Feature

TGFβ1 in liver fibrosis: time to change paradigms?

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1. Introduction

Chronic tissue damage often results in a deregulated wound healing, characterized by an imbalance between extracellular matrix (ECM) synthesis (fibrogenesis) and degradation (fibrolysis), that leads to scar formation. Excessive scarring finally results in architectural distortion and failure of organs such as lungs, kidneys and liver. Hepatic stellate cells (HSC) have been identified as an important cellular source of ECM in liver fibrosis [1]. These cells reside in the perisinusoidal space of Disse that separates hepatocytes from the sinusoidal endothelium. Upon injury (e.g. by toxins or chronic hepatitis) the normally quiescent HSC become activated and start to proliferate. The activated HSC undergo a phenotypic transdifferentiation to contractile myofibroblasts (MFB) that express α-smooth muscle actin and an excess of ECM molecules.

2. Relevance of TGFβ1 in fibrosis

Cytokines of the transforming growth factor (TGF) family influence a wide spectrum of cellular processes including differentiation, proliferation, apoptosis and migration. In most cells TGFβ1 has anti-proliferative activity and is the most potent single profibrogenic factor known. TGFβ1 participates in initiation and maintenance of fibrogenesis in many organs including the liver. Accordingly, tissue and serum levels of active TGFβ1 are elevated in fibrosis, and overexpression of TGFβ1 in transgenic mice and application of exogenous TGFβ1 can induce organ fibrosis [2,3]. Furthermore, experimental fibrosis can be inhibited by anti-TGFβ1 treatments, e.g. with neutralizing antibodies or soluble TGFβ receptors [4,5]. The observed TGFβ1 expression of activated HSC/MFB, the potency of TGFβ1 to upregulate ECM expression, and the expression of TGFβ receptors on HSC has led to a widely accepted model in which persistent auto-/paracrine stimulation of activated HSC/MFB by TGFβ1 is the key fibrogenic response in liver fibrosis. Other cellular sources for TGFβ1 in liver are hepatocytes, (sinusoidal) endothelial cells, platelets, and infiltrating mononuclear cells. Since specific inhibition of TGFβ1 seems to be a promising target for an antifibrotic therapy, the underlying molecular mechanisms of the profibrogenic effects of TGFβ1 are the focus of intense investigations.

3. TGFβ1-induced signal transduction

TGFβ1 is synthesized as a precursor (latent TGFβ1) that has to undergo specific proteolysis, e.g. by plasmin, and dissociation from the latency-associated peptide moiety for activation. Our understanding of TGFβ-dependent signal transduction has been revolutionized in the past few years (see [6] for review). Binding of active TGFβ1 to the cellular, constitutively phosphorylated TGFβ type II receptor (TGFβRII) induces formation of heterotetrameric complexes with type I receptors (TGFβRI) (Fig. 1A). Upon phosphorylation of serine and threonine residues in TGFβRI by ligand-activated TGFβRII the thereby activated serine/threonine kinase domain of TGFβRI phosphorylates the TGFβ-specific signal transducers Smad2 and Smad3. Whereas Smad2 requires presentation to TGFβRI by the adapter protein Smad anchor for receptor activation (SARA), Smad3 works SARA-independent. Phosphorylated Smad2 and Smad3 dissociate from the (receptor) complex and form heterodimeric complexes with Smad4 or heterotrimeric complexes with Smad3 and Smad4. These complexes translocate into the nucleus where they act as transcriptional modulators on TGFβ responsive gene promoters, e.g. those for plasminogen activator inhibitor 1 (PAI-1), the α2 chain of collagen I, and Smad7 [7]. Smad2 seems to be the main signaling molecule for TGFβ1-induced ECM upregulation, since fibroblasts from Smad3 knockout mice still increase their ECM molecule production upon TGFβ1 treatment. A Smad2 gene inactivation is embryonically lethal [7]. The third TGFβ receptor (TGFβRIII or betaglycan) is a proteoglycan that binds TGFβ1 with high affinity, serving as a non-signaling co-receptor for TGFβRI and TGFβRII. Expression of TGFβRIII on the cell surface seems to be downregulated during transdifferentiation of HSC to MFB [8].

4. Distinct TGFβ1 binding by HPCs and myofibroblasts?

Stimulation by TGFβ1 requires binding of the cytokine to its cognate receptors on activated HSC/MFB and subsequent signal transduction to the nucleus. Investigations of the binding properties of HSC and MFB for TGFβ1 led to divergent results. Using HSC isolated from liver subsequent to injection of the hepatotoxin CCl_4, constant cellular TGFβ1 binding within the following 72 h was observed [9]. Cells exhibited an increased secretion of the ECM protein fibronectin and its synthesis could be further stimulated by exogenous TGFβ1. HSC cultured on plastic undergo spontaneous activation and start to transdifferentiate to MFB within 3–5 days. Using 5 day old culture-activated cells Friedman et al. found elevated and saturable TGFβ1 binding compared to HSCs maintained in suspension to prevent spontaneous activation [10]. Fibronectin mRNA expression was only inducible by TGFβ1 in culture-activated HSC, whereas suspension cultured cells showed no response. However, how far HSC held in suspension can substitute for quiescent, normally adherent HSC has to be discussed.

