Chapter 11
The Transforming Growth Factor-Beta (TGF-β) in Liver Fibrosis

Isabel Fabregat and Patricia Sancho

Abstract Liver fibrosis is the final consequence of many chronic liver injuries that later develop in cirrhosis and hepatocellular carcinoma (HCC), which are leading causes of morbidity and mortality worldwide. The transforming growth factor-beta (TGF-β) represents a key cytokine that increases in liver in its activated form upon damage and triggers important cellular events during any progression stage of the disease. TGF-β mediates activation of hepatic stellate cells (HSCs) to myofibroblasts and induces cell death and epithelial mesenchymal transition (EMT) of hepatocytes. Both processes may facilitate extracellular matrix (ECM) deposition and scar formation. Regulatory T cells, important negative regulators of inflammation, depend on TGF-β for terminal differentiation, indicating its impact in the inflammatory response. Oxidative stress plays an essential role in mediating liver fibrosis, and recent studies demonstrate that TGF-β contributes to the reactive oxygen species (ROS) production and oxidative damage. Indeed, the active implication of TGF-β signaling in the progression of liver fibrosis makes this cytokine an attractive therapeutic target. In addition to the increasing number of compounds aimed at direct inhibition of the TGF-β pathway, the recent discovery of new downstream molecules with crucial roles in liver fibrosis development, such as NADPH oxidases, is opening the therapeutic perspectives.

Keywords Cell death • Chronic injury • Epithelial mesenchymal transition (EMT) • Hepatic stellate cell (HSC) • Hepatocyte • Inflammation • Myofibroblast • NADPH oxidases (NOX) • ROS

I. Fabregat (✉)
Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain
e-mail: ifabregat@idibell.cat

P. Sancho
Spanish National Cancer Research Center (CNIO), Madrid, Spain

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Abbreviations

ALK5	Activin receptor-like kinase 5
CLD	Chronic liver disease
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HSCs	Hepatic stellate cells
NASH	Non-alcoholic steatohepatitis
NOX	NADPH oxidase
ROS	Reactive oxygen species

11.1 Introduction

Liver fibrosis is the final consequence of many chronic liver injuries (Brenner 2009) that later develop in cirrhosis and hepatocellular carcinoma (HCC), which are leading causes of morbidity and mortality worldwide. The main etiologies of chronic liver diseases in industrialized countries include chronic hepatitis C virus (HCV) infection, alcohol, and non-alcoholic steatohepatitis (NASH). Regardless of the etiology, all the chronic liver diseases follow a common course: from middle inflammation, to more severe inflammation, to fibrosis and finally to cirrhosis. A complex and multistep process is involved in the progression to a chronic liver injury, which is evidenced by intracellular signal transduction changes, alteration in cell–cell and cell–extracellular matrix contacts and a drastic transdifferentiation of different cell types. During many years, research has been focused on the dissection of these pathways to develop new therapeutic approaches.

One of the cytokines whose levels increase in any kind of chronic liver disease (CLD) is the transforming growth factor-beta (TGF-β), which triggers important cellular events related to fibrogenesis and repair (Dooley and ten Dijke 2012; Hayashi and Sakai 2012). Most liver cells are sensitive to TGF-β, inducing both the canonical Smad-mediated and the non-canonical Smad-independent downstream signals. During the development of fibrosis, hepatic stellate cells (HSCs) respond to TGF-β moving to a myofibroblast phenotype, which in turn produces the higher deposition of extracellular matrix (ECM) proteins. TGF-β also plays essential roles during the inflammatory process linked to liver fibrosis, since it mediates the terminal differentiation of regulatory T cells, important negative regulators of inflammation. TGF-β induces cell death and epithelial mesenchymal transition of hepatocytes, and recent evidences indicate that this process might also contribute to the ECM deposition and scar formation. Activation of liver sinusoidal endothelial cells and

neoangiogenesis is also partially facilitated by TGF-β. Finally, TGF-β contributes to the reactive oxygen species (ROS) production and it is well known that oxidative stress plays an essential role in mediating liver fibrosis.

While the TGF-β role as "master" cytokine in chronic liver diseases is very clear, the complexity of the underlying response in cells and in the organ leading to the drastic changes observed is currently not fully understood. In this chapter, we will update the knowledge about the essential role of TGF-β in liver fibrosis, the proposed molecular mechanisms that mediate its actions, as well as new therapeutic approaches to inhibit its signaling.

11.2 Animal Models for the Study of Liver Fibrosis

During the last years, several mouse models of experimental fibrosis have been used for the study of the pathogenesis and molecular mechanisms associated with the diverse human pathologies leading to liver fibrosis. Among them, chemically induced fibrosis with hepatotoxic agents has been extensively investigated: thioacetamide, dimethylnitrosamine (DMN), and, most importantly, carbon tetrachloride (CCl_4), cause centrilobular parenchymal injury and fibrosis. These agents are processed by the cytochrome P-450 in hepatocytes, which releases damaging products causing massive hepatocyte cell death (Constandinou et al. 2005). In addition, concanavalin A is commonly used as a model for human chronic hepatitis since it triggers immune system-mediated fibrosis with similar histological characteristics (Louis et al. 2000). Finally, bile duct ligation constitutes a very used model for cholestatic fibrosis, triggering extrahepatic biliary atresia and primary sclerosing cholangitis (Constandinou et al. 2005). Most importantly, knockout mice have become a powerful strategy for the last years to study the molecular mechanisms of fibrosis, focusing on the contribution of one or more genes to the pathogenesis of the disease (Hayashi and Sakai 2011). These knockout mice, in addition, have helped to establish new genetic models of liver fibrosis such as the $Mdr2^{-/-}$ mouse, which develops spontaneous sclerosing cholangitis (Fickert et al. 2004).

