Transforming growth factor-β1 downregulation of Smad1 gene expression in rat hepatic stellate cells

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Shen, Hong, Guojiang Huang, Mohammed Hadi, Patrick Choy, Manna Zhang, Gerald Y. Minuk, Yongping Chen, and Yuewen Gong. Transforming growth factor-β1 downregulation of Smad1 gene expression in rat hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 285: G539–G546, 2003. First published June 4, 2003; 10.1152/ajpgi.00436.2002.—Smads are intracellular signaling molecules of the transforming growth factor-β (TGF-β) superfamily that play an important role in the activation of hepatic stellate cells (HSCs) and hepatic fibrosis. Excepting the regulation of Smad7, receptor-regulated Smad gene expression is still unclear. We employed rat HSCs to investigate the expression and regulation of the Smad1 gene, which is a bone morphogenetic protein (BMP) receptor-regulated Smad. We found that the expression and phosphorylation of Smad1 are increased during the activation of HSCs. Moreover, TGF-β significantly inhibits Smad1 gene expression in HSCs in a time- and dose-dependent manner. Furthermore, although both TGF-β1 and BMP2 stimulate the activation of HSCs, they have different effects on HSC proliferation. In conclusion, Smad1 expression and phosphorylation are increased during the activation of HSCs and TGF-β1 significantly inhibits the expression of the Smad1 gene.

THE INTRACELLULAR SIGNALING molecules of the transforming growth factor-β (TGF-β) superfamily are a large and conserved family known as Smads (33). All members of the TGF-β superfamily signal through highly conserved transmembrane receptors and Smads. Binding of ligand to the type II transmembrane serine-threonine kinase receptor recruits and phosphorylates the type I receptor kinase. After activation of the type I receptor, receptor-activated Smads (R-Smads) are recruited as direct substrates for the kinase activity of the type I receptor. Phosphorylated R-Smads then dissociate from the membrane and join common-mediated Smads (Co-Smads) to form heteromeric complexes that translocate into the nucleus, where they mediate transcriptional responses on target genes (1, 22). Interactions between R-Smads and type I receptors are specific. Smad2 and Smad3 interact only with receptors for TGF-β or activin, whereas Smad1, Smad5, and Smad8 interact only with receptors for bone morphogenetic proteins (BMPs) (23). Additionally, all R-Smads appear to share Co-Smad4, and resources of Co-Smad4 are limited within the cell (32).

BMPs are the largest family within the TGF-β superfamily and are involved in several important processes in development. Three Smad proteins have been shown to signal for receptors of the BMP family: Smad5, Smad6, and Smad8. Smad1 was the first mammalian Smad gene to be cloned, whereas Smad5 and Smad8 are two highly conserved vertebrate isoforms. Smad1 is phosphorylated and activated by the BMP receptors BMPR-1A and BMPR-1B, activin receptor (ActR-1), and ActR-like kinase 1 (ALK1) (15, 26). Both Smad1 and Smad5 are important in the induction of ventral mesoderm formation in Xenopus. Targeted disruption of Smad5 indicates a role of this gene in vascular development and patterning, although mutant embryos undergo normal gastrulation (3). Targeted disruption of Smad1 in embryonic stem cells and generation of mutant mice reveals that Smad1-null mice proceed through gastrulation normally but die in midgestation due to defects in allantois development and chorioallantoic placenta formation (14). However, the role of Smad1 in the activation of hepatic stellate cells (HSCs) and the regulation of Smad1 gene expression are poorly understood. In this study, we describe an increased expression of Smad1 during in vitro activation of rat HSCs and TGF-β1 downregulation of Smad1 expression in these cells.

