Smads 2 and 3 Are Differentially Activated by Transforming Growth Factor-β (TGF-β) in Quiescent and Activated Hepatic Stellate Cells

CONSTITUTIVE NUCLEAR LOCALIZATION OF Smads IN ACTIVATED CELLS IS TGF-β-INDEPENDENT

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Hepatic stellate cells are the primary cell type responsible for matrix deposition in liver fibrosis, undergoing a process of transdifferentiation into fibrogenic myofibroblasts. These cells, which undergo a similar transdifferentiation process when cultured in vitro, are a major target of the profibrogenic agent transforming growth factor-β (TGF-β). We have studied activation of the TGF-β downstream signaling molecules Smads 2, 3, and 4 in hepatic stellate cells (HSC) cultured in vitro for 1, 4, and 7 days, with quiescent, intermediate, and fully transdifferentiated phenotypes, respectively. Total levels of Smad4, common to multiple TGF-β superfamily signaling pathways, do not change as HSC transdifferentiate, and the protein is found in both nucleus and cytoplasm, independent of treatment with TGF-β or the nuclear export inhibitor leptomycin B. TGF-β mediates activation of Smad2 primarily in early cultured cells and that of Smad3 primarily in transdifferentiated cells. The linker protein SARA, which is required for Smad2 signaling, disappears with transdifferentiation. Additionally, day 7 cells demonstrate constitutive phosphorylation and nuclear localization of Smad 2, which is not affected by pretreatment with TGF-β-neutralizing antibodies, a type I TGF-β receptor kinase inhibitor, or activin-neutralizing antibodies. These results demonstrate essential differences between TGF-β-mediated signaling pathways in quiescent and in vitro transdifferentiated hepatic stellate cells.

The transforming growth factors β (TGF-βs) are multifunctional peptide growth factors with variable cellular effects, including growth inhibition and matrix induction. They are potent profibrogenic agents, with roles in multiple fibrotic diseases (1). In particular, TGF-β is a key mediator of hepatic fibrosis, as demonstrated by an increase in TGF-β production at sites of matrix deposition, the development of fibrosis in laboratory animals engineered to overproduce TGF-β, and the effectiveness of anti-TGF-β therapies in mitigating experimentally-induced fibrosis (2–9).

Hepatic stellate cells (HSC) are the primary target of fibrogenic stimuli in the diseased liver. In the setting of chronic liver disease, HSC transdifferentiate, adopting a myofibroblast-like phenotype characterized in part by proliferation and the deposition of abnormal matrix. This phenotypic change, termed “activation,” has been modeled in vitro by culturing HSCs on uncoated plastic. Freshly isolated HSC appear undifferentiated and are traditionally termed “quiescent,” whereas cells grown on uncoated plastic for 5 to 7 days become activated. In response to TGF-β, culture-activated cells produce extracellular matrix that is similar in composition to that seen in the fibrotic liver (7, 10–13).

TGF-β signals through the sequential activation of two cell surface serine/threonine kinase receptors, the type II and type I (TβRI). The activated TβRI phosphorylates Smad2 or Smad3, members of the Smad family of cytoplasmic and nuclear signaling molecules, which are specific for the signaling pathways of TGF-β and the related growth factor activin. Phosphorylated Smads 2 and 3 form heteromeric complexes with Smad4, a Smad common to the signaling pathways of multiple TGF-β superfamilies and, move into the nucleus where they join other transcription factors to form transcriptionally active complexes (14). Recent work has also demonstrated a role for the protein Smad anchor for receptor activation (SARA), an FYVE domain linker protein that recruits Smad2 to the TGF-β receptor complex and is required for maximal Smad2-mediated TGF-β signaling (15). Smad2 and Smad3 are clearly functionally distinct, although the details of their differing functions are not well understood. They have different expression patterns, produce different phenotypes in null mice, and demonstrate different effects on at least one promoter (16–20). Smad3 binds DNA directly, whereas Smad2 does not (21–24). Expression of a dominant negative Smad3 but not Smad2 prevents TGF-β-mediated inhibition of adipocyte differentiation (25). Furthermore, studies with passaged fibroblasts derived from Smad2 or Smad3 null mouse embryos suggest that, although both Smads contribute to TGF-β-mediated signaling and plasminogen activator inhibitor (PAI)-1 up-regulation, Smad3 is primarily responsible for the autocrine production of TGF-β and Smad2 for matrix metalloproteinase-2 up-regulation (26).