Studies with transgenic mice that overexpress TGF-β have demonstrated that this cytokine alone is sufficient to induce fibrosis, independently of the primary cause of the disease. The hepatic expression of TGF-β induces upregulation of pro-collagen I and pro-collagen III mRNAs in the hepatic tissue, and deposition of extracellular matrix in the sinusoid (Kanzler et al. 2001). Similar results were obtained in a conditional tetracycline-regulated expression of TGF-β1 in liver of transgenic mice, where fibrosis progressed to an intermediary state (Ueberham et al. 2003). In both cases, activation of HSC was observed. Inversely, in experimental models of liver fibrosis, the fibrogenic process can be attenuated simply by blockade of TGF-β signaling (Ueno et al. 2000). These results together point out to the relevant role played by TGF-β in liver fibrogenesis.

11.3 Effects of TGF-β in Liver Cells: Relevance in Liver Fibrosis

The response to chronic liver injury involves different cell types and undergoes different phases (Dooley and ten Dijke 2012). Initially, liver injury induces epithelial cell stress, which causes cell death either through necrosis or apoptosis. Death-mediated signals and necrotic cells induce a strong inflammation and wound-healing response, as well as activation of HSC. These events may conduct to liver regeneration and repair, but acute setting addresses a fibrogenic process. Below we will discuss the role of TGF-β in these processes.

11.3.1 Role of TGF-β in the Activation of Hepatic Stellate Cells to Myofibroblasts

Regulation of extracellular matrix accumulation in acute and chronic liver injuries involves different mechanisms, but HSCs appear to be the principal effecter in all cases (Friedman 2010). The HSCs are the major storage site of retinoids in the body and are present in the space of Disse in close contact with hepatocytes and the sinusoid. When the HSC is activated, it loses its retinoid content, increases proliferation and motility, expresses new markers, such as smooth muscle actin, and produces ECM proteins. In the normal liver, sinusoidal endothelial cells and Kupffer cells (macrophages) contain relatively high levels of TGF-β mRNA, whereas HSCs express little amounts of TGF-β. However, in response to pro-fibrogenic stimuli HSCs express the three different isoforms of TGF-β and contribute to the development of fibrosis through both autocrine and paracrine loops of TGF-β-stimulated collagen production (Inagaki et al. 2005). The main cell responsible for the fibrosis is the myofibroblast, which produces the fibrous scar found in all chronic liver diseases. Different lines of evidence support the hypothesis that one of the main sources of these myofibroblasts are the quiescent HSC that become activated in response to TGF-β (Fig. 11.1): (1) downregulation of TGF-β expression in liver, by using adenoviruses or genetically modified animals, reveal a failure in HSC activation and fibrogenesis (Hellerbrand et al. 1999; Kanzler et al. 1999); (2) in vitro experiments reveal that HSCs are responsive to TGF-β treatment and transduce a signal that may play important roles in fibrogenesis (Dooley et al. 2000); (3) gene transfer of Smad7, the member of inhibitory Smads, inhibits experimental fibrogenesis, which is coincident with arrested transdifferentiation of primary cultured HSCs to myofibroblasts (Dooley et al. 2003). The response of HSCs to TGF-β, leading to, e.g., induction of α2 (I) collagen expression, is mediated by phosphorylation of Smad2 and Smad3 and subsequent nuclear translocation of a Smad-containing complex (Dooley et al. 2001). Maximal expression of collagen type I in activated HSCs requires Smad3 in vivo and in culture (Schnabl et al. 2001). Interestingly, Smad3 is not necessary for HSC activation as assessed by alpha-SMA expression, but is necessary for inhibition