HSCs are nonparenchymal liver cells, which comprise ~15% of the total number of resident cells within the liver. They play a key role in the development of liver fibrosis and cirrhosis. In normal liver, they are the principle storage sites for retinoids (8). Following liver damage or inflammation, HSCs undergo a process known as activation, which is the transdifferentiation of quiescent, retinoid-storing cells into proliferative, matrix-producing, and contractile myofibroblast-like HSCs (2, 25). One of the characteristic features of activated HSCs is the increased expression and release of transforming growth factor-β; Smad1; regulation

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of TGF-β1, which in turn plays an autocrine role in stimulation of fibrogenic extracellular matrix production and perpetuation of HSC activation (11).

MATERIALS AND METHODS

Materials. Collagenase D, pronase, DNase, and monoclonal antibodies against smooth muscle α-actin (α-SMA) and desmin were purchased from Roche Diagnostics (Laval, QC, Canada). Recombinant human TGF-β1 and human BMP2 were purchased from R&D Systems (Minneapolis, MN). DMEM, FBS, and TRIzol LS Reagent were from Gibco-BRL (Burlington, ON, Canada). Other chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Antibody against Smad1 (06-653) and phospho-Smad1 (Ser463 and Ser465) were purchased from Upstate Biotechnology (Lake Placid, NY). Donkey anti-rabbit IgG (NA934), sheep anti-mouse IgG (NA931), and the enhanced chemiluminescence Western blotting kit were purchased from Amersham Pharmacia Biotech (Baie d’Urfe, QC, Canada). Advantage RT-for-PCR kit, Advantage cDNA PCR kit, and polymerase mix were purchased from Clontec Laboratories (Palo Alto, CA). Human hepatocyte carcinoma cell line (PLC/PRF/5 cells) were kindly provided by Dr. Alan McLachlan (Research Institute of Scripps Clinic at La Jolla, La Jolla, CA).

Rat HSCs. Male Sprague-Dawley rats (450–550 g body wt) were provided by Central Animal Care of the University of Manitoba and maintained under 12:12-h light-dark cycles with food and water ad libitum. During the research described in this report, all animals received humane care in compliance with the institution’s guidelines (Animal Protocol no. 98-053), which are in accordance with the Canadian Council on Animal Care’s guidelines (Animal Protocol no. 98-053), which are in accordance with the Canadian Council on Animal Care’s criteria. HSCs were isolated by two steps of collagenase and pronase methods (29). Briefly, the rat liver was perfused with 0.125 mg/ml collagenase D, 0.5 mg/ml pronase, and 20 μg/ml DNase I in Hanks’ balanced salt solution supplemented with 10 mmol/l HEPEs and 4.2 mmol/l sodium bicarbonate for 15 min. After in situ perfusion, the liver was removed, separated, and incubated with 0.125 mg/ml collagenase D and 0.5 mg/ml pronase for another 15 min with constant low-speed stirring at 37°C. After hepatocytes were removed, HSCs were separated from other nonparenchymal cells by centrifugation on 11.3% Nycodenz with sodium chloride. HSCs were harvested from the interface between suspension buffer and 11.3% Nycodenz solution, washed, and plated on uncoated plastic tissue culture with sodium chloride. HSCs were harvested from the inter-

Northern blot analysis of Smad1 mRNA abundance. TRIzol LS reagent was employed to extract total RNA of HSCs as described in the manufacturer’s manual. Northern blot analysis was performed by using [α-32P]dCTP-labeled full-length rat Smad1 and 18S cDNA probes as previously described (12). Briefly, 40 μg of total RNA was separated through 1% agarose gel, transferred onto GT-zeta nylon membranes (Bio-Rad, Burlington, ON, Canada), hybridized overnight with the probes, and washed as per the manufacturer’s instructions.

