Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart

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Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. Am J Physiol Heart Circ Physiol 282: H1685–H1696, 2002. First published January 17, 2002; 10.1152/ajpheart.00266.2001.—We examined the role of the transforming growth factor (TGF)-β1 signaling inhibitor Smad 7 in cardiac fibrosis. TGF-β1 (10 ng/ml) was found to increase cytosolic Smad 7 expression in primary adult rat fibroblasts and induce rapid nuclear export of exogenous Smad 7 in COS-7 cells. Furthermore, overexpression of Smad 7 in primary adult fibroblasts was associated with suppressed collagen type I and III expression. We detected Smad 7, phosphorylated Smad 2, TGF-β type I receptor (TβRI), and TGF-β1 proteins in postmyocardial infarct (MI) rat hearts. In 2 and 4 wk post-MI hearts, Smad 7 and TβRI expression were decreased in scar tissue, whereas TGF-β1 expression was increased in scar and viable tissue. In the 8 wk post-MI heart, Smad 7 expression was decreased in both scar tissue and myocardium remote to the infarct scar. Finally, we confirmed that these changes are paralleled by decreased expression of cytosolic phosphorylated receptor-regulated Smad 2 in 4-wk viable myocardium and in 2- and 4-wk infarct scar tissues. Taken together, our data imply that decreased inhibitory Smad 7 signal in cardiac fibroblasts may play a role in the pathogenesis of cardiac fibrosis in the post-MI heart.

primary cardiac fibroblasts; transforming growth factor-β1; experimental heart failure; myocardial infarction

MYOCARDIAL INFARCTION (MI) is a common etiology for the development of heart failure and is marked by alteration of the extracellular matrix (ECM) as well as the spatial reorientation of cells and intracellular matrix proteins (44). In the myocardium, cardiac fibroblasts and myofibroblasts are the sole sources for fibrillar collagens, which dominate the cardiac matrix. After MI, the infarct zone is replaced by scar tissue characterized by deposition of the ECM (2). Cardiac fibrosis also occurs in noninfarcted segments of myocardium and contributes significantly to the dysfunction of the failing heart (21). Further elucidation of the mechanism by which myocardial fibrosis is regulated is of great interest as a potential means to limit myocardial remodeling and dysfunction. As a member of a cytokine family that may control a broad range of biological responses on many cell types, transforming growth factor (TGF)-β1 has been shown to be a potent stimulus for matrix deposition by increasing the expression of specific ECM components such as collagen and fibronectin. Furthermore, TGF-β1 also functions to upregulate the expression of ECM protease inhibitors such as plasminogen activator inhibitor and tissue inhibitors of matrix metalloproteinases while simultaneously downregulating proteases that degrade matrix components, such as interstitial collagenase (27, 38). TGF-β1 signaling occurs via ligand-induced heteromeric complex formation of type I and type II serine/threonine kinase receptors (46). After receptor activation, the signal is propagated downstream through the recently identified Smad protein family (23). In the mammalian heart, Smads can be divided into three major groups: the receptor-regulated Smads (R-Smads: Smad 2 and Smad 3), common mediator Smad (Co-Smad: Smad 4), and inhibitory Smads (I-Smads: Smad 6 and Smad 7) (28). Upon TGF-β1 receptor activation, R-Smads are phosphorylated and form a dimer with Co-Smad. The R-Smad-Co-Smad complex translocates to the nucleus, where the dimer can directly or indirectly, through interactions with other transcription factors, regulate specific gene transcription (24).

Smad 6 and Smad 7 have recently been reported to form a stable interaction with the activated TGF-β type I receptor (TβRI), thereby preventing the binding to and activation of R-Smads (16, 17, 31). Smad 7 has been shown to inhibit signal transduction by TGF-β1 and activin receptors (16, 31), whereas Smad 6 has been reported to inhibit bone morphogenetic protein (BMP) signaling (15, 17). All Smad proteins share two regions of sequence similarity: the MH1 domain at the NH2-terminus and the MH2 domain at the COOH-terminus. I-Smads are structurally different from other Smad family members in that they lack the SSXS phosphorylation motif on the MH2 region (15, 17), whereas Smad 6 has been shown to inhibit signal transduction by TGF-β1 and activin receptors (16, 31), whereas Smad 6 has been reported to inhibit bone morphogenetic protein (BMP) signaling (15, 17). All Smad proteins share two regions of sequence similarity: the MH1 domain at the NH2-terminus and the MH2 domain at the COOH-terminus. I-Smads are structurally different from other Smad family members in that they lack the SSXS phosphorylation motif on the MH2 region and they possess shorter MH1 domains (17, 31). Smad 7 expres-
sion is induced by TGF-β1 in several cell types (25, 30), and these findings indicate that Smad 7 may act via an autoregulatory negative feedback loop. With the use of gene disruption methods in mice, Galvin et al. (12) observed that forced expression of lack of function Smad 6 mutants are associated with abnormal cardiac valve morphology, abnormal development and, in the adult cardiovascular system, ossification of aortic tissue.