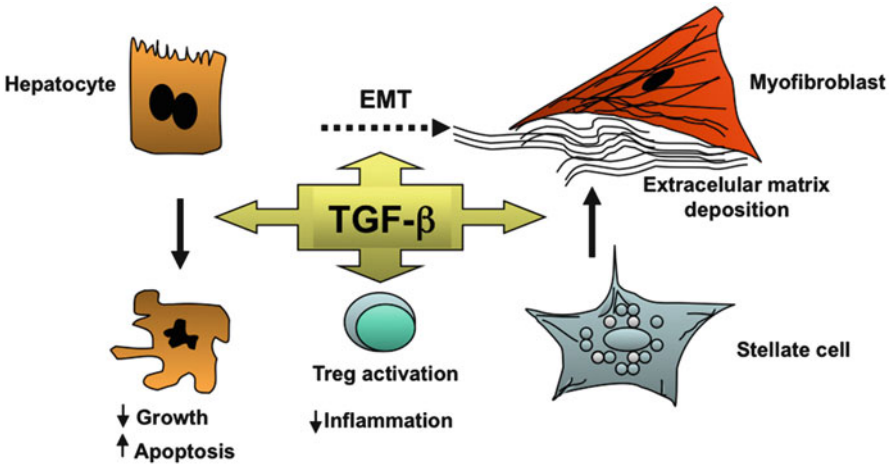

Fig. 11.1 Effects of TGF-β in liver and inflammatory cells. *Effects that may counteract liver fibrosis*: (1) TGF-β triggers activation of hepatic stellate cells to myofibroblats, which are considered the main producers of extracellular matrix proteins. (2) TGF-β induces growth inhibition and cell death of hepatocytes, which impair liver regeneration. (3) The hepatocytes that survive to the inhibitory signals may respond to TGF-β undergoing epithelial mesenchymal transition (EMT). Although controversial, different reports indicate that this process exists and would facilitate ECM deposition and scar formation. *Effects that may counteract liver fibrosis*: regulatory T cells, important negative regulators of inflammation, depend on TGF-β for terminal differentiation, which would have beneficial consequences impairing the fibrotic process

of proliferation of HSCs, which is TGF-β-dependent, and is required for TGF-β1-mediated Smad-containing DNA-binding complex formation in cultured HSCs. These data indicate that HSCs are responsive to TGF-β treatment and transduce a signal that may play an important role in liver fibrogenesis. Myofibroblasts display decreased availability of surface receptors for TGF-β, which could be based on autocrine stimulation. However, lack of activated Smad complexes with DNA-binding activity and absence of α2 (I) collagen transcription inhibition by latency-associated peptide (LAP)/anti-TGF-β antibody raise the possibility of TGF-β signaling independent receptor downregulation in myofibroblasts (Dooley et al. 2000).

11.3.2 Role of TGF-β on Hepatocytes: Relevance in Liver Fibrosis

Chronic liver injuries are characterized by persistent hepatocyte damage and death, induced by chemical toxicity, metabolic overload, viral/microbial infections, etc., which cause metabolic deregulation and oxidative stress. Several modes of cell death have been classified in the damaged liver, including apoptosis and necrosis.

It is now fully accepted that hepatocyte death is critical for hepatic fibrosis (Brenner 2009; Nikolaou et al. 2012). It appears that the primary response to injury would be liver regeneration, but if it is blocked, the default mode will be liver fibrosis. If hepatocytes undergo apoptosis without compensatory proliferation, fibrosis again would result. Indeed, it has been proven that apoptosis and phagocytosis of hepatocytes directly induce HSC activation and initiation of fibrosis (Jiang et al. 2010), and hepatocyte apoptotic bodies during chronic hepatitis C infection amplify stellate cell activation. TGF-β1 might be involved in the impairment of liver regeneration and in amplifying hepatocyte apoptosis (Fig. 11.1). Indeed, TGF-β is an important regulatory suppressor factor in hepatocytes, inhibiting proliferation (Carr et al. 1986) and inducing cell death (Oberhammer et al. 1992). The increase in TGF-β levels in the first stages of liver fibrogenesis may be responsible for an imbalance in the proliferative and survival signals in hepatocytes, contributing to the failure in liver regeneration.

However, paradoxically, in addition to its suppressor effects, TGF-β also induces anti-apoptotic signals in proliferating hepatocytes and hepatoma cells (Valdes et al. 2004; Caja et al. 2007), through activation of the epidermal growth receptor (EGFR) pathway (Murillo et al. 2005). Cells that survive to TGF-β-induced apoptotic signals undergo epithelial mesenchymal transition (EMT) (Gotzmann et al. 2002; Valdes et al. 2002; Caja et al. 2007; 2011; Kaimori et al. 2007), a physiological process during embryogenesis, in which an epithelial cell loses expression of adhesion molecules, such as E-cadherin, and other components responsible for cell polarity. Instead, they express mesenchymal components of cytoskeleton and acquire motility and scattering properties (Thiery et al. 2009). Certain evidences indicate that a crosstalk exists between the genetic programs that control TGF-β-induced growth arrest/apoptosis and those that regulate EMT. Indeed, once hepatocytes undergo EMT they become resistant to TGF-β-induced apoptosis (Valdes et al. 2002), a process in which transcription factors of the Snail family, repressors of the E-cadherin gene, are involved (Franco et al. 2010). A closely related phenotypic conversion is also detected in some models of fibrosis and may be associated with disease progression (Lopez-Novoa and Nieto 2009). In the case of the liver, the role of EMT from hepatocytes to myofibroblasts is perhaps the most intriguing and controversial of recent hypotheses on the origin mechanisms of liver fibrosis (Wells 2011). Strong evidences indicate that hepatocytes from transgenic animals overexpressing Snail (a master gene involved in EMT through its capacity to repress E-cadherin gene, among others) fully undergo EMT (Franco et al. 2010) and might propagate liver fibrosis progression (Rowe et al. 2011). However, under normal genetic background, data from different experimental approaches in animals and humans show controversy. Some reports support a role for EMT in epithelial cells in the liver that might transform into myofibroblasts (Zeisberg et al. 2007; Dooley et al. 2008), whereas others show no evidence of EMT in models of hepatic fibrosis (Taura et al. 2010; Chu et al. 2011). Further experiments are required to fully conclude that TGF-β plays a role in transdifferentiation of hepatocytes to myofibroblasts through EMT processes (Fig. 11.1).

