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ROLES OF TGF-beta IN HEPATIC FIBROSIS

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1. ABSTRACT

TGF-beta has multiple profibrogenic but also anti-inflammatory and immunosuppressive effects. The balance of these actions is required for maintaining tissue homeostasis and an aberrant expression of TGF-beta is involved in a number of disease processes in the liver. In addition to its fibrogenic effects leading to transdifferentiation of hepatic stellate cells into myofibroblasts, TGF-beta is also an important negative regulator of proliferation and an inducer of apoptosis. The major portion of TGF-beta is secreted as part of an inactive complex and the details of the activation process in liver have not yet been elucidated. The initially striking simplicity of the core TGF-beta /Smad signaling pathways is rapidly giving way to a much more complex view of intracellular signal transduction mechanisms and recent work has demonstrated the importance of crosstalk among different signaling pathways to either specify, enhance, or inhibit TGF-beta responses. The ubiquitous pathophysiologic relevance of TGF-beta suggests its measurement in blood as a diagnostic tool. Other approaches aim at inhibition of TGF-beta 1 function or synthesis as a primary target for the development of antifibrotic strategies and recent advances in cell biology have opened several ways to approach the inhibition of TGF-beta action.

2. TRANSFORMING GROWTH FACTOR-BETA IN THE LIVER: STRUCTURE, PROCESSING AND FUNCTION

Transforming growth factor b (TGF-beta) and more than 30 related proteins have been identified as members of the TGF-beta superfamily, which (in mammals) includes three isoforms (beta1, beta2, beta3) of TGF-beta , three isotypes of activins, and nearly 20 isoforms of bone morphogenetic proteins (BMP), which are present with special subtypes in liver tissue (1). They are produced as dimeric precursors, in which the C-terminal portions form active ligands following proteolytic processing. The proform of TGF-beta , a disulfide linked dimeric polypeptide (100 kD), is cleaved intracellularly by the endopeptidase furin into a large N-terminal portion (latency-associated peptide (LAP), 75 kD) and a small C-terminal fraction (mature TGF-beta , 25 kD) (Figure 1). LAP and mature TGF-beta remain non-covalently associated and form the small latent TGF-beta complex, which is biologically inactive (2).

The three-dimensional solution structure of mature TGF-beta 1 has been determined using multinuclear magnetic resonance spectroscopy (Figure 2). Although all TGF-beta isoforms share approximately 80% homology at the level of the amino acid sequence and have an overall similar backbone as well as comparable conformations and flexibilities, the three proteins have distinct and nonoverlapping functions. This was demonstrated in gene deletion studies in the mouse model (3). Most cell types, including rat (4,5) and human hepatic stellate cells (HSC)/myofibroblasts (MFB) (6), Kupffer cells (7), and hepatocytes (5), release the large latent TGF-beta complex (> 225 kD), in which LAP-TGF-beta is linked by disulfide bonds to one of four isoforms of the latent TGF-beta binding protein (LTBP) (8). All four LTBP isoforms were identified in human liver (9,10) and in MFB (6). LTBPs, of which several splice variants exist in the liver (9,10), facilitate TGF-beta secretion, fixation of latent TGF-beta in the extracellular matrix (ECM) by transglutaminase dependent linkage of LTBP to fibronectin and other ECM proteins, and they are structural components of ECM showing about

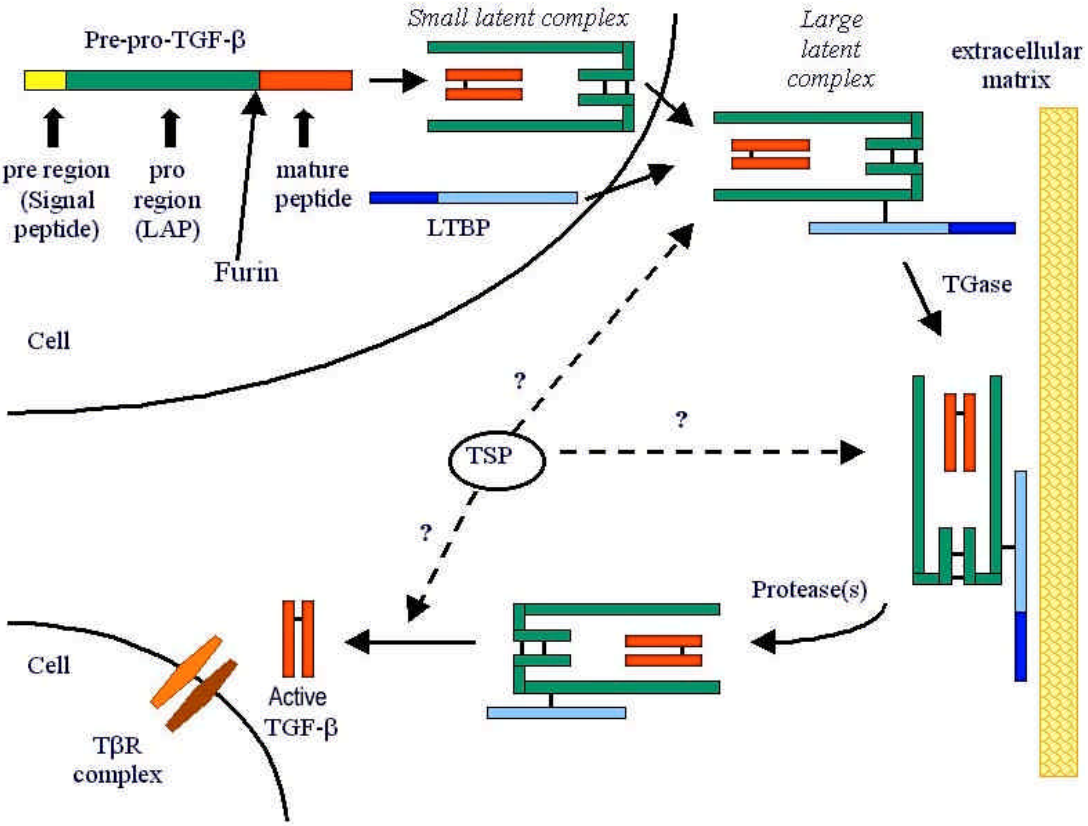

Figure 1. Schematic presentation of the extracellular processing of latent transforming growth factor-beta, (modified after (155)). TGase: Tissue transglutaminase; TSP: Thrombospondin.