Previous work from our laboratory (13) has shown that cardiac Smad 2, 3, and 4 proteins are significantly increased in border and scar tissues, indicating that Smad signaling may be involved in cardiac fibrosis by stimulation of matrix deposition. Furthermore, in the mouse model of bleomycin-induced pulmonary fibrosis, overdriven exogenous I-Smad 7 treatment of lung tissue was associated with an antifibrotic effect (32). Nevertheless, the relationship between I-Smad 7 and the profibrotic effects of TGF-β1 remains unknown. In the present study, we characterized the negative regulation of collagen expression by I-Smad 7 in cultured primary cardiac fibroblasts and noted the Smad 7 expression pattern in post-MI rat hearts at different points in the development of heart failure.

MATERIALS AND METHODS

Primary Adult Cardiac Fibroblast Culture

Adult cardiac fibroblasts cultures were established from the ventricular tissue of male Sprague-Dawley rats according to the methods of Brilla et al. (4) with minor modifications (19). Adult rats (175–200 g body wt) were euthanized, and the hearts were subjected to Langendorff perfusion with a flow of 5 ml/min at 37°C with recirculating Joklik’s medium containing 0.1% collagenase (chaotrophic agent) and 2% bovine serum albumin (BSA) for 25–35 min. Liberated cells were collected by centrifugation at 2,000 rpm for 10 min. Cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM-F-12) and plated on a 100-mm noncoated culture flask at 37°C with 5% CO2 for 2 h. Cardiac fibroblasts attached to the bottom of the culture flask during a 2-h incubation, whereas nonadherent myocytes were removed by washing; i.e., changing the culture medium. The cells were maintained in DMEM-F-12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells used for the study were from the second passage (P2), and the purity of fibroblasts used in these experiments was found to be ≥95% using routine phenotyping methods described previously (19, 34). Briefly, endothelial cells were labeled with the use of a monoclonal antibody against factor VIII, and we found that less than ~1% of cultured cells were positive for this protein. Less than 1% of cells were positive for desmin, which is specific for smooth muscle cells, and less than ~1% of cultured cells stained positively for α-smooth muscle actin, which is produced in smooth muscle cells and myofibroblasts. More than 95% of cells in our P2 cultures stained positively for procollagen type 1, which is a major protein product of fibroblasts.

COS-7 Cell Culture

We wanted to achieve a relatively high percentage of transient transfection with foreign DNA constructs in fibroblasts in a subset of experiments, and for this reason the COS-7 cell line (transformed Green Monkey kidney fibroblasts) was employed. The frozen seed cultures were a kind gift from Dr. Peter Zahiradka (obtained from American Type Culture Collection). COS-7 cells were grown in DMEM-F-12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Experimental Rat Model of MI

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba (Manitoba, Canada) following guidelines established by the Canadian Institutes of Health Research and the Canadian Council of Animal Care (2001). The MI model was produced in male Sprague-Dawley rats (150–175 g body wt) by surgical occlusion of the left coronary artery, as previously described (9). The mortality of the animals operated on in this fashion was ~30% within 48 h. Experimental animals were euthanized after 2, 4, or 8 wk, and cardiac tissue was isolated from two left ventricular (LV) regions: remnant/viable (noninfarcted LV free wall remote from the infarct scar and septum) and infarct scar. Tissues from these regions and the tissues from sham-operated rats were used for Western blot analysis to quantify Smad 7, phosphorylated Smad 2, TβRI, and TGF-β1 expression; these tissues were also used for immunohistochemistry to define the localization of Smad 7 and phosphorylated Smad 2.