In this report we have examined TGF-β signaling in primary...
HSC as they undergo in vitro activation. We demonstrate that there is a dissociation between the cell cycle and matrix effects of TGF-β in HSC, with growth inhibition occurring only in phenotypically quiescent cells while TGF-β-mediated matrix deposition is observed in HSC at all stages. Smad4 in these cells is constitutively expressed in both nucleus and cytoplasm independent of TGF-β treatment. Smads 2 and 3, however, are phosphorylated and translocated into the nucleus in response to TGF-β, although the pattern varies with the state of activation: Smad2 phosphorylation and nuclear translocation occurs primarily in quiescent and intermediate cells, whereas Smad3 activation occurs primarily in activated cells. SARA is expressed in quiescent HSC but is lost with in vitro activation in parallel with the loss of TGF-β-mediated Smad2 phosphorylation. In day 7 HSC, in addition to TGF-β-responsive Smad3 activation, we also observed constitutive phosphorylation and nuclear localization of Smad2. Treatment with a TgRI kinase inhibitor or with TGF-β or activin-neutralizing antibodies does not diminish this constitutive activation. These results demonstrate essential differences between TGF-β-mediated Smad signaling pathways in quiescent and in vitro-transdifferentiated HSC, and suggest that both TGF-β-dependent and-independent Smad activation occurs in activated HSC.

EXPERIMENTAL PROCEDURES

Materials—Media (minimum essential medium and medium 199 (M199)) were obtained from Invitrogen (Grand Island, NY) and Cellgro (Herndon, VA). FBS was from Gemini Biosciences (Woodland, CA), and antibiotics were from Invitrogen. TGF-β1 was from R&D Systems (Minneapolis, MN).

HSC Isolation—Primary rat hepatic stellate cells were isolated from livers of Sprague-Dawley rats weighing 500–700 g by a modification of a method described previously (27). Rats were anesthetized with sodium pentobarbital. In situ liver perfusion and digestion was performed with Pronase E (2.4 mg/ml, Roche Molecular Biochemicals, Chicago, IL) and collagenase B (0.3–0.45 mg/ml, Roche Molecular Biochemicals), and the resulting liver cell suspension was purified by density gradient centrifugation using 8.2% Nycodenz. HSC were plated on uncoated plastic at a density of 5 × 10⁴ per 10-cm diameter plate and maintained in M199/10% FBS supplemented with glutamine and antibiotics (penicillin, 100 units/ml; streptomycin, 100 units/ml; gentamicin, 0.1 mg/ml; and fungizone (Invitrogen), 2.5 µg/ml). Cell viability was greater than 90% as assessed by trypan blue exclusion. Purity was 90–95% as assessed by desmin immunostaining and the typical light microscopic appearance of the lipid droplets. Cells were considered to be quiescent at day 1, 24 h after plating. Cells at day 7 were considered activated, with greater than 95% staining positive for α-smooth muscle actin. For all experiments in which cells at different stages were compared, the cells were from the same isolation. All experiments were repeated with cells from different animals, as noted in the figure legends.

Proliferation Assays—After 1, 4, or 7 days in culture, primary HSC were washed twice with serum-free M199, then incubated in M199/0.3% FBS with or without TGF-β for 24 h. 2 µCi/ml [3H]thymidine was added for the last 18 h of incubation. Labeled cells were washed twice with PBS and lysed in 1 x NaOH; lysates were counted in a liquid scintillation counter.

RNA Isolation and Northern Blotting—A rat Smad2 cDNA probe was generated by reverse transcription (RT)-PCR with primers derived from GenBank™ accession number AB010147 (sense, CGATGCTCAAGGACTGTCCCTA; antisense, CGCTGGTGGTTGACAGTC). A cDNA fragment of rat Smad3 (GenBank™ accession number U66479) was a generous gift of Yun Chen (Indiana University) (28); a rpn1 restriction fragment representing the entire coding region was purified with the QiAquick gel extraction kit (Qiagen, Valencia, CA); the probe for α(I) collagen was generated by restriction digestion of the cDNA and included the entire coding region (29). The probe for rpn1 decorin was generated by RT-PCR using primers from GenBank™ sequence Z12298 (sense, TCCTTCCCTCCCTCCCTCAAT; antisense, GATGCCAGGAGCTCTCTCAGC). Rat β-actin cDNA was from Clontech (Palo Alto, CA). cDNAs were labeled with [32P]dCTP with the Megaprime labeling kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

Western Blotting—For immunoblotting of PAI-1, HSC at days 1, 4, or 7 after isolation were treated with 100 µM TGF-β1 for 24 h in the presence of 0.3% serum. Cells were lysed in hypotonic buffer (20 mM NaCl, 10 mM KCl, 1.5 mM MgCl₂, 10 mM Hepes, pH 7.4, 1% Triton X-100, 1.5 mM phenylmethanesulfonyl fluoride), and lysates were equalized for protein content and separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody against PAI-1 (5 µg/ml, American Diagnostics, Inc., Greenwich, CT).

