INTRODUCTION

Current evidence suggests that regulation of extracellular matrix (ECM) accumulation by fibrogenic transforming growth factor (TGF-β) and platelet-derived growth factor (PDGF) signals involves different mechanisms in acute and chronic liver injuries, even though hepatic stellate cells (HSC) are the principal effector in both cases (Friedman, 2010). HSC characterized by retinoid droplets in the cytoplasm (Figure 1), are present in the space of Disse in close contact with hepatocytes and sinusoidal endothelial cells. When HSC are activated, they lose retinoid, and show increased proliferation, motility, and ECM production. Responsiveness of ECM production to TGF-β is transient in the process of tissue repair such as liver regeneration after acute liver injury (Date et al., 1998, 2000), thus suggesting that some regulatory mechanisms for the TGF-β signal associated with the accelerated ECM accumulation is a common finding in human chronic liver diseases of different etiologies (Kisseleva and Brenner, 2008), indicating that MFB lose their negative regulation for ECM accumulation.

Progress over the past 10 years has disclosed important details of how the TGF-β family elicits its responses (Heldin et al., 1997; Wrana, 2000; Shi and Massague, 2003; Guo and Wang, 2009). Smads, central mediators conveying signals from receptors to superfamily members to the nucleus, are modular proteins with conserved Mad-homology (MH)1, intermediate linker, and MH2 domains (Shi and Massague, 2003). In cell-signaling pathways, various transcription factors are phosphorylated at multiple sites by upstream kinases. Catalytically active TGF-β type I receptor (TβR1) phosphorylates COOH-tail serine residues of receptor-activated Smad (R-Smad), which include Smad2 and the highly similar protein Smad3 (Wrana, 2000). Mitogenic signals alternatively cause phosphorylation of R-Smad at specific sites in their middle linker regions (Kretzschmar et al., 1999; Matsuura et al., 2004; Mori et al., 2004; Kamaraju and Roberts, 2005; Sekimoto et al., 2007; Matsuizaki et al., 2009). After phosphorylated R-Smad rapidly oligomerize with Smad4, this complex translates to the nucleus, where it regulates transcription of target genes. Antagonistic Smad7 acts in opposition to R-Smads and...
Smad4, forming stable associations with the activated type I receptors, and preventing the phosphorylation of R-Smads (Hayashi et al., 1997; Nakao et al., 1997). The transcription of the Smad7 gene is regulated by TGF-β signaling through direct binding of Smad3 and Smad4 to the Smad7 promoter (Nagarajan et al., 1999; von Gersdorff et al., 2000). Therefore, Smad7 functions in a negative-feedback loop to terminate or reduce the strength of the signal.

Monitoring phosphorylation status of signaling molecules is a key step in dissecting their pathways. In Smad signaling, phosphorylation of not only COOH-tail but also linker regions of R-Smad is likely to be important in regulating Smad activity under physiologic and pathologic conditions (Liu, 2006). In this review, we first examine Smad signaling specificity derives from spatial and temporal dynamics of domain-specific R-Smad phosphorylation in response to TGF-β and/or PDGF. We next consider how TGF-β signal together with PDGF promotes collagen synthesis in the activated HSC after acute liver injury. Then, we examine constitutive fibrogenic TGF-β signal in MFB during chronic liver injury. Finally, we consider differential regulation of ECM accumulation between HSC and MFB.

**CANONICAL TGF-β SIGNAL TRANSDUCTION**

Transforming growth factor-β initiates signaling by binding to type I and type II receptor serine/threonine kinase on the cell surface. R-Smads are phosphorylated by the activated TβRI on the C-terminal SXS motif (Derynck and Miyazono, 2008). The activated R-Smads form the complex with the common partner Smad4. The complex translocates to the nucleus, where they regulate target gene expression by interaction with other transcription factors, co-activators, and co-repressors (Heldin et al., 1997; Wrana, 2000; Shi and Massague, 2003; Guo and Wang, 2009). This pathway is regulated by several autoinhibitory feedback loops. In particular, Smad7 interacts stably with activated TβRI to inhibit COOH-tail phosphorylation of R-Smads (Nakao et al., 1997).

**PDGF SIGNAL TRANSDUCTION**

Platelet-derived growth factor is not only a potent mitogen but also a fibrogenic growth factor known to synergize with TGF-β (Pinzani and Macias-Barragan, 2010). Signaling by PDGF begins by interaction with trans-membrane receptor tyrosine kinases (Heldin and Westermark, 1999). Multiple signaling pathways originating from the receptors have been identified. The most prominent pathways are mediated by members of the mitogen-activated protein kinase (MAPK) family, which includes the extracellular signal-regulated protein kinase (ERK) pathway and two stress-activated protein kinase (SAPK) pathways: the c-Jun N-terminal kinase (JNK) and the p38 pathway (Robinson and Cobb, 1997). MAPK is capable of phosphorylating many transcription factors, which are important in initiating cell proliferation and migration.

