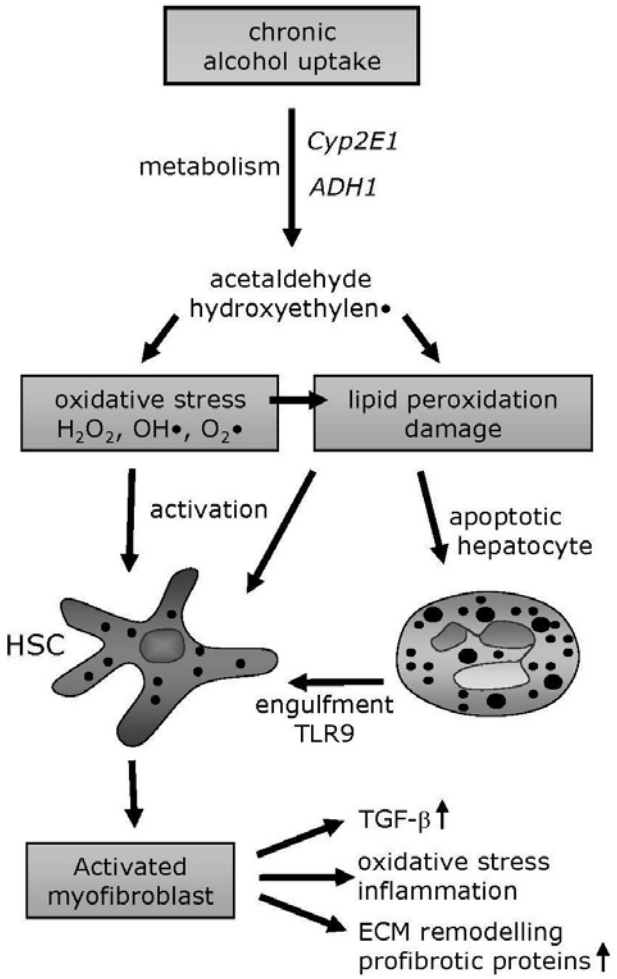

Figure 1. Effects of alcohol uptake in liver cells. Ethanol and its metabolites induce lipid peroxidation and ROS generation with subsequent damage of hepatocytes and HSC activation. Apoptotic hepatocytes are phagocytosed by hepatic stellate cells and trigger their activation (in part by TLR9 signaling), thereby contributing to inflammation and fibrosis (see text for details).

role of TGF-beta and the contribution of hepatocytes to disease.

3. ETHANOL EFFECTS IN LIVER DISEASE

Ethanol displays direct effects on liver cells (especially hepatocytes) and serves as trigger for induction of an orchestrated inflammatory response including all hepatic cell types. Alcohol uptake increases oxidative stress in the liver. Production of ethanol metabolites, particularly acetaldehyde, by alcohol dehydrogenase 1 (ADH1) and Cyp2E1 and enhanced lipid peroxidation result in generation of reactive oxygen species (ROS), namely superoxide, hydroxyl radicals and hydrogen peroxide (Figure 1) (5, 11). Chronic alcohol consumption also impairs the gut barrier function, leading to increased endotoxin levels (mainly lipopolysaccharides, LPS) in the liver (12). Endogenous ligands like heat shock protein Hsp60 as well as LPS itself can bind to Toll-like receptor 4 (TLR4) on Kupffer and hepatic stellate cells (HSCs) and trigger proinflammatory signaling cascades (see Figure 2) (12-15). Activated stellate cells display upregulated TLR4, further increasing sensitivity to endotoxins (13). The impact of TLR4 signaling in alcohol induced liver disease (ALD) has been impressively documented by Thurman and coworkers and is further supported by the association of TLR4 gene polymorphisms with liver fibrosis (16-18).