For immunoblotting of Smad proteins, HSC at days 1, 4, or 7 after isolation were rinsed, serum-starved for 15 min, then treated with or without 100 µM TGF-β1 for 15 min. For 24-h treatments, cells were incubated with or without TGF-β1 for 24 h in the presence of 0.3% serum. Cells were chilled, washed with PBS, and pelleted. Pellets were resuspended in low salt buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM NaVO₄, 1 mM EDTA, 1 mM DTT, 0.2% Nonidet P-40, 0.1% Nonidet P-40, 100 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml Complete (Roche Molecular Biochemicals, Chicago, IL)), incubated on ice for 10 min, and centrifuged (8000 × g, 2 min, 4°C). Supernatants were collected and considered to be the cytoplasmic fraction. Pellets were resuspended in high salt buffer (low salt buffer with 420 mM NaCl, 20% glycerol, and no Nonidet P-40), incubated on ice for 30 min, and centrifuged as before. Supernatants were collected as the nuclear fraction. Protein concentrations were equalized within the two groups (cytoplasmic and nuclear), and equivalent aliquots were separated by reducing SDS-PAGE, transferred to nitrocellulose, blocked with 5% nonfat milk in TBS with 0.1% Tween 20, and immunoblotted in the same buffer with the appropriate antibodies at the following concentrations: HSP-70 (1 µg/ml, Stressgen Biotechnologies Corp., Victoria, BC), proliferating cell nuclear antigen (PCNA, 1:300, Sigma, St. Louis, MO), β-tubulin (0.4 µg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Smad2 (1.25 µg/ml, Transduction Laboratories, Lexington, KY), Smad3 (2.5 µg/ml, Zymed Laboratories Inc., San Francisco, CA), Smad4 (1.0 µg/ml, clone B-8, Santa Cruz Biotechnology, Inc.), and SARA (1.0 µg/ml, Santa Cruz Biotechnology, Inc.). For immunoblotting of total protein (Fig. 4, A and D), cells were lysed with 1% SDS buffer (1% Triton X-100, 0.5% NP-40, 10 M M EDTA, 100 mM NaCl, 0.3% serum, or for shorter periods (0–90 min) in serum-free media. This concentration of TGF-β1 (2–5 ng/ml) is predicted to be saturating. Blots were exposed to x-ray film at 72 h.

Matrix Deposition Assays—Collagen secretion was measured by diffusion of [2,3,4,5-3H]proline for 24 h in the presence of 100 µM TGF-β1. Media were collected, equalized according to the DNA content of the associated cell layer, digested with collagenase, trichloroacetic acid-precipitated, and counted.
Mv1Lu cells, widely used for TGF-β immunoprecipitated with an antibody against fibronectin. For comparison, experiments with fibronectin and PAI-1 performed in parallel with H9252
HSC were treated with TGF-β1 after isolation were treated with 100 pM TGF-β1 for 24 h. The last 16 h in the presence of 1 μCi/ml n-[6-3H]glucosamine HCl (Amersham Biosciences). Media were removed and spun three times in Amicon 10 microcentrifuge tubes to remove excess label, then counted.

Neutralizing Antibody and Inhibitor Treatments—For treatments with neutralizing antibodies and inhibitors, cells were rinsed twice with M199, then incubated with pan-TGF-β-neutralizing antibodies (R&D systems, 1 μg/ml, overnight), activin-neutralizing antibodies (R&D Systems, 0.06 or 0.6 μg/ml, overnight), leptomycin B (Sigma, 20 ng/ml, –60 min), or TβRI kinase inhibitor NPC-34016 (a gift of David Liu, Scios, Inc., 0.1 μM, overnight). After incubation, cells were rinsed, lysed, and prepared for immunoblotting as above. The antibodies and inhibitors had been previously tested and demonstrated to be both effective and non-toxic to HSC at the concentrations used (data not shown).

Smad Activation in Quiescent and Activated HSC

Values (mean ± S.D.; n = 4) are normalized to incorporation in the absence of TGF-β. The data shown are from one of three experiments with similar results (*, p < 0.05; **, p < 0.01).