RT-PCR. Total RNA was isolated as described above from 3-, 6-, and 10-day cultured HSCs. The first-strand cDNA was synthesized by an Advantage RT-for-PCR kit. Briefly, 1 μg of total RNA was dissolved in diethylpyrocarbonate-treated double-distilled water (ddH2O) to achieve a final volume of 12.5 μl. Subsequently, 1 μl oligo(dT) primer, 4 μl of 5 × reaction buffer, 1 μl of 10 mmol/l dNTP mix, 0.5 μl recombinant RNase inhibitor, and 1 μl Moloney murine leukemia virus reverse transcriptase were added and incubated for 1 h at 42°C. At the end of the reverse transcription procedure, the reaction mixture was heated to 94°C for 5 min and brought up to a final volume of 100 μl with ddH2O. PCR was performed by using the Advantage PCR kit, polymerase mix, and oligonucleotides synthesized by Gibco-BRL. The specific primers for rat Smad1 were designed from the rat Smad1 sequence (GenBank accession no. U66478) by the
Oligo 5.1 program on a Macintosh computer. The sense primer was 5\'-H11032-TCCTACCTTTCCGAACCGAAGA-3\' (positions 179–201), and the antisense primer was 5\'-H11032-GAGGTCATCAGTATCACGGATCCTTTAC-3\' (positions 1778–1804). The expected size of products was 1,625 base pairs. The specific rat GAPDH amplimers were from Clontech (5507-3), and the expected size of GAPDH was 986 base pairs. PCR amplification was carried out by applying 32 cycles comprising denaturation at 94°C for 1 min, annealing at 59.5°C (Smad1) and 60°C (GAPDH) for 30 s, elongation at 72°C for 4 min, followed by a final elongation at 72°C for 8 min using an Eppendorf MasterCycler (Eppendorf, Westbury, NY). PCR products were analyzed by electrophoresis in a 1.5% agarose gel. Identity of PCR products was confirmed by sequencing at the DNA-sequencing facility of the Manitoba Institute of Cell Biology.

Real-time RT-PCR quantitation of mRNA. Quantitation of mRNA was performed by LightCycler following real-time RT-PCR protocol provided by Roche Molecular Biochemicals. Briefly, full-length rat Smad1 cDNA was inserted into a pCR2.1 vector (Invitrogen Canada, Burlington, ON, Canada). The sense mRNA of rat Smad1 was synthesized by a Riboprobe Combination System-T3/T7 (Promega, Madison, WI) with T7 polymerase. The synthesized sense mRNA of rat Smad1 was employed as template for a standard curve. Total RNA and the first-strand cDNA were prepared as described above. One microgram of the first-strand cDNA was employed for real-time RT-PCR quantitation of Smad1 with sense primer 5\'-CCGCCTGCTTACCTTCCTCTCTTGA-3\' (positions 685–709) and antisense primer 5\'-GAACCGTTCCGCCACACGTTGT-3\' (positions 863–886). Real-time RT-PCR was performed with LightCycler-DNA Master SYBR Green I kit from Roche Molecular Biochemicals in the following protocol: denaturation at 95°C for 2 min; amplification at 40 cycles of 1 s at 94°C, 10 s at 62°C, and 16 s at 72°C; and melting curve analysis at 58°C for 10 s at the end of each cycle. The amount of Smad1 mRNA was calculated with a computer program and represented as nanograms per milliliter of standard RNA.

Cell proliferation assay. Cell proliferation was measured by a [\(^3\)H]thymidine incorporation method. After isolation of HSCs, primary HSCs were placed in 12-well plates and the

Fig. 1. Morphology of rat hepatic stellate cells (HSCs) and expression of smooth muscle \(\alpha\)-actin (\(\alpha\)-SMA) during primary culture of HSCs. A and B: cell morphology of isolated rat HSCs at days 2 (A) and 9 (B). C: expression of \(\alpha\)-SMA during primary culture of HSCs from days 1 to 12.