Determination of Infarct Size in Experimental Animals

After 2, 4, or 8 wk, respectively, rats were euthanized and hearts were excised. The LV was fixed by immersion in 10% formalin and embedded in paraffin. Six transverse slices were cut from the apex to the base, and serial sections (5 μm) were cut and mounted. The percentage of infarcted LV was estimated after coronary ligation by planimetric techniques, as previously described (20). Animals with an infarct size <40% of the LV free wall were excluded.

Transient Transfection of Fibroblasts

Confluent (60–70%) primary (P2) cardiac fibroblasts as well as COS-7 cells in DMEM-F-12 containing 10% fetal bovine serum were transiently transfected with a Flag epitope-tagged Smad 7 expression vector (the kind gift of Dr. P. ten Dijke, Ludwig Institute for Cancer Research; Uppsala, Sweden) using Effectene (Qiagen; Mississauga, Ontario, Canada), according to the manufacturer’s instructions. Twenty-four hours after transfection, fibroblasts were double stained with anti-Flag antibody and collagen type I/III antibodies. In other experiments, COS-7 cells were incubated with DMEM-F-12 containing 1% fetal bovine serum in the absence and presence of TGF-β1 (10 ng/ml) for 15 min, 30 min, 1 h, and 2 h.

Protein Extraction and Assay

Cardiac tissues from sham-operated LVs, viable LVs, and scar regions were homogenized in 100 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin. This homogenate was sonicated for 5 s (repeated 5 times). To isolate the cytosolic fractions, the samples were centrifuged at 3,000 g for 10 min at 4°C. The resulting supernatant was further subjected to centrifugation at 48,000 g for 20 min at 4°C. The supernatant was used for the cytosolic Smad 7 and TGF-β1 protein assay. The pellet (membrane fraction) was used for immunoreactive TβRI expression analysis. For phosphorylated Smad 2 protein detection,
Western Blot Analysis of Target Proteins

Prestained low-molecular-weight marker (Bio-Rad; Hercules, CA) and 30 μg protein from samples were separated on 10% or 12% SDS gels by SDS-PAGE. Separated protein was transferred onto a 0.45 μM polyvinylidene difluoride membrane that was blocked at room temperature for 1 h or overnight at 4°C in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% skim milk and probed with primary antibodies. The primary antibody against Smad 7 was diluted 1:200 in TBS-T, whereas the TGF-β1 primary antibody was diluted 1:250 in TBS-T, as was the primary phosphorylated R-Smad 2 antibody. The primary antibody for β-tubulin was diluted 1:250 in TBS-T, and the β-tubulin monoclonal antibody was diluted 1:500 in TBS-T. Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-goat IgG for detection of I-Smad 7, anti-rabbit for TGF-β1, βtubulin, and phosphorylated R-Smad 2. All secondary antibodies were diluted 1:10,000 with TBS-T. Protein bands on Western blots were visualized by ECL Plus (Amersham; Arlington Heights, IL) according to the manufacturer’s instructions and were developed on film or using a Molecular Dynamics chemiluminescence detector (Storm 860, Amersham-Pharmacia). Blocking peptides of TGF-β1 were used to identify the band specific to this protein. Relatively even protein loading from cytosolic and membrane fractions was confirmed by a Western blot of β-tubulin and Coomassie blue staining, respectively.

Immunofluorescence Assays

Double staining of cultured primary fibroblasts. Adult cardiac fibroblasts were plated on coverslips and allowed to grow for 24 h. Cells were transiently transfected with Flag-tagged Smad 7 using Effectene (Qiagen). The cells were fixed with 4% paraformaldehyde after 48-h transfection. For double staining, monoclonal mouse anti-Flag antibody (1:1,000 with 3% BSA in PBS) and anti-goat collagen type I/III antibody (1:100 with 1% BSA in PBS) were added to the slides at the same time. After PBS wash, the sections were washed with PBS, incubated with biotinylated anti-goat IgG secondary antibody, and subsequently incubated with fluorescein isothiocyanate (FITC)-labeled streptavidin for 90 min. To distinguish anti-Flag antibody from anti-collagen antibodies, an anti-mouse-linked Texas Red conjugate, and FITC-labeled streptavidin were purchased from Amersham-Pharmacia (Arlington Heights, IL), Collagen type I and type III primary antibodies were from Southern Biotechnology (Birmingham, AL), and anti-Flag antibody was obtained from Sigma (Oakville, Ontario, Canada). TGF-β1 peptide was purchased from R&D Systems (Minneapolis, MN). The Flag protein tag is a trademark of Kodak (New Haven, CT) and was carried out using proprietary reagents.