Somewhat in contrast, Dooley et al. observed high binding

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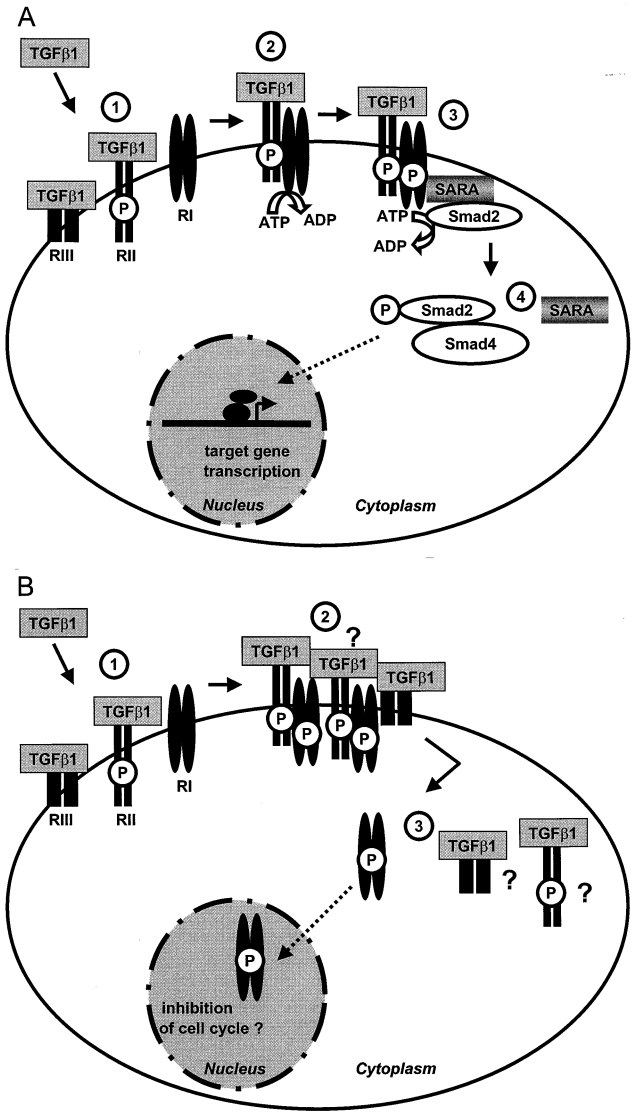

Fig. 1. Scheme of TGFβ1 signaling. A: TGFβ1 that underwent activation binds to TGFβ receptors TGFβRII (RII) and TGFβRIII (RIII, step 1). Upon ligand binding TGFβRII forms a heterotetrameric complex with TGFβRI wherein the constitutively active serine/threonine kinase of TGFβRII phosphorylates and thereby activates TGFβRI (step 2). The activated TGFβRI now phosphorylates the TGFβ-specific signal transducer Smad2, which is recruited and presented by SARA, the SARA (step 3). Smad3 can substitute for the Smad2/SARA complex (not shown). Phospho-Smad2 and SARA separate and after assembling with Smad4 the formed complex translocates into the nucleus where it cooperates with transcriptional activators or repressors to modulate transcription of TGFβ1 responsive genes (step 4). B: Hypothetical alternative signaling pathway for the growth inhibitory effect of TGFβ1. After TGFβ binding the receptors TGFβRI and TGFβRII or TGFβRI, TGFβRII and TGFβRIII form heteromeric complexes that further aggregate on the cell surface (step 1 and 2). The receptor(s) enter the cell with or without bound ligand (not known) by a mechanism distinct from normal endocytosis via clathrin-coated vesicles. TGFβRI translocates into the nucleus where it might influence cell cycle regulation.

of TGFβ1 to short-term cultured (2–3 days) 'quiescent' HSC and an almost absent binding to MFB (one passage) that had been cultured for ≥10 days [8]. Although the TGFβ1 binding decreased, the mRNA levels and protein expression of TGFβRI and TGFβRII increased during early activation of HSC and remained high in MFB. The reduced ability of activated HSC/MFB to bind TGFβ1 was paralleled by a strongly decreased response to the anti-proliferative effects and transcriptional activation of TGFβ1 for Smad7 and the ECM molecules hyaluronan and collagen α2(I). Fully transdifferentiated MFB generated by long term cultivation on plastic or isolated from fibrotic liver showed a constitutive and TGFβ1-independent high expression of collagen I, the major ECM component involved in fibrogenesis.