Fig. 2. Expression of Smad1 during in vitro activation of HSCs and specificity of phospho-Smad1 antibody. Cell extracts were isolated from different times of HSC in vitro culture as indicated. A: Western blot analysis. Sixty micrograms of protein was loaded on SDS-PAGE gel for electrophoresis. Protein was transferred onto nitrocellular membranes, which were incubated with antibodies against Smad1 and phospho-Smad1. Chang liver cells were transiently transfected with rat Smad1 cDNA and incubated with or without 10 ng/ml bone morphogenetic protein (BMP) 2 for 30 min. Sixty micrograms of nuclear protein was loaded on SDS-PAGE gel for electrophoresis. Protein was then transferred onto nitrocellular membranes, which were incubated with phospho-Smad1 antibody.

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medium was first changed after 24 h. HSCs were then incubated with fresh DMEM with 2% FBS and either TGF-β1 or BMP2 for 6 days. The media and reagents were changed every other day. At the end of treatment, HSCs were labeled with 1 μCi of [3H]thymidine (specific activity 45 Ci/mmol; Amersham, Oakville, ON, Canada) for 2 h, fixed in 10% trichloroacetic acid, and lysed in 400 μl of 0.2 M sodium hydroxide. One hundred microliters of the cell lysate was employed to measure [3H]thymidine incorporation with a LKB liquid scintillation counter (Wallac, Turku, Finland), and 10 μl of the cell lysate was used to measure protein content by the Lowry technique.

Statistical analyses. To analyze differences in the treatment groups, we performed ANOVA and Fisher’s protected least significant differences test as a post hoc test using StatView software (version 5.0; SAS Institute, Cary, NC). Differences with P values < 0.05 were judged to be significant.

RESULTS

HSC activation and expression of Smad1. Rat HSCs were isolated and cultured in vitro as shown in Fig. 1. Figure 1A displays rat HSCs at 2 days after isolation, and Fig. 1B shows the cells at 9 days after isolation. One of the features of HSCs is that they can spontaneously activate in vitro when cultured on uncoated plastic dishes. Whole cellular protein was extracted from rat HSCs at different times after isolation, and the expression of α-SMA, a marker of activation of HSCs, was demonstrated in Fig. 1C, which showed the increases in expression of α-SMA after 9- and 12-day culture in vitro.

The effects of TGF-β on HSCs and expression of Smad2, Smad3, and Smad4 in HSCs are the important research areas in liver fibrosis. It has been documented that Smads play an important role in TGF-β regulation of HSC proliferation and activation. However, Smads, which are involved in BMP signaling, have not been of major research interest until recently. By employing antibodies against Smad1, we documented that expression of Smad1 was elevated during the activation of HSCs (Fig. 2A). To demonstrate the specificity of antibody against phospho-Smad1, Chang liver cells were transfected with full-length rat Smad1 cDNA and incubated with or without BMP2 for 30 min. As shown in Fig. 2B, BMP2 increased phospho-Smad1 expression in the nuclear extract of these cells. In addition, specificity of Smad1 antibody was confirmed by transfecting Smad1 into Chang liver cells. As shown in Fig. 2C, transfected cells expressed more Smad1 than non-transfected cells. To further examine whether or not the elevated Smad1 protein was due to an increase in
the expression of Smad1 mRNA, we extracted the total RNA from HSCs and performed RT-PCR with a pair of specific primers for rat Smad1 (Fig. 3A). Quantitative real-time RT-PCR was also performed to document the quantity of Smad1 mRNA in HSCs (Fig. 3B). There was a significant increase in Smad1 RNA transcript after 6-day culture in vitro. The level of Smad1 RNA transcript remained elevated at 9 days after in vitro culture.