30 % amino acid sequence homology to fibrillin-1 and –2 (11-13). Probably most important is the matrix fixation of the large latent complex because it forms a reservoir of latent TGF-beta , from which it is released by proteases, among which the plasminogen/plasminogen activator/plasminogen activator inhibitor system might be the most important one. It is assumed that fixation of the large latent complex in the matrix is a prerequisite for the subsequent activation of TGF-beta (2,14). A proteinase sensitive hinge region was identified as the prefered cleavage point, releasing the remnant TGF-beta complex which subsequently diffuses to the cell surface where the LAP-TGF-beta complex is bound by M6P groups of LAP to the mannose-6-phosphate (M6P)/insulin like growth factor II (IGF-II) receptor of the target cell (15). Details of the activation process of latent TGF-beta in liver have not been elucidated and, therefore, additional proteinases (e. g. metalloproteinases, calpain, mast cell chymase) but also thrombospondins (16), specific integrins (17) and reactive oxygen species (ROS) (18) might be involved in this process. The active fraction of TGF-beta can be bound to and inactivated by a_2-macroglobulin (19) and decorin (20), a small proteoglycan, whose synthesis in HSC is stimulated by TGF-beta (21). Since both proteins are expressed by HSC, this cell type produces in parallel with TGF-beta also scavenger proteins, acting possibly within feedback loops (22).

Hepatocytes of normal and even fibrotic liver contain TGF-beta , LAP and LTBP (23-25), however, they do not synthesize these components, as was shown by absence of corresponding mRNAs (26). The proposed hypothesis is that the latent TGF-beta complex is taken up by hepatocytes and released into the immediate microenvironment by membrane injury (27). Thus, the discharge of TGF-beta by necrotic hepatocytes is likely to be one of the first signals for adjacent HSC leading to their activation and consequent transdifferentiation to MFB (previously defined as the pre-inflammatory step of HSC activation) (28,29).

Activated TGF-beta stimulates the expression of many ECM proteins and downregulates their degradation by matrix metalloproteinases (MMP) through upregulation of tissue inhibitor of metalloproteinases (TIMP) in HSC/MFB (30,31). Aberrant expression of TGF-beta is involved in a number of disease processes including fibrosis and inflammation. This is demonstrated in transgenic mice, which develop multiple tissue lesions including hepatic fibrosis and hepatocyte apoptosis due to an overexpression of active TGF-beta 1 in the liver (32,33). Furthermore, liver regeneration and fibrogenesis are accompanied by an upregulated expression of TGF-beta isoforms (34) reflecting autocrine effects in experimental fibrosis, which can be inhibited by anti-TGF-β treatments like neutralizing antibodies or soluble TβRs (35,36). In addition to its fibrogenic action leading to HSC transdifferentiation into MFB (37), TGF-β is also an

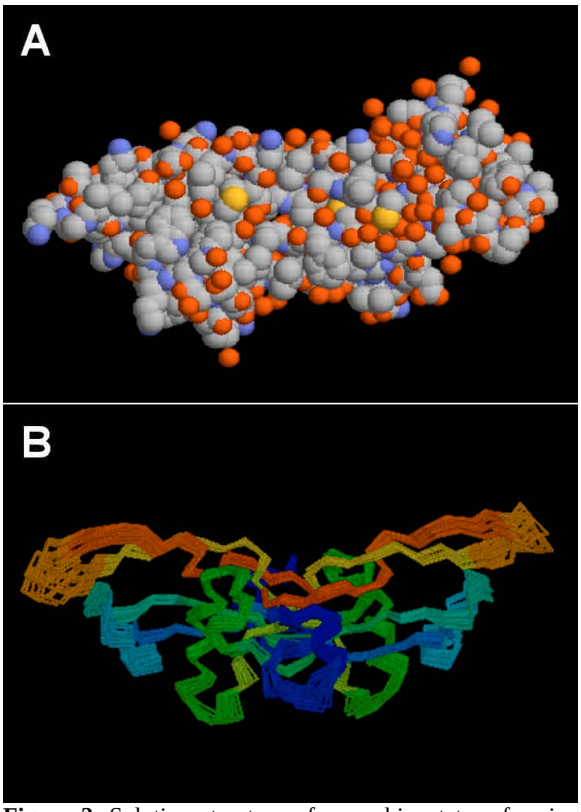

Figure 2. Solution structure of recombinant transforming growth factor-beta1. (A) Spacefill model of human recombinant TGF-β1 derived from the solution structure of TGF-β1 solved by nuclear magnetic resonance (NMR) spectroscopy. (B) Overlay of 17 independent structures of recombinant human TGF- beta1 as determined by heteronuclear NMR showing the mirror image like structure of the homodimer. All backbone heavy atoms (N, Cα, and C') are shown. The models (A, B) were generated using the coordinates deposited in the Brookhaven Protein Databank (PDB) under the accession number 1KLA and the RasWin Molecular Graphics Software (Windows Version 2.7.1.1). The structural characteristics of TGF-beta 1 in solution generally agree closely with the derived crystal structures of TGF- beta 2. For details see (156-160).

important negative regulator of proliferation of hepatocytes (38) and HSC (39) and an inducer of parenchymal cell apoptosis (40). Several experimental and clinical studies suggest that disruption of TGF-β signaling, e. g. by defective processing of the TβR-II promotes hepatocellular tumorigenesis because it leads to an escape of these cells from anti-proliferative effects of TGF- beta (41,42).

Taken together, TGF-β has multiple profibrogenic but also important anti-inflammatory and immunosuppressive effects. The balance of these actions is required for maintaining tissue homeostasis. Both, TGF-β excess and deficiency are causal for the development of fibrotic and autoimmune liver diseases, respectively.