To measure fibronectin production, HSC were treated with 100 pM TGF-β1 in 0.3% serum for 16 h. Cells were then incubated with minimal essential medium lacking cysteine and methionine for 2 h, followed by the addition of [35S]cysteine/methionine (Express, PerkinElmer Life Sciences, Boston, MA) at 100 μCi/ml for an additional 2 h. TGF-β1 was present throughout. The supernatant was collected and immunoprecipitated with antibody against rat fibronectin (1 μl/ml supernatant, Invitrogen). Immunoprecipitants were separated by SDS-PAGE, and gels were fluorographed with 2,5-diphenyloxazole, dried, and autoradiographed.

Glycosaminoglycan secretion was measured by treating HSC with 100 pM TGF-β1 in 0.3% serum for 24 h, the last 16 h in the presence of 1 μCi/ml n-[6-3H]glucosamine HCl (Amersham Biosciences). Media were removed and spun three times in Amicon 10 microcentrifuge tubes to remove excess label, then counted.

Neutralizing Antibody and Inhibitor Treatments—For treatments with neutralizing antibodies and inhibitors, cells were rinsed twice with M199, then incubated with pan-TGF-β-neutralizing antibodies (R&D systems, 1 μg/ml, overnight), activin-neutralizing antibodies (R&D Systems, 0.06 or 0.6 μg/ml, overnight), leptomycin B (Sigma, 20 ng/ml, –60 min), or TβRI kinase inhibitor NPC-34016 (a gift of David Liu, Scios, Inc., 0.1 μM, overnight). After incubation, cells were rinsed, lysed, and prepared for immunoblotting as above. The antibodies and inhibitors had been previously tested and demonstrated to be both effective and non-toxic to HSC at the concentrations used (data not shown).

and counted in a scintillation counter, as described previously (35).

FIG. 1. TGF-β inhibits DNA synthesis in primary HSC at day 1 after isolation but not at days 4 and 7. Primary HSC at days 1, 4, and 7 after isolation were treated with TGF-β1 for 24 h in 0.3% serum then assayed for incorporation of [3H]thymidine, as described under “Experimental Procedures.” Values (mean ± S.D.; n = 4) are normalized to incorporation in the absence of TGF-β. The data shown are from one of three experiments with similar results (*, p < 0.05; **, p < 0.01).

FIG. 2. TGF-β treatment of quiescent and activated cells up-regulates production of matrix proteins. Primary HSC at days 1, 4, and 7 after isolation were treated with 100 pM TGF-β1 as described. A, primary HSC at days 1, 4, and 7 were treated with 100 pM TGF-β1 for 0, 15, 30, or 90 min (left panel) or 0 (−) or 24 h (+; right panel) then analyzed by Northern blotting for collagen α1(I) (top panel) and decorin (middle panel). To analyze loading, the same blot was stripped and re-analyzed with a probe for β-actin (lower panels). B, collagen secretion was measured by differential collagenase sensitivity of [3H]proline-labeled secreted proteins and was increased in response to TGF-β at days 4 and 7 (*, p < 0.05 for both days; six samples for each condition). C, HSC treated (+) or not (−) with TGF-β were lysed, and lysates were immunoblotted with an antibody raised against PAI-1 (upper panel). As a control, aliquots of the same lysate were immunoblotted with an antibody against β-tubulin. D, HSC were treated with TGF-β for 16 h and metabolically labeled, as described under “Experimental Procedures.” Media were collected and immunoprecipitated with an antibody against fibronectin. For comparison, experiments with fibronectin and PAI-1 performed in parallel with Mv1Lu cells, widely used for TGF-β studies, demonstrate a 3- to 4-fold enhancement of synthesis with TGF-β treatment (data not shown). E, HSC were treated with TGF-β1 for 24 h and labeled with [3H]glucosamine to determine secreted glycosaminoglycans, as described. Data are shown as percent increase for plus TGF-β compared with minus TGF-β. **, p < 0.05; *, p < 0.06 for +/− TGF-β comparison. Six replicates were counted for each condition. Results were similar for matrix deposition of glycosaminoglycans (data not shown). Results for each panel are representative of three experiments.
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RESULTS

Culturing rat HSC on uncoated plastic is a well-established in vitro model of HSC activation. HSC at day 1 after isolation demonstrate a quiescent phenotype, with a round shape, abundant lipid droplets, and a lack of α-smooth muscle actin expression. Cells at day 7 are spread out, have few lipid droplets, and express α-smooth muscle actin, consistent with the activated phenotype. Cells at day 4 have an intermediate phenotype. We used this model system to study differences in TGF-β signaling pathways at different stages of phenotypic differentiation. Data in the literature regarding the effects of TGF-β on quiescent and activated cells are contradictory, and the definition of quiescence and activation varies; therefore, as a preface to studying TGF-β signaling in HSC, we systematically defined their response to TGF-β.