**MULTIPLE LEVELS OF CROSSTALK EXIST BETWEEN TGF-β AND JNK SIGNALING PATHWAYS**

Transforming growth factor-β elicits signaling responses through non-Smad pathways that are generally considered as important effector pathways for Ras/MAPK in response to PDGF (Derynck and Zhang, 2003). Notably, JNK activates AP-1 consisting of c-Jun that cooperates with nuclear Smads in mediating TGF-β-induced transcriptional responses (Derynck and Zhang, 2003). Thus, Smad-mediated signaling pathway is controlled by or functions in conjunction with other non-Smad transcription factors governed by JNK.

Although COOH-tail phosphorylation by TβRI is a key event in R-Smad activation, additional phosphorylation can positively and negatively regulate R-Smads pathway. Smad2 and Smad3 contain two conserved polypeptide segments, the MH1 and MH2 domains, joined by a less conserved linker region (Shi and Massague, 2003). The linker domain undergoes regulatory phosphorylation by JNK and cyclin-dependent kinase (CDK) pathways (Matsuura et al., 2004; Mori et al., 2004).

In one set of influential experiments, JNK phosphorylates Smad3 outside its COOH-tail phosphorylation site, and enhances activation and nuclear translocation of Smad3 (Engel et al., 1999). Furthermore, activation of MAPK kinase 1 (MEKK-1), an activator of JNK and ERK MAPK, leads to phosphorylation and activation of Smad2 (Brown et al., 1999). This mechanism may explain the ability of fluid shear stress or some growth factors to activate Smad2 (de Caestecker et al., 1998; Matsuzaki et al., 2009). We further identify phosphorylation sites for JNK in the linker regions of Smad2 and Smad3 (Sekimoto et al., 2007). Thus, multiple levels of crosstalk exist between TGF-β and JNK signaling pathways.
Biologic significance of linker-phosphorylated Smad pathway is therefore difficult to assess in isolation. Antibodies (Abs) reactive with structurally related phosphorylated peptides are emerging as valuable tools for determining phosphorylation sites in vivo, and for investigating their distinct signals via phosphorylated domains. We therefore generated the domain-specific Abs, which selectively determine cell-fate decisions (Matsuzaki, 2011). Immunohistoisoforms, and adds to the repertoire of signaling responses that temporal dynamics for cytoplasmic or nuclear R-Smad phosphorylation. (Matsuzaki, 2009).

In vivo and MFB

Biologic significance of linker-phosphorylated Smad pathway is therefore difficult to assess in isolation. Antibodies (Abs) reactive with structurally related phosphorylated peptides are emerging as valuable tools for determining phosphorylation sites in vivo, and for investigating their distinct signals via phosphorylated domains. We therefore generated the domain-specific Abs, which selectively distinguish between the phosphorylated linker regions and the C-terminal regions of Smad2 and Smad3. Using the Abs, we have investigated for linker-phosphorylated R-Smads pathway, with a particular focus on how JNK modulates TβRII signaling through Smad linker phosphorylation.

Domain-specific phospho-R-Smad Abs have allowed us to reveal that TβRI and JNK/CDK4 differentially phosphorylate R-Smads to create three phosphorylated forms (phosphoisoforms): COOH-terminally phosphorylated R-Smad (pSmad2C and pSmad3C), linker-phosphorylated R-Smad (pSmad2L and pSmad3L), and dually phosphorylated R-Smad (pSmad2L/C and pSmad3L/C; Matsuzaki, 2006, 2012; Sapkota et al., 2006; Wrighton et al., 2009). Except for pSmad2L with cytoplasmic localization (Kretzschmar et al., 1999; Mori et al., 2004), the other phosphoisoforms are localized to cell nuclei (Furukawa et al., 2003; Mori et al., 2004; Yamagata et al., 2005; Yoshida et al., 2005; Matsuzaki et al., 2007; Sekimoto et al., 2007; Alarcon et al., 2009; Matsuzaki, 2009; Murata et al., 2009; Nagata et al., 2009; Kawamata et al., 2011). Linker phosphorylation can modify COOH-terminally phosphorylated R-Smad signaling (Kretzschmar et al., 1999; Matsuura et al., 2004; Mori et al., 2004; Ho et al., 2005; Sekimoto et al., 2007; Matsuzaki, 2009).