3. TGF-BETA SIGNAL TRANSDUCTION IN LIVER CELLS

Binding of TGF-beta to TbR-II triggers heteromerization with and transphosphorylation of TbR-I. The signal is propagated through phosphorylation of receptor associated Smads (Smad2 and 3; R-Smads), which oligomerize with the common mediator Smad4 (co-Smad). Upon TbR activation, phosphorylated Smads2 and 3 and Smad4 translocate into the nucleus, where they affect transcription of target genes via direct DNA binding or by association with numerous DNA binding proteins (43) (Figure 3).

Other signaling pathways have now been shown to either potentiate or inhibit Smad mediated signals. The initially striking simplicity of the core TGF-beta /Smad signaling is rapidly giving way to a much more complex view of cellular regulation by TGF-beta . Positive regulators of TGF-beta signals include both, upstream accessory proteins and several downstream effectors that function as either general or tissue specific transcriptional regulators (44-46).

The first direct cytoplasmic Smad accessory protein to be discovered was Smad anchor for receptor activation (SARA) (47). Unphosphorylated Smad2 is recognized and directed by the SARA Smad binding domain to the membrane in close proximity to TbR complexes. TbR-I activation results in Smad2 phosphorylation, dissociation of SARA and formation of transcriptionally active Smad2-Smad4 heterodimers.

Nuclear Smad binding proteins were identified and the overwhelming majority are transcriptional regulators. In addition to Smad co-factors that positively regulate or enhance transcriptional outputs, a number of proteins have been discovered that attenuate TGF-beta signaling by interfering with Smad functions. These negatively acting Smad partners are required to prevent the inappropriate activation of TGF-beta signaling, or to turn off the pathway following normal activation. The first repressors described were a class of Smad proteins named inhibitory Smads (48-51) (Smad6, 7). Smad7 is transcriptionally induced by TGF-beta and functions as negative feedback inhibitor of TGF-beta signaling. Another way, by which Smad dependent transcriptional activity can be inhibited is *via* direct binding to either transcriptional co-repressors of TGF-beta target genes or to intermediary proteins that recruit such repressors. For example, TGF-beta dependent interaction of Smad2, 3, and 4 with Ski and Sno results in transcriptional repression of several different Smad responsive promoters (52-56). Furthermore, calmodulin, the primary mediator of calcium signaling has also been shown to interact physically with R-Smads and Co-Smads *in vitro*, and to inhibit Smad mediated transactivation of multiple TGF-beta responsive promoters.

Finally, recent work has demonstrated the importance of crosstalk among different signaling pathways

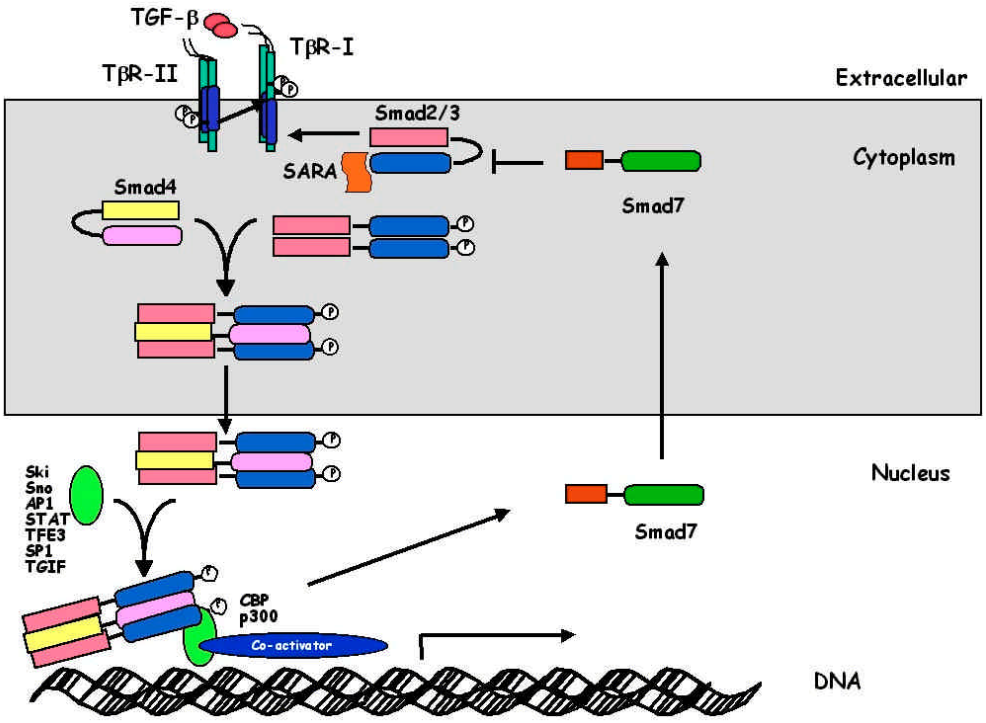

Figure 3: Simplified scheme of the transforming growth factor-b/Smad pathway. Following ligand binding, the TGF-beta type II receptor kinases phosphorylate cytoplasmic domains and thereby activate transforming growth factor type I receptor (TbR-I). The Smads then act as TbR-I activated signaling effectors, which, following receptor induced phosphorylation and interaction with Smad4, translocate into the nucleus and activate transcription of selected target genes. Specific mechanisms at nearly all levels have been identified that activate or repress TGF-beta signaling. The adaptor protein Smad anchor for receptor activation (SARA), antagonistic Smad7, several transcription activators and repressors and cofactors, which cooperate with activated Smad complexes are indicated (for reviews see (44-46)).

to either specify, enhance, or inhibit TGF-beta responses. Signaling by interferon-g (IFN-g) is mediated by cytokine receptors that activate janus kinase (Jak) tyrosine kinases and subsequently signal transducers and activators of transcription (STAT) proteins. IFN-g inhibits TGF-beta signaling by direct STAT mediated transcriptional induction of Smad7 (57). In liver, the potential of IFN-g to counteract activation of HSC has been shown (58,59). Due to the profibrogenic role of TGF-beta , a direct link to the TGF-beta pathway is obvious but has not yet been investigated in the liver. Additionally, the classical mitogen-activated protein kinase (MAPK) pathway has been implicated in both positive and negative regulation of TGF-beta signaling (58-60).