TGF-β-mediated Inhibition of DNA Synthesis—We first characterized the growth response to TGF-β. As assayed by [3H]thymidine incorporation, quiescent HSC (day 1) demonstrated marked inhibition of DNA synthesis in response to TGF-β (10–500 pM), with a decrease in incorporation of up to 83% and an IC50 of 10 pM (Fig. 1), similar to what has been reported for phenotypically quiescent cells by other groups (36). In contrast, cells at days 4 and 7 after isolation demonstrated no change in DNA synthesis even with 500 pM TGF-β, although these cells displayed a 3.4-fold higher baseline rate of thymidine incorporation in day 7 than in day 1 cells (Fig. 1).

TGF-β-mediated Matrix Deposition—We also characterized TGF-β-mediated changes in the expression of various matrix components. Collagen mRNA synthesis and secretion are modestly but significantly increased in response to TGF-β at days 4 and 7 (Fig. 2, A and B). Similar findings were seen for deposited collagen (data not shown). PAI-1 protein expression was increased (1.4- to 2.3-fold) with TGF-β stimulation (data not shown). This blot is representative of results obtained in independent experiments. We consistently see two faster bands in the cytoplasm at day 4, with a corresponding reduction in the intensity of the major signal. The significance of proteolysis of Smad4, especially at day 4, is not known, although it has been observed by others (68). B, primary HSC at day 7 after isolation were placed in serum-free media,

Smad4 is constitutively present in the nucleus and cytoplasm, independent of TGF-β. A, primary HSC at days 1, 4, and 7 were treated plus or minus 100 pM TGF-β1 for 15 min and then lysed. Lysates were separated into nuclear and cytoplasmic fractions and were immunoblotted with antibodies against Smad4 (top panel) and, to confirm the purity of the fractions, HSP70 (middle panel) and PCNA (lower panel). Sizes are indicated in kilodaltons. All three blots were prepared from the same lysate. The results are representative of five independent experiments. We consistently see two faster bands in the cytoplasm at day 4, with a corresponding reduction in the intensity of the major signal. The significance of proteolysis of Smad4, especially at day 4, is not known, although it has been observed by others (68). B, primary HSC at day 7 after isolation were placed in serum-free media, then treated with leptomycin B (20 ng/ml) for 0,15,30, or 60 min, or with TGF-β1 (100 pM; β) or TGF-β and leptomycin B (2β) for 15 min, then lysed and immunoblotted with antibodies against Smad4. There was no significant nuclear accumulation of Smad4 with leptomycin B treatment. Serially stripping the membrane and re-immunoblotting with antibodies against Smad2 and Smad3 gives similar negative findings (data not shown). This blot is representative of results obtained in three separate experiments.

Smad4 Activation—To study the activation of Smad4 in HSC, cytoplasmic and nuclear lysates from cells at different stages of activation were immunoblotted with an antibody against the entire Smad4 coding sequence. There was no significant change in overall levels of Smad4 in cells 1, 4, or 7 days after isolation (Fig. 3A). Treatment with TGF-β for 15 min resulted in minimal increases in nuclear Smad4 at days 1 and 4, although Smad4 was present in the nucleus even in the absence of TGF-β. Immunodetection of blots with cytoplasmic (HSP-70) and nuclear (PCNA) marker proteins confirmed the purity of the different fractions.

A recent study reported the existence of widely expressed Smad4 isoforms that constitutively shuttle between the nucleus and cytoplasm and are able to form complexes with Smad2 in the nucleus (41). To determine whether this accounts for the Smad4 we observed constitutively in the nucleus, we treated day 7 cells with leptomycin B, an inhibitor of CRM1-mediated nuclear export. There was no significant accumulation of Smad4 in the nucleus after leptomycin B treatment, indicating that the shuttling observed by Pierreux et al. (41) does not explain our observations.