5. TGFβ1 signaling in HPCs and myofibroblasts

In this issue of the journal Dooley et al. present a more detailed analysis of the TGFβ1 signaling in quiescent HSC (cultured for only 2 days) and MFB [11]. In TGFβ1 responsive 2 day cultured quiescent HSC, phosphorylation and nuclear translocation of Smad2 was induced by exogenous TGFβ1, whereas this event was reduced in culture-activated HSC (7 days) and absent in MFB. TGFβ1 also induced phosphorylation of Smad3 in quiescent HSC, but not in MFB. Since Western blot analysis demonstrated comparable protein expression of Smad2 and Smad3 both in quiescent HSC and MFB, the absence of phosphorylated Smad2 and Smad3 in MFB provides further evidence for reduced TGFβ1 binding to its receptors or a postreceptor blockade in MFB. A low response to TGFβ1 was also reported for CFSC-2G cells, an activated HSC line derived from a cirrhotic rat liver displaying a high constitutive expression of ECM proteins [12]. Since in CFSC-2G cells ECM production could be further stimulated by TGFβ1, they might not be fully activated HSC. Accordingly, TGFβ1 induced phosphorylation, subsequent nuclear translocation of Smad2 and transcriptional activation of ECM promoters. However, in untreated CFSC-2G cells Smad3 was constitutively phosphorylated and already present in the nucleus.

Transfecting MFB with a recombinant adenovirus encoding a constitutively active TGFβRI Dooley et al. completely restored TGFβ1 signaling [11]. In these modified MFBs phosphorylated Smad2 and transcriptional activation of a Smad responsive promoter were clearly detectable, demonstrating that the downstream signaling is functional in MFB. Consequently, the different TGFβ1 response of quiescent HSC and MFB appears to be caused by a variant activity of their TGFβ receptors. Recent investigations using modified mink lung epithelial cells (Mv1Lu-DR26) expressing a kinase-domain mutated and functionally inactive TGFβRII indicated that growth inhibitory and transcription regulatory roles of TGFβ1 might be signaled independent of each other [13]. In these cells TGFβ1 failed to inhibit proliferation and to upregulate the cyclin-dependent kinase inhibitor $p15^{INK4B}$, while transcriptional activation of the TGFβ1 responsive promoter of a profibrogenic protein (PAI-1) was not affected. On the other hand, mutations in the TGFβRI have been defined that only block the growth inhibitory effects of TGFβ1, while transcriptional activation of PAI-1 and fibronectin remains unaffected [14]. In summary these results suggest a major role of TGFβRII in the regulation of ECM gene expression, while TGFβRI appears to mainly transduce regulatory effects of TGFβ1 on proliferation.

In exploring the fate of the TGFβ receptors after ligand binding, Zwaagstra et al. found that downregulation of cell surface TGFβ receptors was due to internalization. This downregulation is a cooperative event with participation of

all three receptors and consisting of two phases: the receptor complexes first form aggregates on the cell surface, followed by internalization by a mechanism that is distinct from normal endocytosis via clathrin-coated vesicles [15,16]. Receptor internalization was prevented when ligand binding took place at 4°C and subsequent warming to 37°C decreased cell surface bound TGFβ1. Since the authors show that this observation was not caused by internalization of TGFβ1, they hypothesized that TGFβ receptor aggregation could lead to decreased receptor affinity.

The signal transduction mechanism underlying the TGFβ1-induced growth arrest is still not completely understood. Surprisingly, in immunohistochemistry the majority of TGFβRI was found intracellularly and accumulated in the nucleus after TGFβ1 treatment [17]. Since the cell cycle (cyclin-dependent kinase) inhibitor olomoucine also induced nuclear localization of TGFβRI, it might be speculated that signaling of growth arrest is conferred by TGFβRI translocation into the nucleus (Fig. 1B). Thus ligand-induced receptor downregulation and reduced receptor affinity, apart from altered receptor stoichiometry, could be an explanation for a decreased TGFβ binding to and responsiveness of MFB compared to quiescent HSC [18].

6. Time to change paradigms?

In summary, the results presented by Dooley et al. [8,11] demonstrate a TGFβ1 insensitivity of MFB that strongly argues against the continuous auto-/paracrine stimulation of these cells by TGFβ1, as postulated by the currently accepted model of fibrogenesis. The authors provide strong evidence that the lost TGFβ1-sensitivity of MFB results from altered properties of the (signaling) TGFβ receptors. Nonetheless, TGFβ1 still plays a pivotal role in the pathogenesis of organ fibrosis, especially in liver. Although apparently not directly affecting MFB, TGFβ1 can induce activation of quiescent HSC and their subsequent transdifferentiation to MFB. Furthermore, continuous TGFβ1 secretion of MFB supports spreading of the fibrogenic reaction into previously unaffected tissue areas. A currently unresolved problem is how the TGFβ1-insensitive MFB manage to stay activated. Other profibrogenic factors, e.g. platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF-2), and endothelin-1 may preserve MFB activation. However, interpretation of the available experimental data on HSC activation requires caution, since this process was mainly studied in vitro using culture-activated cells. Activation of HSC and the resultant MFB phenotype may differ significantly in vivo and in vitro, as exemplified by divergent expression of some HSC activation markers [19,20]. In addition, a recently performed proteome analysis of quiescent HSC, in vivo activated rat HSC/MFB isolated from fibrotic liver, and in vitro activated HSC/MFB revealed non-matching protein expression changes in about 40% of identified proteins ($n = 43$) between in vivo and in vitro activated HSC [21]. Therefore, future efforts will have to focus on those events that most reliably represent the in vivo fibrogenic activation.

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