11.4 Crosstalk Between TGF-β and Inflammatory Signals

Inflammation plays an essential role in the development of liver fibrosis. When a chronic injury takes place, a large infiltration of mononuclear cells, which include macrophages, lymphocytes, eosinophils, and plasma cells, occur. Mobilization of lymphocytes produces lymphokines that activate macrophages, which, in turn, stimulate lymphocytes, fibroblasts, and other inflammatory cells, thus setting the stage for persistence of an inflammatory response (Wynn and Barron 2010). Furthermore, macrophages produce pro-fibrotic mediators, including TGF-β1 and PDGF, and control extracellular matrix turnover by regulating the balance of various matrix metalloproteases and tissue inhibitors of metalloproteases. Examples of knockout mice that are resistant to fibrosis because they have less inflammation include those with gene deletions of TNF-α or Toll-like receptor 4 (TLR4), among others (Kitamura et al. 2002; Seki et al. 2007).

Crosstalk between TGF-β and inflammatory signals occurs at different levels. On one side, from studies in different tissues including the liver, TGF-β is believed to play an important role in the regulation of the immune system. Indeed, it activates the differentiation of regulatory T cells (Treg) (Hammerich et al. 2011), a unique subset of $CD4^+$ T-helper cells that control effector T-cell responses to prevent autoimmune reactions. Activated Treg produce the anti-inflammatory cytokine IL-10, which would have beneficial effects in a pro-fibrotic process (Fig. 11.1). However, on the other side, perturbation of TGF-β signaling by pro-inflammatory cytokines in liver cells contributes to both fibrogenesis and carcinogenesis (fibro-carcinogenesis). Smad proteins have intermediate linker regions between conserved Mad homology (MH) 1 and MH2 domains. TGF-β type I receptor and pro-inflammatory cytokine-activated kinases differentially phosphorylate Smad2 and Smad3 to create phosphoisoforms that are phosphorylated at the COOH-terminal (C), linker (L), or both (L/C) regions (Matsuzaki 2009). TGF-β and pro-inflammatory cytokines synergistically enhance collagen synthesis by activated hepatic stellate cells via pSmad2L/C and pSmad3L/C pathways. During chronic liver disease progression, pre-neoplastic hepatocytes persistently affected by TGF-β together with pro-inflammatory cytokines come to exhibit the same carcinogenic (mitogenic) pSmad3L and fibrogenic pSmad2L/C signaling as do myofibroblasts, thereby accelerating liver fibrosis while increasing risk of HCC (Matsuzaki 2009). c-Jun N-terminal kinase (JNK) activated by pro-inflammatory cytokines is mediating this perturbed hepatocytic TGF-β signaling (Yoshida et al. 2005). Under normal conditions, to avoid unlimited extracellular matrix deposition, Smad7 induced by TGF-β negatively regulates its pro-fibrogenic response. In the presence of pro-inflammatory cytokines and activation of the JNK and MAPKs pathways, Smad7 cannot be induced by the pSmad3L pathway (Yoshida and Matsuzaki 2012). Another example of modulation of TGF-β signals by pro-inflammatory cytokines comes from studies in the TLR4-chimeric mice (Seki et al. 2007). In quiescent HSCs, TLR4 activation not only upregulates chemokine secretion and induces chemotaxis of Kupffer cells, but also downregulates the TGF-β pseudoreceptor Bambi, to sensitize HSCs to TGF-β-induced signals

and allow unrestricted activation by Kupffer cells. Clinical relevance of the crosstalk between TLR4 and the TGF-β/Bambi signaling has been demonstrated in studies of liver fibrosis progression in hepatitis C and hypercholesterolemic patients (Guo et al. 2009; Teratani et al. 2012). Finally, there is evidence that Th2 cytokines cooperate with TGF-β to induce fibrosis (Wynn 2008). IL-13 activates the production of latent TGF-β in macrophages and upregulates the expression of proteins that cleave the Latent Association Protein (LAP), which contributes to the release of active TGF-β (Lee et al. 2001).

11.5 Reactive Oxygen Species in Liver Fibrosis: Connection with the TGF-β Pathway

ROS, including H_2O_2, OH·, and O_2^-, are critical intermediates in both the normal physiology and pathological conditions of liver cells. When the equilibrium between ROS generation and the antioxidant defense of the cell is disrupted, it results in an oxidative stress process (Sies and Cadenas 1985). As commented above, fibrosis has been well documented in many chronic liver diseases, usually beginning with an inflammatory phase which progresses to fibrosis after chronic oxidative stress (Diesen and Kuo 2010). ROS play a central role in the development of liver fibrosis/cirrhosis by both alcohol and hepatitis virus core proteins (Perlemuter et al. 2003; Dionisio et al. 2009). In addition, oxidative stress markers have been detected in the serum of and biopsy samples from liver cirrhosis patients and in experimental liver fibrosis/cirrhosis animals (Yadav et al. 2002; Pawlak et al. 2008). Moreover, in liver biopsies, areas of fibrosis were localized to areas with increased 4-hydroxy-2′-nonenal (4-HNE), a marker of lipid peroxidation (MacDonald et al. 2001; Seki et al. 2005).