Cytokine production mediating proinflammatory and profibrogenic responses is induced by alcoholic liver injury. Among these factors, TNF-alpha, PDGF and TGF-beta play pivotal roles in liver disease and progression from initial liver inflammation to chronic and destructive stages (7, 19-21). This review focuses especially on TGF-beta, delineating the underlying mechanisms of liver fibrosis and cirrhosis for several reasons. Firstly, ethanol metabolism activates and upregulates TGF-beta, while subsequent ethanol-induced apoptosis of hepatocytes primes Kupffer cells and HSCs to upregulate TGF-beta (22-24). Secondly, active TGF-beta has been shown to be sufficient to induce fibrosis in transgenic mouse models (25, 26). Thirdly, TGF-beta synergizes with alcohol in inducing oxidative

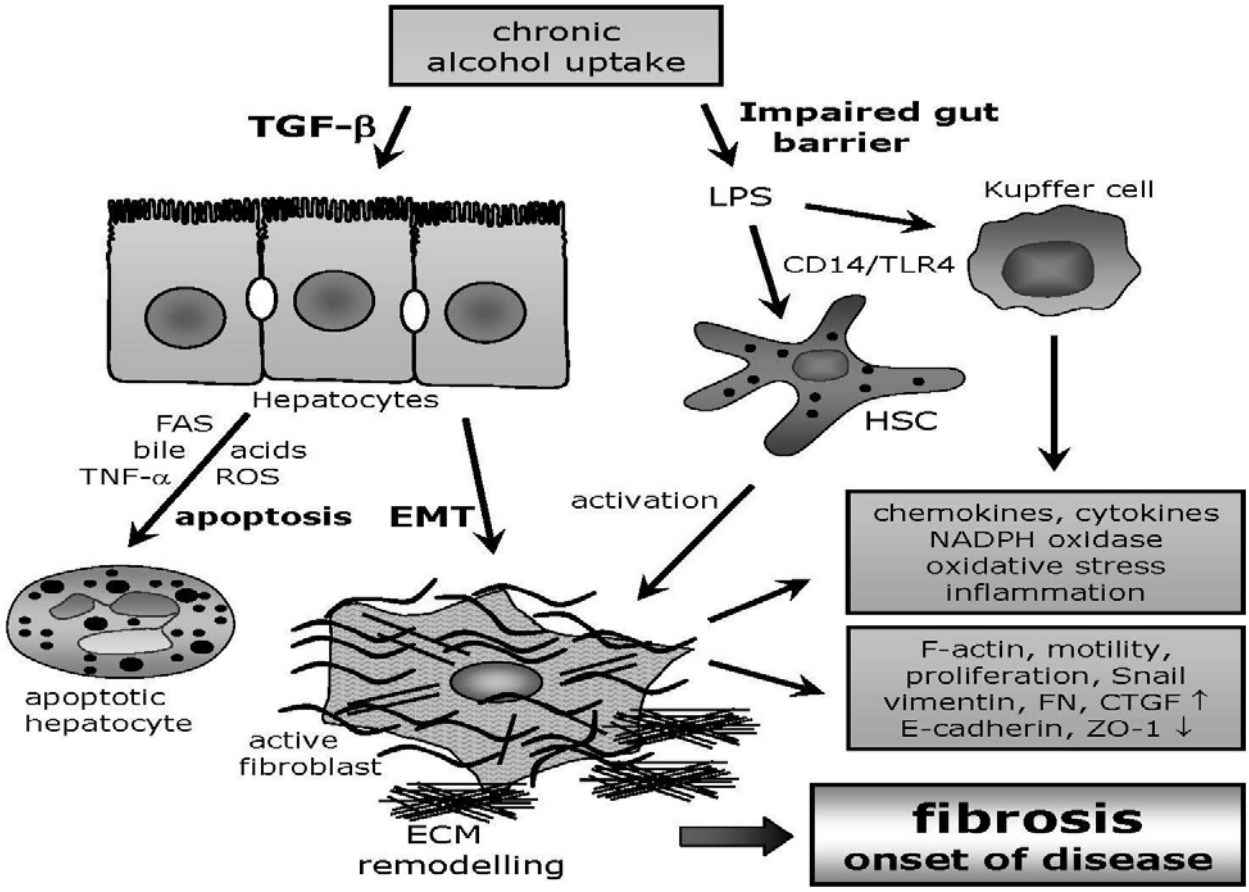

Figure 2. Mechanisms contributing to EMT and fibrosis. Alcohol challenge is accompanied with an impairment of the gut barrier function which leads to elevated endotoxin levels in the liver. LPS triggers HSC and Kupffer cell activation by TLR4 and CD14 signaling. Present TGF-beta stimulates hepatocytes to undergo apoptosis or an epithelial to mesenchymal transition (EMT). The described mechanism of hepatocyte EMT upon TGF-beta signaling actively participates in inflammatory and fibrotic processes. A considerable fraction of the active, profibrogenic fibroblasts derive from hepatocytes (see text for details). FN: fibronectin

stress thereby elevating alcohol induced damage (27). By a combination of these properties and the above mentioned role of endotoxins in liver disease, TGF-beta synergistically increases the inflammatory response to endotoxins (15).