Differential localization of kinases and phosphatases in the cytoplasm or nucleus raises the intriguing possibility of different temporal dynamics for cytoplasmic or nuclear R-Smad phosphoisoforms, and adds to the repertoire of signaling responses that determine cell-fate decisions (Matsuzaki, 2011). Immunohistochemical and immunofluorescence analyses using specific Abs in human tissues can examine clinical significance of context-dependent and cell type-specific signaling mediated by R-Smad phosphoisoforms, by comparison of tissue/cellular localization of these phosphoisoforms in various pathologic specimens.

DIFFERENTIAL PHOSPHO-Smad SIGNALING BETWEEN HSC AND MFB IN VITRO

Liu (2006) group previously reported that Smad3 is phosphorylated by CDK4 in vivo and in vitro. CDK4-mediated phosphorylation of Smad3 at its linker region inhibits its transcriptional activity and the anti-proliferative activity of TGF-β in fibroblasts (Matsuura et al., 2004; Wang et al., 2009). Importantly, COOH-tail phosphorylation of Smad3 is necessary for TGF-β-induced phosphorylation of Smad3 at its linker region in fibroblasts (Wang et al., 2009; Matsuura et al., 2010). Consistent with these observations concerning Smad3, we have confirmed that the nuclear cyclin D1/CDK4 complex of fibroblasts activated by TGF-β and PDGF signaling directly phosphorylates the linker segment of pSmad2C to produce pSmad2L/C (Matsuura et al., 2009). The expression of c-Myc in fibroblasts is initially repressed by TGF-β, but subsequent cyclin D1/CDK4 undergoes a complete functional change to stimulate c-Myc (Matsuzaki et al., 2009). TGF-β inhibits cell growth by down-regulating the c-Myc via the pSmad2C and pSmad3C pathways (Figure 2A, left). However, signaling activated by TGF-β and PDGF can enhance fibroblast growth by up-regulating c-Myc via the CDK4-dependent pSmad2L/C and pSmad3L/C pathways in cell nuclei (Figure 2A, right). Moreover, Hayashi et al. (2003) reported that pSmad3L/C increases collagen I synthesis in human mesangial cells. We further reported that co-treatment of primary cultured HSC with TGF-β and PDGF enhances transcription of collagen I gene via pSmad3L/C pathway (Furukawa et al., 2003; Yoshida et al., 2005). Importantly, TGF-β-mediated Smad3 in HSC terminates the fibrogenic signals via the pSmad2L/C and pSmad3L/C pathways (Takahashi et al., 2002; Yoshida et al., 2005).

Primary cultured HSC are activated into MFB-like cells after being cultured on plastic dishes. The morphologic features of the cell line closely resembled a MFB-like morphology characterized by spindle shape, cell enlargement, and reduction of the size of intracellular vacuoles (Rockey et al., 1993). In MFB, PDGF induces pSmad3L/C via the activated JNK pathway (Yoshida et al., 2005). The JNK-mediated Smad3L phosphorylation leads to hetero-complex of Smad3 with Smad4 in the nucleus (Mori et al., 2004; Sekimoto et al., 2007). On the other hand, activated JNK retains most Smad2 protein in the cytoplasm (Kretzschmar et al., 1999; Sekimoto et al., 2007). Smad2 can accumulate in the nucleus only if its C-terminus is phosphorylated under conditions of sustained linker phosphorylation by JNK. After COOH-tail phosphorylation of cytoplasmic pSmad2L by TβRI, pSmad2L/C undergoes translocation to the nucleus where it binds to the pSmad3L and Smad4 complex (Furukawa et al., 2003; Matsuzaki et al., 2009; Figure 2B, right), which then in turn stimulates plasminogen activator inhibitor (PAI)-1 transcription (Furukawa et al., 2003). The level of PAI-1 is significantly elevated in fibrotic liver, and PAI-1 overexpression contributes to excessive accumulation of collagen and other ECM proteins, while lack of PAI-1 protects liver fibrosis in response to injury-related profibrotic TGF-β signals (Hu et al., 2009). In contrast to Smad7 induction in HSC via pSmad3C pathway, pSmad3L cannot induce Smad7 transcript in MFB (Takahashi et al., 2002; Takahashi et al., unpublished data). Collectively, fibrogenic TGF-β signal can constitutively accelerate ECM accumulation in MFB by upregulation of PAI-1 protein under a low level of Smad7.
expressions of TGF-β and its receptors in HSC and MFB during acute and chronic CCl₄ intoxication (Date et al., 1998, 2000). The finding, however, merely shows an elevated TGF-β expression in both cases, and no differences in the expression of their
receptors between HSC and MFB. This implies that a modification of postreceptor TGF-β signal in MFB is a more important step for the process of liver fibrosis during chronic injury.