In relation to TGF-β, ROS play a complex role promoting fibrosis progression. On one side, they constitute a commonly known downstream effector implicated in TGF-β signaling (Liu and Gaston Pravia 2010). On the other side, ROS may also promote fibrosis activating latent TGF-β through either LAP direct oxidation and subsequent release of the cytokine (Pociask et al. 2004) or via MMP activation (Wang et al. 2005). Indeed, LAP/TGF-β1 complex has been proposed to function as an oxidative stress sensor (Jobling et al. 2006). Finally, ROS can also stimulate the expression and secretion of TGF-β in a positive feedback loop in many types of cells, including hepatic stellate cells and hepatocytes (Proell et al. 2007; Boudreau et al. 2009).

11.5.1 Subcellular Sources of ROS in Liver Fibrosis

The primary cellular sources of oxidative stress during the inflammatory phase of liver fibrosis are mainly neutrophils, Kupffer cells, and, specially, hepatocytes. Although for many years the mitochondria have been considered as the major

source for ROS in the living cells, we have to consider two additional ROS-producing systems playing determinant roles in the liver pathophysiology, such as the P450 system in hepatocytes and the NADPH oxidases (NOX) proteins in different liver cells.

Mitochondria play a central role for ROS production in the liver, since hepatocytes contain hundreds of these organelles and the mitochondrial electron transport is disrupted in a great number of pathophysiological circumstances, resulting in increased electron leak (Murphy 2009). Indeed, several reports have suggested a central role for mitochondrial ROS in hepatic toxicity in models of hepatic cholestasis (Graf et al. 2002; Fang et al. 2004) and alcoholic disease (Kukielka et al. 1994; Zhu et al. 2012). Most importantly, several reports have shown that direct treatment with TGF-β induces a prolonged mitochondrial ROS production in rat hepatocytes (Albright et al. 2003; Herrera et al. 2004). This fact can be attributed to its capacity of downregulating the expression of several antioxidant enzymes, such as glutaredoxin, catalase, superoxide dismutase, and glutathione peroxidase (GPx) (Franklin et al. 2003; Herrera et al. 2004).

CYP2E1, the hepatocytic member of the cytochrome P450 oxidase system, is involved in the metabolism of xenobiotics in the body. Most drugs and hepatotoxins are detoxified by CYP2E1, which can generate ROS as a byproduct of the oxidative reaction. Both in vitro experiments and animal studies in vivo have demonstrated that CYP2E1 is an important source of ROS in alcohol-induced liver injury, and its expression is inducible by alcohol (Zhu et al. 2012). Importantly, it has been reported that TGF-β enhances hepatocyte toxicity in cells overexpressing CYP2E1 upon ethanol exposure (Zhuge and Cederbaum 2006).

Other main source of ROS implicated in TGF-β signaling and fibrosis is the NOX family of NADPH oxidases. This family has been discovered for homology to $gp91^{phox}$, the phagocytic oxidase. Nowadays, the NOX family includes seven different members NOX1 to NOX5, DUOX1, and DUOX2 (Bedard and Krause 2007) whose main function is active ROS production. NOX proteins have been previously related to fibrosis in several organs such as lung (Hecker et al. 2009), pancreas (Masamune et al. 2008), kidney (Sedeek et al. 2010), and heart (Cucoranu et al. 2005). In the liver, several reports have demonstrated a key role for NOX proteins in the progression of hepatic fibrosis (De Minicis et al. 2010; Cui et al. 2011; Paik et al. 2011; Jiang et al. 2012; Sancho et al. 2012). The isoforms expressed by the different resident populations of the liver are mainly NOX1, NOX2, and NOX4 (Paik et al. 2011).

11.5.2 Implication of ROS in the Molecular Mechanisms Mediating Liver Fibrosis

One of the most studied mechanisms of fibrogenesis actually influenced by ROS is myofibroblast activation. In the liver, stellate cell transdifferentiation into myofibroblast is inhibited by antioxidants (Foo et al. 2011; Abhilash et al. 2012). Indeed,