Differences in Smad2 and Smad3 Activation—Smad2 phosphorylation in response to TGF-β changed dramatically as cells underwent in vitro activation (Fig. 4). This is shown for both total lysates and nuclear and cytoplasmic fractions. Treatment of cells at days 1 and 4 with TGF-β for 15 min resulted in a dramatic increase in phosphorylation of Smad2 (Fig. 4, A and B) as well as a shift from cytoplasm to nucleus (Fig. 4B). In contrast, Smad3 was phosphorylated and translocated to the nucleus in response to TGF-β primarily at days 4 and 7 (Fig. 4, A and B). Interestingly, there was a significant portion of constitutively phosphorylated and constitutively nuclear Smad2 and, to a lesser extent, Smad3, present at days 4 and 7.

Consistent with the stable total protein levels of Smads 2 and 3 (Fig. 4B and data not shown), no change in the message levels of either Smad was seen with short (0–90 min) or long (24 h) term treatment of cells with TGF-β (Fig. 4C), although an additional transcript for Smad2 was seen at days 4 and 7. This band was specific: an identical pattern was also seen on different blots probed with a different, non-overlapping probe (data not shown). Two or more bands specific for Smad2 by Northern blotting have been reported previously (23, 42, 43), although their significance is not known. A splice variant of Smad2 that, unlike the wild type, is able to bind DNA, has been reported (24) and may account for this additional band, although one recent report demonstrated neither multiple bands for Smad2
nor changes in the splice variant with HSC activation (45). In contrast to other reports (45), we consistently observed a single transcript for Smad3 in HSC.

The FYVE Protein SARA Decreases with Activation—The linker protein SARA recruits Smad2 to the TGF-β receptor complex and is necessary for maximal Smad2-mediated TGF-β signaling (15). The relationship between SARA and Smad3 is less clear, and one report suggests that SARA is required for Smad2- but not Smad3-mediated TGF-β signaling (46). We therefore examined HSC undergoing in vitro activation for changes in SARA expression, looking for correlations between SARA expression and Smad activation. By Western immunoblotting, we noted a dramatic decrease in SARA levels in HSC from day 1 to day 4 after isolation; SARA was not detectable in HSC cultured for 7 days (Fig. 4D).

Constitutively Phosphorylated and Nuclear Smads Do Not Result from Autocrine TGF-β Signaling—We observed a significant amount of constitutively phosphorylated Smads 2 and 3, particularly Smad3, present in the nucleus at days 4 and 7 (Fig. 4B). This does not represent contamination of the different fractions (see controls in Fig. 3A); additionally, two different TGF-β-responsive cell lines (L6 myoblasts and LLC-PK1, proximal tubule epithelial cells) demonstrated phospho-Smad2 only in nuclear fractions.2 One possibility is that this represents a response to autocrine TGF-β production, although increased production of active (as opposed to latent) TGF-β has not been observed in culture-activated cells (12, 47–49), and the responsiveness of our cells to exogenous TGF-β in other assays argues against saturation of the TGF-β signaling machinery. To answer this question definitively, we treated HSC with both TGF-β-neutralizing antibodies (Fig. 5, A and B) and with a specific TβRI kinase inhibitor (Fig. 5, A and B). Neither treatment altered the baseline nuclear localization or constitutive phosphorylation of Smad2 or Smad3, indicating that it is not the result of autocrine TGF-β production.

Autocrine Activin Signaling Does Not Cause Constitutive Smad Activation—It has been reported that the TGF-β superfamily member activin is produced in an autocrine fashion by activated HSC (50). Because activin downstream signaling pathways share Smad2 and Smad3 with TGF-β signaling, it is possible that it is not the result of autocrine TGF-β production.

DISCUSSION

Our data demonstrate in an in vitro model of HSC activation that: 1) there is a dissociation between the growth regulatory and matrix-inducing effects of TGF-β as HSC become activated; 2) activation is associated with a shift in TGF-β signaling pathways such that TGF-β predominantly activates Smad2 in quiescent cells and Smad3 in activated cells; 3) Smad4 is present constitutively in the nucleus and cytoplasm of HSC, and its distribution does not change in response to TGF-β; 4)
overnight with antibodies against TGF-β1, 4, and 7 were treated overnight with 0.1 μg/ml activin A10 (0.06 μg/ml activin A1) and analyzed as in Fig. 2 and data not shown) or against activin A1 or activin signaling. Although the relationship between receptor expression and Smad activation is not well understood, varying receptor expression could explain the decrease in TGF-β-mediated Smad phosphorylation in cells from day 1 to day 7 after isolation, although this does not explain why Smad3 is not phosphorylated in response to TGF-β treatment in day 1 cells. We have noted significant changes in the population of all three TGF-β receptors in HSC at days 1, 4, and 7 of in vitro culture.3 Although the relationship between receptor expression and Smad activation is not well understood, varying receptor expression is a potential cause of differential Smad activation.