4. THE TGF-BETA SIGNALING CASCADE

TGF-beta is a cytokine of the TGF-beta superfamily comprising more than 35 structurally related proteins, among them activins, nodals and bone morphogenetic proteins (BMPs). It forms homodimers and upon activation it regulates numerous cellular responses like proliferation and apoptosis, differentiation and migration as well as innate immunity (28-30). Binding of TGF-beta to the receptor complex of TbetaRII and ALK5 induces signaling via Smad proteins. The serine/threonine kinase receptor ALK5 phosphorylates Smad2 and Smad3 and these R-Smads then heteromerize with Smad4. The resulting Smad complexes translocate to the nucleus to regulate gene transcription in cooperation with other transcription factors (31). The relevance of R-Smad signaling has been intensively studied in liver cells and is well established, but besides the canonical Smad signaling, TGF-beta can transduce signaling via other pathways (30, 32-34). TGF-beta is playing a prominent role during development and tissue homeostasis, and thus, its signaling has to be tightly controlled. The complexity of TGF-beta induced signaling mirrors in cofactors' binding to and associating with signaling components as well as in cross talks with other signaling pathways. An updated picture of the TGF-beta pathway interactome was recently given by Taylor and Wrana (35). With regard to hepatic fibrosis, p38 MAPK was shown to considerably contribute to extracellular matrix production by constitutive phosphorylation of Smad3 in hepatic stellate cells (36).

Smad signaling is regulated by several adaptor proteins, like the Smad anchor for receptor activation (SARA) or promyelocytic leukaemia (PML) tumor supressor, both promoting the pathway (37, 38). Furthermore, the TGF-beta signaling cascade induces several factors modulating signaling (positive and negative feedback mechanisms) (39-41). Smad7 provides a negative feedback regulation, exerting its role at different stages and locations of TGF-beta signaling (31, 42-45).

5. RESIDENT LIVER CELL TYPES INVOLVED IN WOUND REPAIR AND DISEASE PROGRESSION

While HSCs are still considered the main mediators of liver fibrogenesis, the paradigm of activated HSCs as the sole source of myofibroblasts contributing to hepatic fibrogenesis has come under intense scrutiny over the last years (46). In intact liver, HSCs are quiescent, vitamin A storing cells that undergo a differentiation into myofibroblasts upon activation. The differentiated cells are motile and contractile in contrast to quiescent HSCs. Further, this cell type is producing a variety of cytokines and factors contributing to hepatic fibrogenesis (9, 47-49). Substantial progress has been achieved in identifying and understanding the wide variety of signaling pathways and effector expression in HSC-derived myofibroblasts driving fibrosis and inflammation. Adding further complexity, other cell types have been identified that can differentiate into active, profibrogenic fibroblasts. Portal fibroblasts can substantially contribute to liver fibrosis via autocrine TGF-beta signaling (50-52). Further, infiltrating cells from the bone marrow play a role in hepatic injury (53, 54). The impact of each cell type on development of fibrosis is most likely depending on the etiology of the disease and may also represent a temporal pattern during disease progression. Recent findings introduce hepatocytes as a further source of active, profibrogenic, fibroblastoid cells. During liver fibrosis, hepatocytes have been recognized as cells contributing to inflammation via ROS production and apoptosis, but emerging evidence shows an epithelial to mesenchymal transition (EMT) process turning hepatocytes into fibroblasts during chronic liver disease (45, 55). Essentially, transitioned hepatocytes lose their susceptibility to TGF-beta (56). This change is driven by active TGF-beta signaling and the recent findings will be discussed in more detail in the following.