In the liver, TGF-beta potently suppresses proliferation of hepatocytes, stimulates production of ECM, and can mediate apoptosis. Liver injury by a variety of means results in a rapid induction of TGF-beta synthesis, predominantly in HSC, consistent with a ubiquitous role for TGF-beta in wound healing. Concomitant with increased TGF-beta production, HSC increase production of collagen, and it is suggested that unbalanced TGF-beta activity during wound repair could lead to damaging fibrotic responses and scaring. However, because TGF-beta suppresses the cellular immune response, it is considered a potent anti-inflammatory cytokine and may also be anti-fibrogenic under some circumstances. Therefore, in addition to altered cytokine production, changes in the multiplicity of components that control the TGF-beta signaling pathway may underly the onset of the pathological condition.

Connective tissue growth factor (CTGF) is a profibrogenic peptide induced by TGF-beta , that stimulates the synthesis of collagen type I and fibronectin and may mediate some of the downstream effects of TGF-beta . It is upregulated during activation of HSC, suggesting that its expression is another determinant of a fibrogenic response to TGF-beta (61-64). However, a direct regulatory role of TGF-beta for CTGF expression in HSC, as it was reported, e. g., for cardiac fibroblasts and cardiac myocytes, was not found (65,66).

Furthermore, involvement of ROS and lipid peroxidation products can be clearly demonstrated in fundamental events of hepatic fibrogenesis, like activation of HSC and expression of MMP and TIMP (67). The important outcome of such findings in regard to pathogenesis of liver fibrosis derives from the observation of a consistent and marked oxidative stress condition in many if not all chronic disease processes affecting hepatic tissue. Hence, reactive oxygen species (ROS) are likely to contribute to both onset and progression of fibrosis as

induced by alcohol, viruses, iron, or copper overload, cholestasis, and hepatic blood congestion. Expression and synthesis of TGF-beta have been reported to be modulated through redox sensitive reactions but molecular details of this connection have not yet been determined.

TGF-beta dependent inhibition of hepatocyte proliferation is in part mediated by inhibition of extracellular regulated kinase (ERK2) and p70 S6 kinase activity (68). In primary cultured rat hepatocytes, TGF-beta 1 is capable of decreasing the level of cyclin A mRNA in a dose dependent manner, while it has little effect on the level of cyclin D1 mRNA. p21 mRNA expression was greatly induced by TGF-β1, while p27 mRNA expression was not affected (69).

The specificity of TGF-beta results from various distinct signaling events, involving many different regulatory components. This enables TGF-beta to be a "plasticity" factor. It will be necessary to investigate molecular details of TGF-beta signal transduction from cell membrane to the nucleus in the various cell types of the liver, to determine the TGF-beta target genes leading to, e. g., TGF-beta dependent fibrogenesis, HSC activation, or inhibition of hepatocyte proliferation and induction of apoptosis.

4. HEPATIC STELLATE CELL ACTIVATION

HSC comprise about 5 % of the total number of resident liver cells. In normal liver, they are the major storage site for retinoids. Following liver injury of any etiology, HSC undergo a response known as activation, which is the transition of quiescent cells into proliferative, fibrogenic, and contractile MFB (70-76). In the liver, TGF-beta is a very potent profibrogenic mediator of cellular responses leading to tissue repair, ECM production, growth regulation, and apoptosis (77). During fibrogenesis, tissue and blood levels of active TGF-beta are elevated and overexpression of TGF-beta 1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited with neutralizing antibodies or soluble TbR-II (see below). These findings along with the potency of TGF-beta to upregulate ECM expression and the presence of functional TbR on the surface of HSC, has led to a widely accepted model, in which persistent autocrine stimulation of activated HSC/MFB by TGF-beta is a key mechanism in liver fibrogenesis (78). Based on the identification of downstream events of TGF-beta signal transduction during the past few years, molecular mechanisms underlying the profibrogenic effects of TGF-beta signal transduction are subject of intense investigations. Many of these studies were performed with primary cultured HSC, which were spontaneously activated by contact to the plastic surface of the culture well. In this *in vitro* model of fibrogenesis, HSC are strongly responsive to TGF-beta dependent Smad phosphorylation during initial stages of activation, whereas fully transdifferentiated MFB are insensitive to treatment with TGF-beta 1 (39). Thereby, HSC transduce TGF-beta 1 dependent signals, which result in growth inhibition of the cells and transcription of TGF-beta target genes. MFB instead are neither growth inhibited nor do they display activation of TGF-beta dependent transcription. TbR-I and TbR-II, as well as Smad2 and Smad4 are expressed in similar amounts in HSC and MFB. TGF-beta dependent stimulation of Smad7 expression was found specifically in HSC and increased expression of Smad3 was detected in MFB. Furthermore, TGF-beta 1 dependent phosphorylation of Smad2/3, subsequent nuclear translocation of activated Smad complexes, and DNA binding to and activation of a strongly responsive TGF-beta response element (TRE) were found to be limited to HSC (79). Ectopic expression of a constitutively active TbR-I in MFB was able to overcome TGF-beta insensitivity and to restore the signaling pathway, leading to activation of the TRE driven reporter construct (79). This indicates, that the principal machinery, necessary to transmit TGF-beta signals is functional in MFB. Furthermore, the results point to the availability of TbR at the surface of the cells as a cause for TGF-beta insensitivity, a model, which was confirmed by the finding that ligand binding to cell surface receptors is diminished in MFB, due to reduced expression of TbR-II (80) and/or cell surface availability of expressed receptors, respectively (39).

Upregulation of collagen synthesis during activation is among the most striking molecular responses of HSC to injury. Current research displays some evidence that increased collagen type I expression in culture activated primary HSC/MFB and in permanent HSC lines, may be regulated independently from TGF-beta 1 (81,82). Therefore, at least in cell culture models of fibrogenesis, the direct target genes for the HSC activating and profibrogenic effect of TGF-beta need to be determined, using detailed molecular analyses of downstream signal transduction and the commonly accepted role of TGF-beta in regulating ECM expression in MFB needs to be thought over. Potential TGF-beta target genes could be selected members of MMP or TIMP for example. Expression of, e. g., TIMP-1 is upregulated during activation of HSC (83). Induction of TIMP-1 expression does not by itself result in liver fibrosis, but strongly promotes ongoing fibrotic development by inhibiting enzymes that possess ECM degrading activity (84). A direct link between TGF-beta signal transduction and TIMP-1 was found in dermal fibroblasts, where a combined cDNA microarray/promoter transactivation approach for the identification of direct TGF-beta target genes was used (85). In HSC a direct link between TGF-beta signal transduction and Smad dependent TIMP-1 transcription has not yet been characterized.