Fig. 5. Constitutive activation of Smads 2 and 3 at day 7 is not the result of autocrine TGF-β or activin signaling. A, HSC at days 1, 4, and 7 were treated overnight with 0.1 μg/ml NPC-34016 TβRI inhibitor, then lysed and analyzed by immunoblotting with antibodies against phospho-Smad2, total Smad2, or total Smad3 (the same blot was stripped and reprobed sequentially). A separate blot was probed with antibody against phospho-Smad3. B, HSC at day 7 were treated with either the inhibitor (as in A) or with 1 μg/ml of a pan-TGF-β-neutralizing antibody (T) and analyzed as in A. C, HSC were treated overnight with antibodies against TGF-β (as in B) or against activin (0.06 μg/ml A1 or 0.6 μg/ml A10) overnight, then lysed and immunoblotted as above with antibodies against Smad2 or (using the same lysates) Smad 3. All blots shown are representative of at least three independent experiments.

We clearly demonstrate in this physiological system that there is a dissociation between the cell cycle and matrix effects of TGF-β. TGF-β treatment resulted in growth inhibition in HSC only at day 1, but caused increased expression of matrix components in cells at all stages of in vitro transdifferentiation; although the background expression of matrix components was increased in culture-activated HSC, the -fold induction by TGF-β did not change significantly. Our results are consistent with descriptions of the behavior of HSC in bile duct-ligated rats and smooth muscle cells in human atherosclerotic lesions (3, 51). Interestingly, although there are several reports of engineered or malignant cell lines that have lost TGF-β-mediated growth inhibition while retaining matrix responses, there are no reports of the reverse scenario (52–55), and it has been suggested that there is a higher threshold of Smad activation required for growth inhibition than for matrix induction (3, 52, 56). Whether this is correct has yet to be determined.

In activated rat HSC in culture, TGF-β treatment results in the net deposition of abnormal matrix by up-regulation of multiple matrix components, including proteoglycans, collagen, and fibronectin, and protease inhibitors (11, 13, 37, 40, 57). The effects of TGF-β on quiescent HSC in vitro has not been extensively studied. Our results demonstrating that TGF-β up-regulates matrix production modestly (less than 3-fold) are consistent with the published results of other investigators (13, 36–40), although the various studies use HSC at different time points after isolation. These data and our finding that TGF-β up-regulates matrix production to similar degrees in quiescent and in vitro activated cells (Fig. 2 and data not shown) are consistent with the hypotheses of other investigators that autocrine TGF-β signaling by activated cells is an incomplete explanation for the action of TGF-β in liver fibrosis (49, 59). Although HSC at day 1 after isolation have almost certainly started to transdifferentiate by virtue of being in culture, the data nonetheless show that TGF-β has significant matrix effects well before full activation. The effects on quiescent cells are potentially important in early liver disease and may explain in part why TGF-β is necessary and sufficient for in vivo stellate cell activation and liver fibrosis (2, 4, 5, 60–62).

This is the first description of differential Smad activation in HSC during the process of transdifferentiation. The mechanism for the switch from Smad2 to Smad3 is not obvious, because the total populations of Smads 2 and 3 do not change at the protein or RNA level. In the context of the finding of Goto et al. (46) that SARA is required for Smad2- but not Smad3-mediated TGF-β signaling, the loss of SARA expression as HSC activate could explain the decrease in TGF-β-mediated Smad2 phosphorylation in cells from day 1 to day 7 after isolation, although this does not explain why Smad3 is not phosphorylated in response to TGF-β treatment in day 1 cells. We have noted significant changes in the population of all three TGF-β receptors in HSC at days 1, 4, and 7 of in vitro culture.3 Although the relationship between receptor expression and Smad activation is not well understood, varying receptor expression is a potential cause of differential Smad activation.

Dooley et al. (49) have shown TGF-β-responsive phosphorylation and nuclear localization of Smads 2 and 3 in day 2 HSC but not passaged cells. The same group demonstrated a peak in TGF-β-mediated activation of a Smad3/4-specific luciferase reporter construct in day 4 HSC, although Smad activation was not examined directly in this second report (45). Other investigators, using a spontaneously immortalized line of activated rat HSC, report that Smad2 is activated in response to TGF-β, whereas Smad3 is constitutively activated (63). Some of the differences between these results and ours may relate to the use of passaged or immortalized versus primary activated HSC.