6. TGF-BETA EFFECTS ON HEPATOCYTES IN FIBROGENESIS

6.1. Apoptosis

In the past, hepatocyte apoptosis has been considered a major effect upon progression of hepatic diseases through loss of functional liver parenchyma. This process not only removes functional liver tissue, but further enhances liver inflammation via activation of stellate cells able to phagocytose hepatocyte debris (57, 58). The engulfed hepatocyte DNA then triggers Toll-like receptor 9 signaling to increase inflammation by upregulation of TGF-beta1 and enhancement of fibrosis via expression of type I collagen in HSCs (see Figure 1) (59). The induction of apoptotic cascades in hepatocytes can be triggered by TNF-alpha/TNF receptor, Fas/Fas ligand or TGF-beta signaling, ligands which are all present in inflamed and damaged liver (60, 61). Further proapoptotic stimuli are bile acids accumulating in parenchymal cells during disease in certain instances, thereby regulating expression of bile transporters (62-64). TGF-beta signaling induces apoptosis via canonical Smad signaling in conjunction with transcription factors of the AP-1 complex to express TNF-related apoptosis-inducing ligand (TRAIL) in hepatoma cells (61). In non-transformed hepatocytes, ROS production by NADPH oxidases, e.g. isoform NOX4, followed by loss of the mitochondrial transmembrane potential, release of cytochrome c and activation of caspases is a main mechanism of TGF-beta induced apoptosis (65-67). Nevertheless, ROS also originate from ADH1 and Cyp2E1 metabolism. The impact of NADPH oxidase produced ROS is pronounced by the report of Kono and coworkers using the Tsukamoto-French model showing that $p47^{phox}$ (a subunit of the NADPH oxidase complex) knockout mice did not develop liver inflammation and steatosis (68).

6.2. Epithelial to mesenchymal transition (EMT)

Although the proapoptotic function of TGF-beta and several underlying molecular mechanisms are of major relevance, not all hepatocytes follow this route. Partial hepatectomy as a model of liver regeneration defined TGF-beta as an important cytokine terminating replication and proliferation of hepatocytes to avoid an excess of liver mass without instructing resident hepatocytes to undergo apoptosis (69, 70). To conclude, TGF-beta induced hepatocyte apoptosis and proliferation inhibition has to be strictly regulated, as an overexpression of TGF-beta otherwise would lead to hepatic failure due to massive cell loss (71).

Noteworthy, the TGF-beta mediated anti-proliferative and proapoptotic behaviour of hepatocytes is changed under certain circumstances. Especially in cancer, the TGF-beta growth inhibitory capacity is often lost leading to facilitation of proliferation and migration making the cells more prone to infiltrate surrounding tissue and enhance the formation of metastasis (72). A recent study provided evidence for laminin-5 and TGF-beta acting together in the transition of non-invasive hepatocellular carcinoma cells to an invasive tumor type. This phenotypical change was driven by TGF-beta and sustained by increased PDGF expression (73, 74). The TGF-beta mediated epithelial to mesenchymal transition is not only documented in hepatocellular carcinoma cells, but also in adult, non-transformed hepatocytes.

A recent study from our lab demonstrated that TGF-beta induces apoptosis only in a minor fraction of cultured hepatocytes, whereas the majority of cells lose their epithelial characteristics and acquire a mesenchymal phenotype (EMT), regulated by a switch in the cells' expression pattern (45). Interestingly, we also observed that primary hepatocytes strongly differ in their response to TGF-beta depending on their differentiated state *in vitro*, where induction of EMT is associated to activation of survival pathways such as Akt and Ras-ERK1/2, which antagonize TGF-beta pro-apoptotic effects while supporting its ability to induce EMT (75). These data illustrate the diverse responses of hepatocytes to TGF-beta, probably depending on the cellular and surrounding context. In a study from Behrns and colleagues, isolated hepatocytes from CCl_4 treated animals (murine cirrhosis model) expressed epithelial markers such as vimentin and type I collagen. Further, the isolated hepatocytes had a changed signaling pattern with regard to survival pathways (76). In line with these findings are data from the same group unravelling another mechanism of hepatocytes to

avoid apoptosis through a ROS dependent mechanism, although in most cases, elevated ROS levels are detrimental for the cells (77). Additionally, activation of NF-kappaB by different means might preserve hepatocytes from apoptosis (78, 79). These observations are indicative of a complex change in gene expression and signaling profile during liver damage and onset of disease in hepatocytes bypassing cell death.