Several reports suggest a prominent role of Smad3 in wound repair. In a model of cutaneous wound healing, Smad3 deficient mice have a reduced number of monocytes and neutrophils and the amount of TGF-beta at the site of injury was diminished, leading to increased keratinocyte proliferation. In contrast, lack of Smad3 does not diminish efficient ECM production, resulting in increased wound healing (86). *In vitro* transdifferentiated MFB display increased Smad3 expression in comparison to HSC. Additionally, studies with wild type and Smad3 heterozygous or Smad3 homozygous knock out mice reveal that maximum expression of collagen type I in activated

HSC *in vivo* and in culture requires Smad3 (87); the data further indicate that Smad3 is required for TGF-beta dependent growth inhibition and TGF-beta 1 mediated formation of Smad containing DNA binding complexes in cultured HSC. Interestingly, there is no influence of Smad3 on HSC activation as assessed by α-smooth muscle actin (a-SMA) expression. A potential profibrogenic role of Smad3 is further confirmed by the finding that activated HSC lines display a significant amount of constitutively activated Smad3 (81,82).

Most of the data, delineating fibrogenic TGF-beta signaling in HSC were established *in vitro*, using activated primary cells or permanent HSC lines. Fibrogenic activation of HSC and the resultant MFB phenotype may differ significantly *in vivo* and therefore, the reported findings should be focussed in animal models of fibrogenesis.

5. TGF-BETA AND ACTIVIN INDUCED PARENCHYMAL CELL APOPTOSIS

TGF-beta and activin are potent inhibitors of the growth of hepatocytes *in vitro* and in regenerating liver *in vivo* where it may serve as the terminator of the replicative response to partial hepatectomy (88-90). Recently, TGF-beta was identified as the principle mediator responsible for the maintenance of constant liver mass (42) supporting the view that parenchymal TGF-beta regulates hepatocyte proliferation (34). Besides blocking cell growth in intact liver, TGF-beta also induces apoptosis in cultured hepatocytes (91-94). Massive overexpression of bioactive TGF-beta is lethal to rats that underwent hepatectomy, and histological examination revealed hepatic failure from massive apoptosis (40). The execution of TGF-beta induced apoptosis in parenchymal liver cells may be initiated by many different signals (95) and it appears that cell arrest in primary rat hepatocytes and human hepatoma cells is linked to suppression of phosphorylation of the retinoblastoma gene product pRb (96). A further mechanism of TGF-beta induced apoptosis might be the induction of pro-apoptotic genes such as p53 and bax (97). During TGF-beta induced apoptosis in cultured rat hepatocytes the activities of CPP32-like proteinase (caspase 3) and caspase 8 were shown to increase in a time dependent manner and preceed the onset of apoptosis (91,98-100). Parenchymal cell apoptosis is also associated with an increase in intracellular ROS and a lowering of the level of reduced glutathione (101,102). Furthermore, induction of cytosolic tissue transglutaminase was directly linked to TGF-beta 1 induced apoptosis in a rat hepatoma cell line (103). *In vivo* the increase of the apoptotic rate is obvious two hours after injection of TGF-beta 1, while in cultured hepatocytes it requires at least 16 hours to induce the apoptotic program. TGF-beta 1 acts synergistically on cells already primed for apoptosis and therefore the effects of this cytokine may be that of an executor rather than a primary inducer of apoptosis. Apoptosis triggered by TGF-beta 1 in cultured hepatocytes can be effectively blocked by dexamethasone, phenobarbital, bacterial lipopolysaccharide, cyproterone acetate, peroxisome proliferators (nafenopin), epidermal growth factor (EGF), nuclear transcription factor kB (NFkB), and other inhibitors of apoptosis (104-106). Apoptotic death of hepatocytes was also confirmed in a transgenic mouse model directing the hepatic expression of mature TGF-beta 1 (32). In experimentally injured liver tissue and liver hyperplasia induced by the antiandrogen cyproterone acetate, TGF-beta mRNA and protein levels increase and hepatocytes become positive for TGF-beta 1-LAP (107,108).

Activin A was also shown to inhibit DNA synthesis and to induce apoptosis in hepatocytes but this member of the TGF-beta family has much lower apoptosis inducing potential than TGF-beta . Because it is synthesized by proliferating hepatocytes it may act as an autocrine death factor (109), which maintains constant liver mass by tonically blocking cell growth in intact liver. Consequently, infusion of follistatin, a natural activin antagonist, into the portal vein induced liver cell growth and increased normal liver mass (110). *Vice versa*, administration of recombinant human activin A *via* implanted minipumps was sufficient to evoke a dose- and time dependent decline in relative liver mass (111).

Apoptosis has been recognized in various liver diseases including viral hepatitis, primary biliary cirrhosis and alcoholic liver disease. TGF-beta might be one important mediator of this phenomenon since it was demonstrated that MFB, i. e. activated HSC, secrete sufficient (latent) TGF-beta , which after activation is capable of inducing liver cell death (112). Since these cells are in close proximity to hepatocytes, apoptotic reduction of parenchymal cells might be very effective (112). In addition, autocrine TGF-beta mediated parenchymal cell suicide is conceivable since this cell type contains TGF-beta , which can be released under certain conditions (27). Because TGF-beta (like tumor necrosis factor a (TNF-a)) suppresses apoptosis in activated HSC, possibly by activation of intracellular anti-apoptotic mechanisms and reduction of CD95L-expression (113), the hypothesis is put forward that this cytokine could promote the expansion of proliferating and activated HSC into the hepatocyte depleted tissue surrounding them.