The implications of the observed differential Smad activation for TGF-β signaling specificity are not clear. Although we demonstrate a correlation between TGF-β-mediated growth inhibition and the activation of Smad2, the requirement for individual Smads in TGF-β-mediated responses in HSC is not known, and knockouts of the different Smads in HSC at different time points will be required to determine definitively the signaling pathways required for each response. Roberts et al. (64) hypothesize that Smad3 is a critical element in fibrogenesis in general, although it is notable that HSC from Smad3 null mice undergo normal activation on plastic and show only a minor decrease in collagen α1(1) mRNA (65). Our data demonstrating

3 M. D. A. Gaça and R. G. Wells, manuscript in preparation.
that Smad3 activation occurs late in transdifferentiation in vitro are consistent with the findings from Smad3 null mice, although both results raise questions about the actual function of Smad3 in activated HSC.

The expression of the linker protein SARA in HSC has not previously been described. This protein, which binds phosphatidylinositol 3-phosphate through its FYVE domain, is important for localization of the Smads and their recruitment to the TGF-β receptor complex (15). Our data, showing loss of TGF-β-mediated Smad2 activation coincident with the loss of SARA expression, are consistent with the finding that SARA is required for Smad2- but not Smad3-mediated TGF-β signaling (46). In addition, the recent report that localization of SARA to early endosomes is required for Smad-mediated signaling has interesting implications for understanding TGF-β signaling in HSC (66); basic mechanisms of TGF-β signaling may be different in quiescent cells, which have SARA, and activated cells, which have lost it.

Our data demonstrate constitutive Smad 2 activation in day 7 HSC (Figs. 4 and 5). This could result from endogenous TGF-β production, although our Smad3 data clearly demonstrate that the TGF-β response is not saturated (Fig. 4). We have shown in two different ways, with a TGF-β signaling inhibitor and with neutralizing antibodies, that autocrine TGF-β is not the cause (Fig. 5). This is consistent with published data (12, 47–49) showing that, although there is a significant amount of latent TGF-β produced by transdifferentiated HSC, there is little active ligand produced. Constitutive phosphorylation of Smad2 in HSC from acutely injured rat livers and in immortalized, highly passaged HSC has been reported by Tahashi et al. (67), although this group reports inhibition of the phosphorylation with TGF-β-neutralizing antibodies. Of note, there is a significant amount of phospho-Smad2 in the cytoplasm of HSC, the function of which is not known, although cytoplasmic phospho-Smad2 has been observed in other cells (41). Feldmann et al. (68) have reported sustained phosphorylation of Smads 2 and 3 in both the cytoplasm and nucleus of splenocytes and lymphocytes and have found that this is correlated with resistance to TGF-β-mediated growth inhibition. Our observations in HSC are consistent with the data of Inagaki et al. (63) using an immortalized line of HSC. It was recently reported that there are widely expressed and functional Smad4 splice variants that shuttle constitutively between nucleus and cytoplasm (41). We did not observe accumulation of Smad4 in leptomycin B-treated cells, however, making this an unlikely explanation. Smad2 and Smad4 can form active complexes in the nucleus as well as the cytoplasm, suggesting that Smad4 could play a role in TGF-β signaling in HSC even in the absence of significant cytoplasmic to nuclear translocation (41).

In summary, we demonstrate that Smad signaling pathways can be differentially activated during cellular differentiation, specifically in primary HSC in culture. We are currently examining whether differential Smad activation is responsible for the differences in TGF-β-mediated growth inhibition and matrix induction in quiescent and activated HSC. The results from these studies will be important to our understanding of TGF-β signaling as well as to the design of rational anti-fibrotic therapies.

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TGF-β-induced Smad4 nuclear translocation in our system was minimal, especially in activated HSC. A similar observation has been made in an immortalized line of activated HSC (63). This finding may indicate that TGF-β signaling in HSC is Smad4-independent, as has been shown for some TGF-β responses in other cell systems (44, 73). We also observed a significant fraction of Smad4 in the nucleus constitutively, consistent with the data of Inagaki et al. (63) using an immortalized line of HSC. It was recently reported that there are widely expressed and functional Smad4 splice variants that shuttle constitutively between nucleus and cytoplasm (41). We did not observe accumulation of Smad4 in leptomycin B-treated cells, however, making this an unlikely explanation. Smad2 and Smad4 can form active complexes in the nucleus as well as the cytoplasm, suggesting that Smad4 could play a role in TGF-β signaling in HSC even in the absence of significant cytoplasmic to nuclear translocation (41).
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