First hints for an active involvement of hepatocytes and TGF-beta in fibrosis and matrix remodelling came from a study in AML12 cells, an immortalized non-transformed murine hepatocyte cell line, demonstrating that TGF-beta strongly induces PAI-1 in a rapid and transient manner (80). PAI-1 as an acute-phase protein is a major inhibitor of tissue-type plasminogen activator and the urokinase-type plasminogen activator thereby regulating fibrinolysis via controlling activation of plasminogen (81). By inhibiting the plasminogen activating system, PAI-1 directly modulates other ECM proteins like laminin and type IV collagen (82). Next, PAI-1 affects activity of matrix metalloproteases (MMPs) and further contributes to matrix remodelling. Besides its function in hepatic fibrosis, PAI-1 has been linked to hepatic steatosis and also to hepatic inflammation (81). In ongoing liver fibrosis and cirrhosis, extracellular matrix remodelling is accompanied by scar formation and increased secretion and deposition of fibril forming collagens (like type I collagen and others). The conversion to a fibrilar collagen matrix additionally requires degradation of normal liver ECM. These orchestrated actions are regulated by expression and activation of MMPs as well as by tissue inhibitors of metalloproteinases (TIMPs) (6). Noteworthy, metabolites and cytokines like acetaldehyde and TGF-beta directly contribute to this process, in part by downregulation of MMPs and upregulation of type I collagen (6).

6.3. Loss of cell polarity in EMT

In healthy liver, hepatocytes are highly polarized and this polarity enables them to fulfil their diverse and spatially directed physiological roles, such as substance uptake from blood, metabolism of body matter including carbohydrate homeostasis, catabolism and detoxification, as well as bile secretion. This is accomplished by an impermeable layer of hepatocytes connected by cell-cell connections, mainly tight junctions.

The epithelial phenotype disappears upon TGF-beta induced cell transition; tight junctions are dissolved and several epithelial markers like zonula occludens-1 and E-cadherin are downregulated. In parallel, mesenchymal markers emerge: Among others, vimentin and type I collagen are induced and this transition is accompanied with a change in morphology towards a fibroblastoid shape (see Figure 2). Remarkably, alpha-smooth muscle actin, another mesenchymal marker for myofibroblasts (or mesenchymal cells) originating from stellate cells has not been found in transdifferentiated hepatocytes to date, arguing for the existence of different fibroblast populations in the damaged liver. Nevertheless, it was proposed that alpha-smooth muscle actin should not be considered as a marker for EMT (83, 84). Another property of cellular transition towards a mesenchymal phenotype is the gain in migratory capacity, and TGF-beta can promote migration under certain conditions.

Very importantly, the TGF-beta signaling outcome differs tremendously between polar and dedifferentiated hepatocytes. *In vitro* culture systems exist which allow analysis of polar, bile canaliculi forming hepatocytes (in a 3D soft collagen environment) and also of hepatocytes spontaneously transdifferentiating (cultured on a stiff collagen matrix). With the loss of polarity in the latter system, TGF-beta is capable to activate the pro-survival AKT pathway. Besides this, integrin mediated focal adhesion kinase and Src signaling contribute further to apoptosis resistance via the AKT pathway. Together with elevated activity of the ERK 1/2 pathway in this system, TGF-beta directs hepatocytes to undergo EMT (75). In contrast, polar hepatocytes do not show basal and TGF-beta mediated AKT signaling but instead, TGF-beta increases p38 activity which ultimately results in apoptosis (75). Current evidence for *in vivo* relevance of a hepatocyte shift in the signaling outcome emerged. A recent study demonstrated that hepatocytes originating from chronic CCl_4 treated mice are less sensitive to apoptosis than control hepatocytes from intact livers (76). Hence, damage of liver cells changes the hepatocyte response to cytokines. Although polarity of hepatocytes seems to be of importance for the decision whether to undergo EMT or in apoptosis upon TGF-beta challenge, with favouring the latter, also under polar conditions (the *in vivo* situation), a priming of hepatocytes might occur in specific damage situations or environments shifting the outcome of TGF-beta signaling towards EMT.