6. THE POTENTIAL OF CIRCULATING TGF-beta AS A DIAGNOSTIC TOOL

The pathophysiologic relevance of TGF-beta in inflammatory (114,115), fibrogenic (116) and malignant (117) liver disease, its increased expression in chronically injured liver tissue and reversal during interferon a-therapy (118), the possible relationship between circulating TGF-beta and immunosuppressive drug therapy in human transplant recipients (119,120), and the major role of the liver in the clearance of circulating TGF-beta (121) suggest measurement of TGF-beta in blood as a diagnostic tool. This might allow estimation of liver insufficiency, fibrogenesis, malignancy, and immunosuppressive drug treatment in liver transplant patients. Indeed, studies have reported elevated concentrations of plasma TGF-beta 1 in patients with chronic active hepatitis B and C, HBV related and alcoholic liver cirrhosis (122). The degree of elevation could be correlated with scores of the Child-Pugh classification (123) and a number of biochemical liver function tests (124) in cirrhotic patients. In addition, patients with chronic liver diseases and (much more

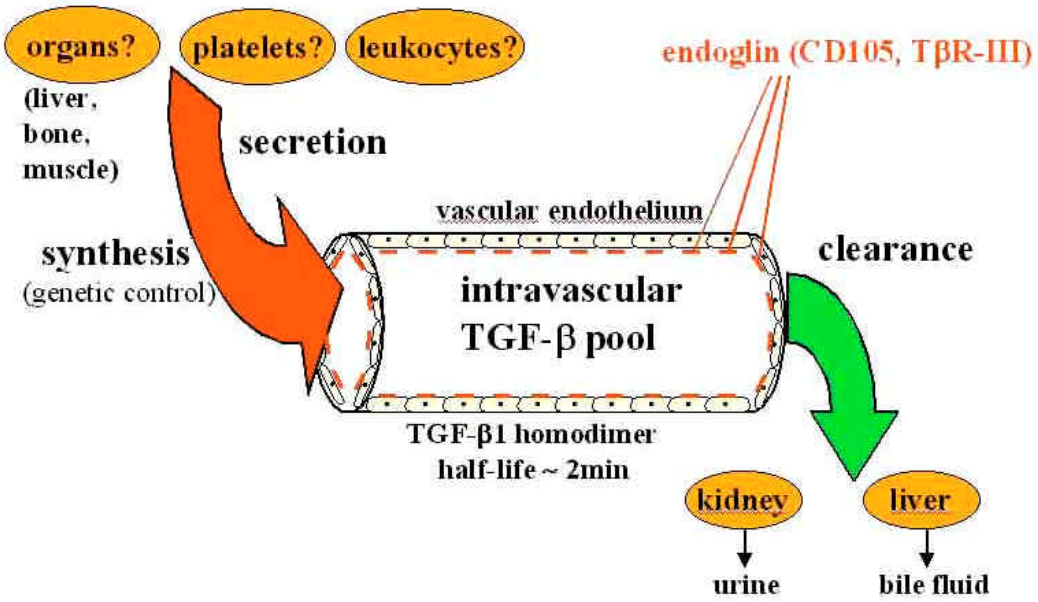

Figure 4. Determinants of the steady state level of plasma transforming growth factor-beta. Possible sources and clearance organs are indicated. Nearly the total plasma TGF-beta is present in a latent (inactive) form and integrated into a large complex containing alpha$_2$-macroglobulin. Endoglin binds TGF-beta to the surface of vascular endothelial cells.

pronounced) with hepatocellular carcinoma have elevated urinary excretion rates of TGF-beta 1 (125), which was correlated with the severity of HCV related chronic liver disease (126). This study further demonstrates a relationship between urinary TGF-beta 1 and circulating levels of the aminoterminal propeptide of procollagen type III, which is used as a marker of ongoing fibrogenesis. Similarly, a correlation between elevated levels of TGF-beta 1 and disease activity in autoimmune hepatitis was reported (127), which points to a role of this cytokine in the pathogenesis of autoimmune liver disease due to its immunoregulatory effects on NK-cells (128), i. e. pit cells in the liver. Although the majority (albeit not all) of clinical studies reveal changes in circulating TGF-beta 1 levels in chronic liver diseases and liver malignancies, due to analytical and (patho-) physiological reasons the diagnostic and prognostic value of this parameter is not yet firmly established. The amount of intravascular TGF-beta is determined by the rate of supply and clearance, respectively, and is suggested to be under genetic control (129) (Figure 4). Presently, it is not known which organs (liver, bone, muscle) and cells (platelets, leukocytes) are the major contributors, and to which degree the liver and kidney play a role in the clearance of systemic TGF-beta (130). Furthermore, a substantial fraction of this cytokine might be reversibly fixed to the TbR-III (endoglin, CD105) (131) of the vascular endothelial surface. In addition, TGF-beta is associated with circulating blood cells, which show an isoform specific expression of this cytokine. Probably the most significant analytical problem derives from the complex structure of circulating TGF-beta , which is almost completely in the latent, biologically inert form (132). Before assaying, the sample has to be activated, e. g. by transient acidification in order to dissociate the latent complex, to measure "total" TGF-beta by enzyme linked immunosorbent assay (ELISA) technique, receptor binding assay or bioassay, respectively. It is not known which supramolecular structure circulating TGF-beta might have and whether the molecular architecture changes in certain disease conditions, however, binding of the small or even large latent complex to a_2-macroglobulin (133,134), decorin (20) and other scavenger proteins is likely. Thus, all these variables have to be carefully considered in the performance and interpretation of TGF-beta measurements and are the reason for the wide range of reference values reported so far for physiological TGF-beta concentrations in human blood (0.5 to 25.0 µg/l, mean values) and for some of the discrepancies encountered measuring plasma TGF-beta in liver diseases.

Future clinical significance of TGF-beta might eventually come from the assessment of TGF-beta 1 gene polymorphisms since it was reported that the TGF-beta 1 Arg/Arg-genotype at codon 25 is associated with more severe fibrosis in hepatitis C than other genotypes (135). In a recent study the heterozygous Arg/Pro genotype of codon 25 was found to predict a significantly faster fibrotic progression of chronic hepatitis C than other genotypes, which was estimated by the METAVIR-Score (136). However, all these data need further confirmation in large scale population studies before clinical use of TGF-beta 1 genotyping can be recommended.