**TGF-β regulation of Smad1 gene expression in HSCs.** One of the most important findings in this study is that TGF-β1 decreased the expression of Smad1. When rat HSCs were incubated with TGF-β1 for 3, 6, and 12 days, the abundance of Smad1 and phospho-Smad1 was reduced in a consistent manner (Fig. 4). To further confirm TGF-β1 regulation of Smad1, we isolated rat HSCs and treated these cells with TGF-β1. The abundance of Smad1 mRNA and protein were documented by Northern and Western blot analyses. After rat HSCs were cultured for 6 days and incubated with TGF-β1 in fresh culture media without serum, Smad1 RNA transcript was decreased in a time-dependent manner (Fig. 5). The level of Smad1 mRNA reached a nadir after 24 h of TGF-β1 treatment. The TGF-β1 inhibition of Smad1 mRNA level was not observed in a human hepatocellular carcinoma cell line (PLC/PRF/5 cells). When rat HSCs were treated with different concentrations of TGF-β1 for 12 h, the abundance of Smad1 mRNA and protein was decreased with an increase in TGF-β1 concentrations (Fig. 6). In addition, the antibody employed in this experiment was the antibody against Smad1, not phospho-Smad1, which indicates that TGF-β1 downregulated Smad1 protein but not phosphorylation of Smad1.

Smads are usually cytoplasmic proteins. However, after Smads are activated by BMPs or TGF-β receptors, Smads will migrate to the nuclei. To document whether Smad1 was translocated in the nuclear compartment with or without TGF-β1 treatment, HSCs after 9-day in vitro culture were treated with or without TGF-β1. Nuclear and cytoplasmic proteins were isolated and analyzed for both Smad1 and phospho-Smad1. As shown in Fig. 7, phospho-Smad1 was only present in the nuclear fraction but Smad1 can be observed in both nuclear and cytoplasmic fractions of cells.

**TGF-β and BMP regulation of HSC proliferation and activation.** Since Smad1 is the signaling molecule of BMP and TGF-β1, TGF-β regulation of Smad1 expression may affect the biological activity of TGF-β on HSCs. We examined the regulation of HSC prolifera-
tion and activation by TGF-β1 and BMP2. Primary HSCs were treated with different concentrations of TGF-β1 and BMP2 for 6 days. TGF-β1 significantly inhibited HSC proliferation, whereas BMP2 did not affect the proliferation of HSCs (Fig. 8). In addition, after primary HSCs were incubated with TGF-β1 or BMP2 for 6 days, both cytokines increased the abundance of α-SMA protein, with BMP2 tending to induce more α-SMA than TGF-β1 in these cells (Fig. 8).

**DISCUSSION**

Smad1 is activated by the receptors for BMPs as well as TGF-β1 (18, 19). The function of Smad1 is to mediate signal transduction of BMPs and TGF-β. Recent studies from Smad1 transgenic mice reveal early postnatal lethality in transgenic mice and impaired cell cycle in embryonic fibroblasts derived from these animals (24). Smad1 knockout mice die in midgestation due to defects in allantois development and chorioallantoic placenta formation (14). The observation of increased expression of Smad1 during the culture of HSCs suggests a role for Smad1 in the activation of HSCs. It is not clear whether Smad1 directly mediates the activation of HSCs or acts through the signal transduction of BMPs. The expression and role of BMP in the activation of HSCs are poorly understood. In this study, we observed that BMP2 had no effect on HSC proliferation but that it did elevate the level of α-SMA protein in HSCs. These results suggest that BMPs may play an important role in transdifferentiation of HSCs. This is consistent with the function of BMPs in development, because BMPs and Smad1 are required for bone and heart formation in vertebrates (30).

Studies investigating the regulation of Smad1 are limited to Smad1 phosphorylation. It is known that Smad1 is phosphorylated after binding of BMPs to their receptors (20). Studies from Mulder’s group (20) indicate that Smad1 can be phosphorylated by TGF-β1. Moreover, they reveal that Ras and MEK are partially required for Smad1 phosphorylation by both TGF-β1 and BMPs (35). In addition, it has been documented that epidermal growth factor and hepatocyte growth factor could phosphorylate Smad1 through an ERK-mediated pathway to inhibit BMP-mediated nuclear accumulation and transcriptional activation of Smad1 (17). Our observations of TGF-β1 downregulation of Smad1 mRNA and protein levels indicate a new regulatory mechanism of Smad1 gene expression. The role of Smad1 in HSCs remains to be further explored; however, it has been documented that Smad2, Smad3, and Smad4 remained constant during the activation of HSCs (7, 31).