Features of EMT were shown by Kalluri and colleagues for hepatocytes treated with TGF-beta (55). In their very elegant study using double transgenic mice they demonstrate that a considerable fraction of FSP-1 positive (fibroblast specific protein 1) fibroblasts originates from hepatocytes in a CCl_4 induced liver fibrosis model. Snail, a critical regulator of E-cadherin expression is a TGF-beta target gene and upon induction facilitates the downregulation of the cell junction protein E-cadherin. Our recent study revealed upregulation of Snail in a TGF-beta dependent manner in hepatocytes around inflamed and fibrotic tissue, further endorsing the idea of hepatocyte transition and profibrogenic drive in liver diseases. To draft a more precise and detailed picture of TGF-beta induced profibrotic and EMT patterning in hepatocytes, gene expression profiling was accomplished of primary cells treated with TGF-beta. Using this approach, we confirmed expression of known target genes involved in growth control and apoptosis. Additionally, we identified genes related to EMT (vimentin, Snail, E-cadherin, ZO-1, beta-catenin and others), fibrosis (CTGF, collagen type I, TIMP-1 and others) and to alcohol metabolism (ADH1 and Cyps). Connective tissue growth factor (CTGF) is a strong profibrotic mitogen and until recently was accepted to be expressed by activated stellate cells in liver diseases (85). CTGF is an early target of the TGF-beta signaling cascade and displays sustained expression in hepatocytes. Increased CTGF expression has been associated with severity of

fibrotic diseases and might offer a therapeutical node for treating chronic liver fibrosis (86, 87). Therefore, it was a surprise to find this cytokine expressed in hepatocytes of hepatitis B virus infected livers as well as in CCl_4 treated mice (8). Noteworthy, expression of CTGF was mainly mediated by TGF-beta/ALK5/Smad3 signaling, although alternative pathways have been described to contribute to TGF-beta mediated CTGF expression in other cells types (88-91).

7. *IN VIVO* IMPACT OF TGF-BETA SIGNALING IN HEPATOCYTES TOWARDS FIBROSIS

BMP7 is a member of the TGF-beta superfamily and stimulates hepatocytes to proliferate and differentiate during development (92). Therefore it counteracts the effects of TGF-beta, and indeed BMP7 co-administration with TGF-beta1 prevented the EMT process *in vitro*, depicted by prevention of albumin down-regulation and FSP-1 induction. This result could be verified *in vivo* using recombinant BMP7 in CCl_4 treated animals showing a significant prevention of FSP-1 expression in hepatocytes. Liver function was less affected and fibrotic lesions were reduced by BMP7 treatment (55).

To investigate the role of TGF-beta signaling in hepatocytes in liver fibrosis directly a transgenic mouse model with an inducible, hepatocyte-specific Smad7 element was used. The induction of Smad7 abrogates TGF-beta signaling and is therefore used as an inhibitory system. In this study, hepatocyte Smad7 overexpression strongly reduced the fibrotic response upon CCl_4 induced damage. Active Smad2 signaling was found reduced in Smad7 overexpressing cells. Further, histological stainings of liver tissues showed reduced interstitial collagen deposition and alpha-smooth muscle actin expression compared to controls. Liver damage was also monitored by alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase levels in serum and verified disease reduction by ectopic Smad7 expression. Inhibition of TGF-beta signaling by overexpression of Smad7 in hepatocytes also influenced the innate immune reaction. Leukocyte infiltration as a typical response to tissue inflammation, was markedly reduced in Smad7 overexpressing mice and liver apoptosis was strongly reduced (45).

In summary, these reports highlight a more active role of hepatocytes in liver disease, both in context of hepatocyte plasticity (transdifferentiation into fibroblastoid cells by TGF-beta) and contribution to hepatic fibrosis and inflammation. Further effort will delineate the precise role of hepatocytes in alcohol induced liver injury and hence may open new possibilities of interventional strategies in liver fibrosis and cirrhosis.

8. ACKNOWLEDGEMENTS

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Send correspondence to: Steven Dooley, II. Medical Clinic, Faculty of Medicine Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany, Tel: 49-0-621-383-4983, Fax: 49-0-621-383-1467, E-mail: steven.dooley@medma.uni-heidelberg.de