7. THERAPEUTIC ANTAGONISM OF TGF-BETA

Blockade of TGF-beta 1 synthesis or signaling is a primary target for the development of antifibrotic

Table 1. Therapeutic Antagonisms for TGF-betaFunction in the Liver

Factor	Reported Action	General Mechanisms	References
Binding proteins			
Alpha2-macroglobulin	Scavenger of TGF-beta	Binding of TGF-beta	145
decorin	Scavenger of TGF-beta	Binding of TGF-beta	20
Drugs			
Camostat mesilate	Suppression of plasmin activity	Serine protease inhibitor	143
Perindopril	Suppression of TGF-beta1 expression	ACE inhibitor	144
Candesartan	Suppression of TGF-beta1 expression	AT_1-R blocker	144
Antioxidants			
Glutathione	Glutathione antagonizes TGF-beta and oxidant synergism	Antioxidant	137
Alpha-tocopherol	Suppression of fibrosis	Antioxidant	139
Resveratrol	Suppression of fibrosis	Antioxidant	140
Quercetin	Suppression of fibrosis	Antioxidant	140
N-acetylcysteine	Suppression of fibrosis	Antioxidant	140
Herbal compounds			
Sho-saiko-to	Reduces experminatally induced fibrosis	Antioxidant baicalin, baicalein	141
salvia miltiorrhiza	Reduces experimentally induced fibrosis	Suppression of TGF-beta 1expression	142
Soluble receptors			
Dominant negative Tbr-II	Block of experimentally induced fibrosis	Binding of mature TGF-beta	35, 36
Soluble Tbr-II	Acceleration of chemically induced hepatocarcinogenesis	Binding of mature TGF-beta	151
	block of experimentally induced fibrosis	Binding of mature TGF-beta	149
TGF-beta synthesis blocker			
Hepatocyte growth factor	Suppression of TGF-beta 1 synthesis	Block of TGF-beta 1expression	146
Antisense mrna	Suppression of TGF-beta 1 synthesis	Block of TGF-beta 1expression	149

approaches and modern hepatology has facilitated the design of drugs removing this causative agent. Although a definitive antagonistic therapy for TGF-beta 1 in the treatment of liver fibrosis has not been developed yet, recent advances in cell biology have opened several ways to approach the inhibition of TGF-beta action. These include administration of antioxidants, specific drugs, herbal compounds and neutralizing antibodies, the expression of TGF-beta binding proteins like dominant negative and soluble receptors and decorin, application of antagonistic cytokines or suppressors of apoptosis, and blockade of synthesis by antisense oligonucleotide based strategies (Table 1).

The rational for the use of antioxidants is the finding that oxidative stress is associated with increased collagen production, which overlaps in this regard with the biological effects of TGF-beta 1. In respect to liver fibrosis, De Bleser and coworkers demonstrated that treatment with TGF-beta increased the production of H_2O_2 in activated HSC and *vice versa* H_2O_2 induced the production of TGF-beta in these cells (137). As one consequence, TGF-beta mediated accumulation of H_2O_2 was shown to result in activation and binding of a C/EBPb containing transcriptional complex to the a1(I) collagen gene promoter (138). Thus, antioxidants may have therapeutic impact in chronic liver injury by interfering with oxidative signal cascades, in which TGF-beta plays a key role. In line with these findings it is evident that the use of antioxidants such as a-tocopherol (vitamin E), resveratrol, quercetin, and N-acetylcysteine provides a means to suppress fibrogenesis (139,140). Also the antifibrotic mechanism of diverse herbal compounds (e.g. Sho-saiko-to) may be based on their antioxidative activity, involving baicalin and baicalein as active components (141). Other herbal medicines like *salvia miltiorrhiza* (Dan-shen) were shown to reduce experimentally induced hepatic fibrosis in animal models and to suppress expression of TGF-beta 1 (142). Another promising approach is to inhibit proteolytic release and activation of latent TGF-beta . The serine protease inhibitor camostat mesilate (FOY 305, CMM) is able to suppress HSC activation and prevents hepatic fibrosis at least in part by inhibiting the generation of biologically active TGF-beta . In porcine serum induced rat hepatic fibrosis this drug supresses the generation of TGF-beta by inhibiting hepatic plasmin activity (143). The drugs perindopril and candesartan are antagonists of the renin-angiotensin system by blocking the angiotensin converting enzyme (ACE) or the angiotensin-II type 1 receptor (AT_1-R). In recent investigations it was shown that both drugs suppress expression of TGF-beta 1 and induce cell proliferation in activated HSC and may therefore provide an effective new strategy for treatment of patients with chronic liver disease and fibrosis (144). Other possibilities to functionally block TGF-beta have been studied, including neutralizing antibodies or TGF-beta sequestering proteins such as a2-macroglobulin or LAP. Both proteins bind TGF-beta and are able to reduce the paracrine and autocrine stimulation of HSC in culture (7,27,145). In experimental glomerulosclerosis the small proteoglycan decorin was able to antagonize the action of TGF-beta (20), which was proposed to be useful as a tool for antifibrotic therapies. Transduction of the hepatocyte growth factor (HGF) gene also suppresses the increase of TGF-beta 1, inhibits fibrogenesis and hepatocyte apoptosis, and generates a complete resolution of fibrosis in the cirrhotic liver, thereby

improving the survival rate of rats with this severe illness (146). A deletion variant of HGF was previously shown to effectively downregulate mRNA expression of procollagens and TGF-beta 1 and to inhibit HSC activation *in vivo* (147). Furthermore, it is well established that the administration of IFN-a in patients with chronic hepatitis also results in sustained clinical responses with normalization of hepatic TGF-beta 1 mRNA expression levels, which did not differ from the expression in untreated normal control patients (118).