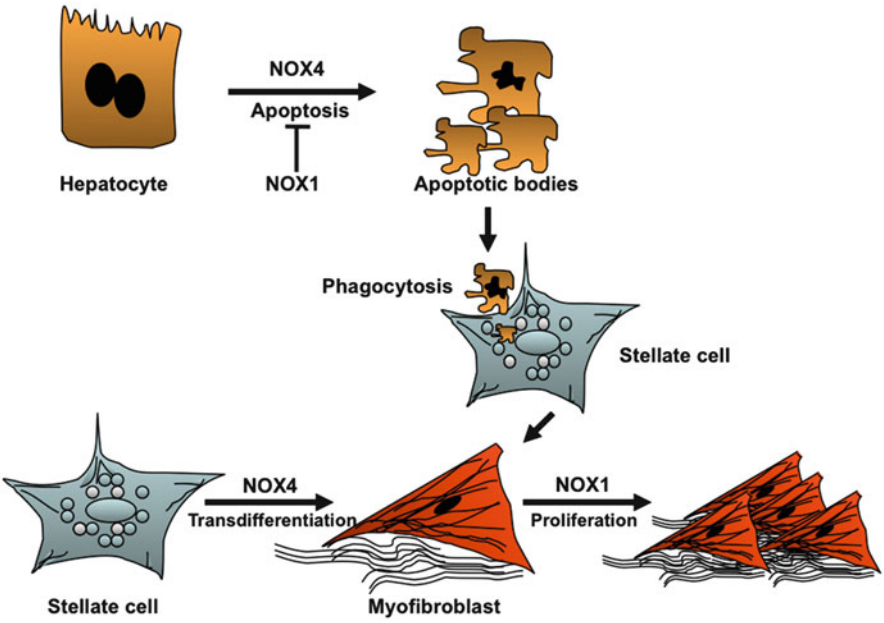

Fig. 11.2 NOX proteins play crucial different roles during liver fibrosis development. Opposite functions of NOX1 and NOX4 in hepatocytes: NOX1 protects cells from pro-apoptotic stimuli and mediates proliferation, while NOX4 promotes TGF-β-induced cell death. Afterwards, stellate cells can phagocytose the resulting apoptotic bodies, which functions as a triggering signal for activation. Primed stellate cells can also suffer transdifferentiation into myofibroblasts in response to TGF-β, a process where NOX4 plays a determinant role controlling the acquisition of the activated phenotype. Finally, and once fully activated, NOX1 favors myofibroblast proliferation, contributing to fibrosis development

NOX4 downstream TGF-β has been described as the main mediator for myofibroblast activation in different organs such as heart (Cucoranu et al. 2005), lung (Hecker et al. 2009), kidney (Bondi et al. 2010), and diseased prostatic stroma (Sampson et al. 2011). Equivalently, it has been demonstrated in cultured HSC that TGF-β-induced transdifferentiation is accompanied by NOX4-derived ROS (Proell et al. 2007), which can be a useful target for therapeutic approaches (Ikeda et al. 2011). Very recently, two different reports have described a key role for NOX4 in hepatic myofibroblasts activation downstream TGF-β (Jiang et al. 2012; Sancho et al. 2012) both in vivo and in vitro (Fig. 11.2). In these works, HSC activation was attenuated either by NOX4 downregulation or in a $Nox4^{-/-}$ genetic background, and, importantly, the myofibroblast-activated state could be also reversed by NOX4 downregulation (Sancho et al. 2012). However, the role of NOX proteins in liver fibrogenesis is not only circumscribed to NOX4. Thus, studies performed in $Nox1^{-/-}$, $Nox2^{-/-}$, or $p47phox^{-/-}$ mice have pointed out the importance of NOX1 and NOX2 in fibrosis development (De Minicis et al. 2010; Jiang et al. 2010; Cui et al. 2011; Paik et al. 2011). Concretely, NOX1 promotes myofibroblast proliferation by PTEN

inactivation to positively regulate an Akt/FOXO4/p27 signaling pathway (Cui et al. 2011). Indeed, NOX1 seems to mediate the pro-fibrogenic effects exclusively in endogenous liver cells, while NOX2 could be implicated in both endogenous liver cells and bone marrow-derived cells (Paik et al. 2011), possibly acting in the process of phagocytosis of dead hepatocytes (Jiang et al. 2010) (Fig. 11.2).

Finally, promotion of hepatocyte apoptosis constitutes also a crucial mechanism influenced by TGF-β-induced ROS. In fact, and as mentioned before, TGF-β induces apoptosis through ROS that is derived from both mitochondria and NOX activity (Herrera et al. 2004). Indeed, pretreatment with antioxidants block apoptosis (Sanchez et al. 1996; Herrera et al. 2001). Recently, it has been described that hepatocytes express different members of the NOX family, mainly NOX1, NOX2, and NOX4 (Murillo et al. 2007), which play opposite roles in the control of hepatocyte survival and death. Indeed, NOX4 is necessary to mediate apoptosis induced by TGF-β (Carmona-Cuenca et al. 2008; Caja et al. 2009), but the pro-apoptotic effect of the cytokine can be attenuated when NOX1 is active (Sancho et al. 2009; Sancho and Fabregat 2011; Ortiz et al. 2012) (Fig. 11.2). In addition, $Nox4^{-/-}$ hepatocytes are also resistant to apoptosis induction by other stimuli, such as FasL and TNF-α/actinomycin D (Jiang et al. 2012). In addition, NOX1 activity might further contribute to the inflammatory process promoting COX-2 expression and prostaglandin synthesis in hepatocytes (Sancho et al. 2011). Interestingly, dual NOX4/NOX1 pharmacological inhibition with GKT137831 is able to diminish both the apparition of fibrogenic markers and hepatocyte apoptosis in vivo upon bile duct ligation (Jiang et al. 2012), reinforcing the relevant role of NOX1 and NOX4 in liver fibrosis and opening new perspectives for its treatment.