The causative role of TGF-β in hepatic fibrosis has been provided by studies in transgenic mice and also in genetic transfer of active TGF-β1 by adenoviral vector. Chronic expression of active hepatic TGF-β1 in trans-
genic mice through the hepatocyte-specific promoters phosphoenolpyruvate carboxykinase (4) and albumin (16) resulted in hepatic phosphoenolpyruvate carboxykinase (4) and albumin been provided from studies of HSCs in vitro and in vivo. It remains to be determined whether these actions will alter Smad1 expression.

The causative role of TGF-β in HSC activation has been provided from studies of HSCs in vitro and in vivo. It has been documented that TGF-β induces transdifferentiation of HSCs into myofibroblasts (13) and inhibits proliferation of HSCs (27). However, contrasting observations regarding TGF-β regulation of HSC proliferation and transdifferentiation exist. It has been documented that TGF-β1 inhibits quiescent HSC proliferation but does not affect activated HSC proliferation (6). Loss of the TGF-β1 inhibition of activated HSC proliferation appears to be related to loss of TGF-β1 binding on surface receptors of the cells. A similar effect of TGF-β1 regulation of collagen I expression was observed in quiescent and activated HSCs (13). However, our results indicate that TGF-β1 still inhibits subcultured (for 18 days) HSC proliferation. Moreover, TGF-β1 slightly increases α-SMA expression in HSCs. The discrepancies may be due to the culture condition and the state of HSC differentiation. In our experiments, 10% FBS was employed, whereas in other experiments 0.1% FBS was used (6). TGF-β1 may just inhibit the stimulated effect of FBS or growth factors in the PBS on HSCs. It has been known that TGF-β1 reverses the stimulating effect of platelet-derived growth factor on HSC proliferation (5). Moreover, the role of TGF-β1 in the HSCs is still unclear, because in mice with TGF-β1 gene knockout HSCs can still be activated when cultured in vitro (13). In addition, it has been documented that Smad3 knockout mice have an increased proliferation rate when stimulated with FBS or with platelet-derived growth factor compared with wild-type HSCs (28). It is expected that Smad3 is necessary for inhibition of culture-activated HSC proliferation. Therefore, alteration of Smad1 gene expression by TGF-β1 may strengthen the role of Smad3 or Smad2 in TGF-β signal transduction, which may determine HSC responses to TGF-β1.

Although TGF-β downregulation of Smad1 in HSCs may be of benefit to TGF-β profibrogenic action on HSCs, it may also alter the transdifferentiation of HSCs. Since Smad1 expression is increased during transdifferentiation of HSCs and BMP2 is more potent in induction of α-SMA (a transdifferentiation marker of HSCs) than TGF-β1, the reduction of Smad1 could interfere with the transdifferentiation of HSCs. This may ultimately alter the response of HSCs to TGF-β1. In addition, TGF-β1 can activate the Smad1 signaling pathway, Smad2/Smad3 signaling, and the MAPK pathway (21). TGF-β1 inhibition of Smad1 gene expression could inhibit the signaling events mediated by Smad1 while strengthening the signaling events mediated by the other signaling molecules. Therefore, the findings in our study indicate complex effects of TGF-β on HSCs.

In conclusion, we have documented that Smad1 mRNA and protein are elevated during in vitro activation of HSCs and that TGF-β1 downregulates Smad1 mRNA and protein in HSCs. These findings reveal an important role of Smad1 in HSC activation and an important molecular mechanism in Smad1 gene regulation. In addition, TGF-β downregulation of Smad1 may be important in the balance of different TGF-β biological effects.

DISCLOSURES

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