Another way to interfere with TGF-beta signaling is the direct blockade of TGF-beta 1 synthesis. Constitutive expression of an antisense mRNA *in vitro* was shown to lower the overall concentration of TGF-beta 1, to increase the rate of HSC proliferation and to induce differential gene expression in MFB (148). Presently, potential gene therapies using dominant negative or soluble TGF-beta receptor type II (TbR-II) are under close investigation. Because TbR-II is the primary binding receptor for TGF-beta , overexpression of an inactive TbR-II construct counters TGF-beta actions. The development of hepatic fibrosis by dimethylnitrosamine (DMN) in rats was markedly reduced by adenoviral vectors expressing either a truncated human TbR-II injected *via* the portal vein (35) or soluble human TGF-beta receptors (a chimeric protein between an entire ectodomain of human TbR-II and the Fc portion of human immunoglobulin G) injected intramuscularly (149). Impressively, in these experiments a single injection of adenovirus expressing the truncated receptor, given prior to DMN administration, appeared to prevent both hepatic injury and the development of hepatic fibrosis. In a subsequent study, the same adenoviral vector was administered to animals with on-going fibrosis after 3 weeks of DMN in order to determine whether reversal of fibrosis occurs with this agent. The results were similar with lack of progression and possibly some regression of hepatic fibrosis in rats that received the dominant negative receptor (150). The antifibrogenic potential of soluble TbR-II was also demonstrated in the rat bile duct ligation model by slow infusion of the chimeric proteins into the femoral vein (36). In these approaches the intact extracellular domain and the residual intracellular portion of the receptor allow binding of ligand and recruitment of the TbR-I, but phosphorylation of the TbR-I receptor does not occur, and signal propagation is blocked. The truncated (kinase-deleted) TbR-II is termed a dominant negative inhibitor in that it exerts its effect by competing with the wild type receptor for recruitment of the TbR-I. Furthermore, in transgenic mice overexpression of a dominant negative TbR-II was sufficient to accelerate chemically induced hepatocarcinogenesis (151). In addition to the involvement of TGF-beta in hepatic fibrogenesis, these results give *in vivo* evidence for a tumor suppressor activity of the endogenous TGF-beta system in the liver during chemically induced hepatocarcinogenesis. Another option to inhibit TGF-beta function is to interfere with postreceptor signaling. Overexpression of Smad7, a natural antagonist of TGF-beta signaling, prevents bleomycin induced pulmonary fibrosis in mice (152) but there are presently no reports available describing the administration of this mediator in liver. Although constitutive overexpression of Smad7 can be the cause of inflammatory diseases (153), controlled gene delivery of Smad7 could have potential therapeutic implication for liver diseases in the future, especially if enhanced TGF-beta 1 production or defective TGF-beta 1/Smad signaling contributes to chronic inflammation. It is notable that there exist different transgenic models for hepatic expression of mature TGF-beta 1, providing an appropriate paradigm for testing new therapeutic interventions *in vivo* aimed at neutralizing the detrimental effects of this important cytokine (32,33,154). Although many of the discussed approaches to block TGF-beta are effective in experimental models, their efficacy and safety in human liver fibrosis remains unknown.

8. OUTLOOK AND FUTURE PERSPECTIVES

TGF-beta is pivotal in nearly all aspects of inflammation, immune surveillance and neoplasia and, thus, has a most relevant pathophysiologic impact on a wide spectrum of liver diseases. It affects not only HSC transdifferentiation, parenchymal cell apoptosis and hepatocyte proliferation but also matrix synthesis and degradation and immunogenic and tolerogenic immune responses. It is conceivable that the disturbance of the homeostasis of TGF-beta activity either in the deficient or excessive direction is pathogenetically most relevant for the development of fibrosis and for the control of normal liver cell mass, liver regeneration, growth and metastasis of hepatocellular carcinoma, and the development of autoimmune liver diseases. Thus, TGF-beta is and will be an important target for therapeutic interventions to restore the balance of the active to inactive (latent) cytokine. Our understanding of TGF-beta mediated signal transduction by way of the recently discovered Smad proteins, the identification of mutational changes of TbRs, the elucidation of mechanisms of extracellular TGF-beta activation, and knowledge of transcriptional regulation and posttranslational processing of TGF-beta has been revolutionized in the past few years. Based on this greatly augmented knowledge we will gain further insight into disease specific molecular aberrations but simultaneously enlarge our repertoire to antagonize excess production of TGF-beta with drugs and gene therapeutic devices or to restore lost TGF-beta sensitivity of target cells. Although very promising experimental data have been collected with TGF-beta neutralizing antifibrotic trials, these regimens have to consider the important positive aspects of this cytokine as an anti-inflammatory, immune- and tumor suppressive, and wound healing agent. Thus, a general inhibition of this factor, even if it is limited to the liver, is likely to have severe adverse effects during long term treatment. Therefore, more differentiated forms of interventions have to be developed, e. g. by considering possible (not yet known) specialized functions of the TGF-beta isoforms *in vivo*, by selecting specific Smads (e. g. Smad2 *versus* Smad3) as therapeutic targets, by modulation of the hitherto not well understood function of betaglycan, the TbR-III, and by selective interference with extracellular activation mechanisms using LTBP competitive synthetic peptides, thrombospondin competitors, inhibitors of specific receptors (IGF-II, integrins), or other drugs. Only these or similar sophisticated devices provide a firm basis of a successful, safe and effective approach to the treatment

of fibrogenic and non-fibrogenic liver diseases using TGF-beta as a therapeutic modality.

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Abbreviations: BMP, bone morphogenetic protein(s), CTGF, connective tissue growth factor, DMN, dimethylnitrosamine, ECM, extracellular matrix, EGF, epidermal growth factor, ELISA, enzyme linked immunosorbent assay, ERK, extracellular regulated kinase, HGF, hepatocyte growth factor, HSC, hepatic stellate cell(s), IFN-g, interferon-g, IGF, insulin like growth factor, Jak, janus kinase, LAP, latency-associated peptide, LTBP, latent TGF-beta binding protein, MAPK, mitogen activated protein kinase, MFB, myofibroblast(s), MMP, matrix metalloproteinase(s), M6P, mannose-6-phosphate, NFkappaB, nuclear transcription factor kappa B, ROS, reactive oxygen species, SARA, Smad anchor for receptor activation, STAT, signal transducers and activators of transcription, a-SMA, a-smooth muscle actin, TGF-beta , tranforming growth factor beta, TIMP, tissue inhibitor of metalloproteinase(s), TNF-alpha, tumor necrosis factor alpha, TRE, TGF-beta response element, TbR, TGF-beta receptor

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