11.6 TGF-β Pathway Inhibitors as a Promising Therapy in Liver Fibrosis

During the last years, after the role of TGF-β signaling in cancer and other pathologies, including fibrosis, became established, a great effort has been made in order to develop different approaches to inhibit TGF-β pathway. Thus, the number of possible compounds used either in preclinical or clinical studies related to fibrosis is continuously growing, thanks to previous experiences in other pathologies. The different strategies to block the TGF-β pathway can be classified as: (1) ligand traps, which include blocking antibodies and inhibitory peptides; (2) antisense oligos; (3) receptor kinase inhibitors; (4) Smad inhibitors; and (5) indirect inhibitors (Table 11.1). However, the list of compounds tested for liver fibrosis is rather reduced when compared with all the available inhibitors, since clinical efforts have concentrated for the last few years in blocking the underlying pathology specific for each type of fibrosis.

One of the most studied strategies for inhibiting the TGF-β pathway related to liver fibrosis is the ligand trapping, either by soluble receptors or inhibitory peptides. Indeed, several studies have demonstrated the antifibrotic potential of a

Table 11.1 Current preclinical and clinical TGF-β-based therapeutic strategies

Class	Drug	Target	Disease	References/Trial ID
Blocking antibodies	CAT-192	TGF-β_1	Systemic sclerosis	NCT00043706
			Myelofibrosis	NTC01291784
	CAT-152	TGF-β_2	Trabeculectomy, renal fibrosis	Hill et al. (2001), Grehn et al. (2007), Khaw et al. (2007)
	LY238770	TGF-β_1	Diabetes	NTC01113801
	GC1008	Pan-TGF-β	Systemic sclerosis	NCT01284322
			Myelofibrosis	NCT01291784
			Idiopathic pulmonary fibrosis	NCT00125385
			Glomerulosclerosis (FSGS)	NCT00464321
Peptide inhibitor	sTβRII	Pan-TGF-β	Liver fibrosis	Sullivan et al. (2010), Yao et al. (2010), Nakamuta et al. (2005), Cui et al. (2003), Yata et al. (2002), Ueno et al. (2000), George et al. (1999)
	P144	TGF-β_1	Skin fibrosis	NCT00781053
			Myocardial and liver fibrosis	Ezquerro et al. (2003), Hermida et al. (2009)
Kinase inhibitors	GW388788	TβRII/ALK5	Infarction, renal and skin fibrosis	Lagares et al. (2010), Petersen et al. (2008), Tan et al. (2010)
	SKI2162	ALK5	Peyronie's disease	Piao et al. (2010)
	GW6604	ALK5	Liver fibrosis	De Gouville et al. (2005)
	LY2109761	TβRII/ALK5	Several cancers, pulmonary fibrosis	Connolly et al. (2011), Fransvea et al. (2008), Flechsig et al. (2012), Ganapathy et al. (2010), Lacher et al. (2006), Zhang et al. (2010, 2011)
	SD208	ALK5	Scleroderma	Chen et al. (2006)
	SM16	ALK5	Vascular fibrosis	Fu et al. (2008)
	IN-1130	ALK5	Renal fibrosis, Peyronie's disease	Moon et al. (2006), Ryu et al. (2009)
Smad inhibitors	HSc025	Smad-dependent transcription	Systemic sclerosis	Hasegawa et al. (2009)
			Liver fibrosis	Higashi et al. (2011)
	SiS3	Smad3	Liver fibrosis	Matsubara et al. (2011)

(continued)

Table 11.1 (continued)

Class	Drug	Target	Disease	References/Trial ID
Indirect inhibitors	STX-100	αvβ6 integrin	Idiopathic pulmonary fibrosis	NCT01371305
	Tranilast	Not known	Rheumatoid arthritis	NCT00882024
			Lupus (LMDF)	Koike et al. (2011)
			Crohn's disease	Oshitani et al. (2007)
			Diabetes	Martin et al. (2005), Kelly et al. (2007)
			Hypertension	Hocher et al. (2002), Kagitani et al. (2004)
			Liver fibrosis	Ikeda et al. (1996), Uno et al. (2008), Said et al. (2012)

soluble type II receptor. This antagonist, consisting of a chimeric IgG containing the extracellular portion of the TGF-β type II receptor, was able to inhibit several fibrosis markers when tested in the model of bile duct ligation in mice (George et al. 1999). Importantly, it also showed to be dose-dependently effective in models of chemical liver fibrosis with carbon tetrachloride or DMN at either short- or long-term evaluation (Yata et al. 2002; Nakamuta et al. 2005). Interestingly, in this last publication authors demonstrated that remote delivery of the compound in the muscle is effective in inhibiting hepatic alterations. Recently, effectiveness of this compound has been improved using a novel strategy consisting of a fusion protein formed by the soluble portion of TβRII and IFN-γ administered intraperitoneally in a model of experimental fibrosis in rats (Yao et al. 2010). Alternatively, several studies have exploited a genetic approach using adenovirus containing the ectodomain of the TβRII alone or fused with other proteins. This approach was tested in vitro by infection of primary rat HSC, showing an inhibitory effect on the autocrine TGF-β production concomitant with transdifferentiation into myofibroblasts (Cui et al. 2003). In addition, the adenovirus strategy has shown to be effective in vivo with no apparent side effects. In this case, adenovirus expressing the entire ectodomain of the human TβRII fused to the Fc portion of human IgG (AdTbeta-ExR) was injected in the skeletal muscle in rats (Ueno et al. 2000).