Statistics

All values are expressed as means ± SE. One-way ANOVA followed by Student-Newman-Keuls methods were used for comparing the differences among multiple groups (SigmaStat). Significant differences among groups were defined by a probability ≤ 0.05.
RESULTS

Localization of Endogenous Smad 7 and Phosphorylated Smad 2 After TGF-β1 Induction in Primary Cardiac Fibroblasts

In quiescent fibroblasts (COS-1 cells), Smad 7 is known to localize in the nucleus and undergo export to the cytoplasm upon cotransfection of TβR-I, in the cytosol, Smad 7 may inhibit activation of receptor-regulated Smads by TβR-I (18). To determine the effect of TGF-β1 on endogenous Smad 7 expression and localization in primary cardiac fibroblasts, both nuclear and cytoplasmic proteins were extracted from cells treated with TGF-β1 (10 ng/ml) at 15 min and 1, 2, and 6 h and examined by Western blot analysis. In the cytoplasmic fraction, we observed a stimulatory effect on Smad 7 expression in the presence of TGF-β1 at various times. Lane-to-lane protein loading normalization was carried out using laser densitometry and the band specific for actin. The values of the signal ratio of Smad 7 to actin is 0.082 in the absence of TGF-β stimulation, 0.169 after 1-h TGF-β1 stimulation, and 0.089 at 2-h TGF-β1 stimulation. Therefore, Smad 7 protein induction (shown in Fig. 1) in the cytosolic fraction peaked at 60 min after TGF-β1 treatment and then returned to the basal level at 2 h after stimulation, suggesting that Smad 7 was rapidly and transiently induced in the cytoplasmic fraction by TGF-β1 treatment. These data did not allow us to resolve whether this change was a translocation event or that of simple induction. To clarify this issue, subcellular localization of Smad 7 was investigated by indirect immunofluorescence in primary fibroblasts in the presence and absence of TGF-β1. The results (shown in Fig. 2) revealed relatively low but detectable levels of endogenous cytoplasmic Smad 7 in quiescent primary cardiac fibroblasts in the absence of TGF-β1 (Fig. 2A). After 1-h TGF-β1 stimulation, we observed an increase in the staining intensity for Smad 7 protein (Fig. 2F). With this treatment, almost all Smad 7 staining remained localized in the cytoplasmic space, indicating that TGF-β1 treatment does not markedly alter subcellular endogenous Smad 7 localization in primary cardiac fibroblasts in the presence of TGF-β1. For the purpose of comparison, we investigated phosphorylated Smad 2 localization using the same cultured cells under similar conditions. As indicated in Fig. 2, A and B, 1-h TGF-β1 treatment of fibroblasts caused a relative translocation of phosphorylated Smad 2 into the nuclei, in contrast to the Smad 7 staining pattern. The phosphorylated Smad 2 translocation pattern depicted is representative of four independent experiments, confirming our previous results (14), and showed a high degree of reproducibility.

Exogenous Smad 7 Translocation After TGF-β1 Stimulation

To further understand TGF-β1-dependent subcellular Smad 7 activation and translocation in fibroblasts, we transfected COS-7 cells with a Flag-Smad 7 expression construct. Localization of Smad 7 in transfected cells was investigated in the presence of TGF-β1 (10 ng/ml) treatment at durations of 15 and 30 min and 1 and 2 h by indirect immunofluorescence analysis. In contrast to our results obtained with endogenous Smad 7 in primary fibroblasts, ectopic Smad 7 was localized primarily in cell nucleus of COS-7 cells in the absence of TGF-β1 (Fig. 3A). No significant change of Smad 7 localization was noted after 15-min TGF-β1 treatment (Fig. 3B). However, exogenous Smad 7 was almost completely translocated to the cytoplasm after exposure to TGF-β1 for 30 min (Fig. 3C). Immunoreactive Smad 7 staining indicated a trend of movement back to the nucleus after 1-h exposure to TGF-β1 (Fig. 3D), which is indicated by perinuclear and nuclear localization. At 2 h after the onset of TGF-β1 stimulation, Smad 7 was observed to localize primarily within the nucleus; the staining pattern at this time was similar to that of the control and 15-min results (Fig. 3E).