To elucidate how cytostatic TGF-β signaling in HSC takes on collagen-producing features within inflammatory microenvironments during acute liver injury, we focus on the Smad pathway investigating localization of pSmad2L/C and pSmad3L/C in chemically injured rat livers. Nuclear localization of pSmad2L/C and pSmad3L/C is seen in the activated HSC (Yoshida et al., 2005). In particular, strong Smad2/3 phosphorylation at the COOH-tail and threonine residues in the linker regions is observed in the activated HSC (Yoshida et al., 2005). pSmad2L/C and pSmad3L/C signaling may mobilize HSC from the space of Disse to sites of damage, where the activated HSC contribute to tissue repair by producing large amounts of collagen. In HSC after acute liver injury, TβRII activated by endogenous TGF-β signal phosphorylates Smad3C, further up-regulating Smad7 transcription (Figure 2A, right). Subsequently, Smad7 terminates the fibrogenic TGF-β signal mediated by pSmad2L/C and pSmad3L/C pathways. Smad7 could be involved in tight restriction of R-Smads activation in HSC, and regulates the intensity and duration of the TGF-β responses during acute liver injury (Dooley et al., 2000; Tahashi et al., 2002).

The above regulatory mechanism, which avoids unlimited accumulation of ECM proteins that promote liver fibrosis, is disrupted in MFB during chronic liver injury. During transdifferentiation from HSC to MFB in culture, pSmad3C-mediated signal decreases while the pSmad3L pathway predominates (Furukawa et al., 2003; Figure 2B left). The observations fully support the finding of pSmad3L rather than pSmad3C in nuclei of α-smooth muscle actin (SMA)-immunoreactive MFB in portal tracts of chronically HCV-infected liver specimens (Matsuzaki et al., 2007). The presence of α-SMA is associated with transdifferentiation of HSC into scar-forming MFB, an event that is considered pivotal in the fibrogenic response (Pinzani and Macias-Barragan, 2010). In contrast to a transient increase in Smad3L in the activated HSC after acute liver injury, Smad7 remains at a low level in MFB throughout chronic liver injury (Tahashi et al., 2002). Because Smad7 cannot be induced by the pSmad3L pathway (unpublished data), the lack of Smad7 induction in MFB during chronic liver disease might lead to constitutive fibrogenic TGF-β (Dooley et al., 2000; Stopa et al., 2000; Tahashi et al., 2002). Accordingly, Smad7 overexpression results in less accumulation of interstitial collagens and improves liver fibrosis (Dooley et al., 2003). Moreover, IFN-γ displays antifibrotic effects by upregulation of Smad7 expression (Weng et al., 2009).

CONCLUSION AND FUTURE PERSPECTIVES

Because TGF-β involves a variety of physiologic processes such as liver regeneration, unraveling the molecular mechanisms of the TGF-β signal in a pathologic condition is critical to our understanding of its role in disease and the development of its therapies (Bataller and Brenner, 2001). This review summarizes Smad phosphoisoform-mediated signals in HSC, showing differences between in acute and chronic liver injuries. After acute liver injury, TGF-β and PDGF synergistically enhance collagen synthesis by activated HSC via the pSmad2L/C and pSmad3L/C pathways. In recovery stage of acute liver injury, Smad7 induced by TGF-β negatively regulates the fibrogenic TGF-β signaling. In contrast, PDGF-mediated pSmad3L cannot induce Smad7 in MFB even if high concentration of TGF-β is observed in chronic liver diseases. Under a low level of Smad7, MFB can constitutively exhibit the mitogenic pSmad3L and fibrogenic pSmad2L/C signaling, thereby accelerating liver fibrosis.

Transforming growth factor-β activates both Smad and non-Smad signals, which crosstalk with JNK pathway at multiple levels to provide context-dependent outcome (Derynck and Akhurst, 2007). Hence, context-specific effects in HSC can be generated by combined assemblies and spatiotemporal dynamics of R-Smad phosphoisoforms and non-Smad pathway activation, changes of interacting protein partners, and availability of repressors/activators. Although our understanding of the molecular mechanism of liver fibrosis in MFB has significantly advanced during the past decade, much work is needed to define the transcriptional regulatory networks in a context-specific way.

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