Additionally, a soluble form of the type III receptor (betaglycan) has also been tested both at the preclinical and at the clinical levels for treatment of different fibrosis-related diseases, such as pulmonary, cardiac, and skin fibrosis (Liu et al. 2002; Hermida et al. 2009). Regarding the liver, a short peptide derived from this receptor, P144, showed in vitro efficacy blocking TGF-β-dependent stimulation of the human α2(I) collagen promoter. Importantly, intraperitoneal administration of P144 was able to diminish histological fibrosis markers and the number of

myofibroblats in rats treated with carbon tetrachloride, with no apparent side effects (Ezquerro et al. 2003).

Although inhibiting the kinase activity of TGF-β receptors is clearly the most recurrent strategy used in current preclinical and clinical therapies related to this cytokine, no significant studies referring to liver fibrosis have been performed. However, a great number of inhibitors of the ATP binding site of TβRI kinase (activin receptor-like kinase 5: ALK5) have been designed and preclinically tested in various fibrosis-related diseases and, may be eventually, used for liver fibrosis treatment. Up to date, only one kinase inhibitor, GW6604, has shown beneficial effects preventing liver damage in both an acute model of liver disease and a chronic model of dimethylnitrosamine (DMN)-induced liver fibrosis (de Gouville et al. 2005). In this last chronic model, where DMN was administered for 6 weeks and GW6604 dosed for the last 3 weeks, mortality was prevented, which correlated with reduced matrix deposition and decreased liver function deterioration. Another compound, IN-1130, has also potential applicability for liver fibrosis treatment. Indeed, this compound, which has been positively tested for renal fibrosis and Peyronie's disease (Moon et al. 2006; Ryu et al. 2009), is preferentially accumulated in the liver upon oral administration (Kim et al. 2008).

Alternatively, new compounds have been recently discovered which act by inhibiting Smad-dependent transcriptional inhibition. The indol-derivative SiS3 is a specific inhibitor of Smad3 phosphorylation and activity, and it was first described to be effective in inhibiting the activated phenotype of scleroderma fibroblast (Jinnin et al. 2006). Regarding liver fibrosis, only one in vitro preclinical study using a cholestatic disease model has been published so far. In this work, SiS3 treatment was able to inhibit the expression of several genes related to cholestasis development (Matsubara et al. 2011). In addition, the compound Hsc025 is a Smad-dependent transcriptional inhibitor that has been effectively tested in mice models of skin, pulmonary and hepatic fibrosis by oral administration (Hasegawa et al. 2009; Higashi et al. 2011). Indeed, in vitro treatment with HSc025 significantly suppressed collagen gene expression in cultured HSC, while oral administration of HSc025 improved liver injury and hepatic fibrosis degree in mice treated with carbon tetrachloride (Higashi et al. 2011).

Although its effects at the molecular level are not fully understood, tranilast is a drug mainly described as a collagen expression inhibitor, thus possessing antifibrotic properties. Indeed, while it can also inhibit the production of other cytokines, the major effect described for tranilast is the inhibition of both TGF-β expression and action (Miyazawa et al. 1995; Ikeda et al. 1996; Platten et al. 2001). This compound showed potential for hepatic fibrosis treatment several years ago, when it was described that tranilast treatment inhibited the expression of pro-collagen and TGF--β (Ikeda et al. 1996). Importantly, this compound also was effective in two different in vivo models of liver fibrosis. First, using a dietary model of NASH where obese diabetic and nondiabetic rats were fed with a methionine-deficient and choline-deficient diet, treatment with tranilast was effective at two different levels (Uno et al. 2008): on one side, it was able to inhibit fibrosis development and the activation of stellate cells, downregulating the expression of TGF-β, pro-collagen, and

plasminogen activator-1; on the other side, it attenuated hepatic inflammation and Kupffer cell recruitment, downregulating the expression of TNFα. In the second model, tranilast was able to improve hepatic fibrosis due to schistosomal infection, proved by a significant improvement of hepatic functions, reduction of the histopathological changes and collagen content, and decreased TGF-β1 levels in serum (Said et al. 2012).

11.7 Conclusions

Liver fibrosis is one of the main causes of mortality worldwide. Nowadays, a lot of effort is being made in order to increase the knowledge of the molecular mechanisms underlying this complicated disease, in which TGF-β seems to play a determinant role. Indeed, the active implication of TGF-β signaling in the progression of liver fibrosis, regardless of its original etiology, makes this cytokine an attractive therapeutic target for the development of new treatments. In addition to the increasing number of compounds aimed at direct inhibition of the TGF-β pathway, the recent discovery of new downstream molecules with crucial roles in liver fibrosis development, such as NADPH oxidases, is opening the therapeutic perspectives. Indeed, specific targeting of these molecules could be an important step forward in the treatment of the disease, since its inhibition may be effective enough avoiding the possible side effects of TGF-β systemic inhibition.

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