Effect of Overdriven Smad 7 on the Expression of Fibrillar Collagens in Primary Cardiac Fibroblasts

TGF-β1 is known to stimulate the transcription of collagen genes and deposition of collagen protein and is involved in the progression of fibrosis in cardiovascular disease. The recent identification and investigation of Smad proteins has provided novel insights regarding TGF-β1 signaling in these cells (13, 14). In this set of experiments, we investigated whether overdriven Smad 7 exerts a direct influence on fibrillar collagen expression in primary cardiac fibroblasts. Flag epitope-labeled Smad 7 was transiently transfected into primary cardiac fibroblasts (P2 generation). The transfection efficiency was estimated at 10% via calculation of positively stained cells after probing with anti-Flag antibody. We observed that ectopic Flag-labeled Smad 7 was localized in both the nucleus and cytoplasm of these cell cultures. The relative expression of fibrillar collagen I or collagen III in these Smad 7-transfected fibroblasts was detected using double immunofluorescence analysis. We found that Smad 7-transfected fibroblasts showed decreased protein expression of collagen type I (a representative transfected fibroblast is...
shown in Fig. 4A) and type III (Fig. 4B) in direct comparison with neighboring nontransfected fibroblasts. This result was consistent between transfected cells with an incidence of ~90% in positive transfec-
tants. Thus transient overexpression of Smad 7 is associated with attenuation of the expression of fibril-
lar collagens in primary cardiac fibroblasts.

Fig. 2. Immunofluorescent staining of phosphorylated receptor-regulated Smad (R-Smad) 2 (left) and I-Smad 7 (right) in cultured adult cardiac fibroblasts stimulated with TGF-β1 (10 ng/ml) for 1 h. A: untreated fibroblasts stained for phosphorylated R-Smad 2; B: TGF-β1-treated fibroblasts stained for phosphorylated R-Smad 2; E: untreated fibroblasts stained for I-Smad 7; F: TGF-β1-treated fibroblasts stained for I-Smad 7. C, D, G, and H: nuclei (Hoechst 33342 staining) of identical sections corresponding to A, B, E, and F, respectively. Original magnification, ×400.

Smad 7, Phosphorylated Smad 2, TGF-β1, and TβRI Expression in Different Stages of In Vivo Cardiac Fibrosis

To investigate the association between Smad 7 and the development of cardiac fibrosis in vivo, male Sprague-Dawley rats were used to generate experi-

Fig. 3. Immunofluorescent staining of ectopically expressed Flag-Smad 7 in COS-7 cells treated with TGF-β1 (10 ng/ml). COS-7 cells were transfected with Flag-labeled Smad 7. A: untreated COS-7 cell transfected with Smad 7; B–E: TGF-β1-treated COS-7 cell at indicated times; F–J: nuclei (Hoechst 33342 staining) of the identical fields corresponding to A–E, respectively. Original magnification, ×400.
mental MI as previously described (9). The experimental model was characterized by the presence of large MI with an average total infarct scar weight of 0.28 ± 0.026 g, which is comparable to values reported earlier (13). We have previously determined that graded fibrosis is present at 2, 4, and 8 wk post-MI and that this pattern is positively correlated to the development of frank heart failure. Immunofluorescent staining of experimental tissue revealed that Smad 7 protein was mainly localized to cells occupying the cardiac interstitium and that the expression of Smad 7 was decreased in those cells within the infarct scar compared with both sections taken from age-matched sham-operated hearts and viable (LV myocardium from infarcted hearts remote to the infarct scar) in 8 wk post-MI hearts (Fig. 5). Western blot analysis was used to determine the cytosolic Smad 7 protein expression from sham-operated, viable, and scar groups in 2, 4, and 8 wk post-MI hearts. In addition, the dimeric immunoreactive form of TGF-β1 (25-kDa band) as well as the cytosolic phosphorylated Smad 2 (62-kDa band) were detected in 2 and 4 wk post-MI hearts. The expression patterns of TGF-β1 and phosphorylated Smad 2 in 8 wk post-MI hearts have been characterized in previous work from our lab, and this data was confirmed but not reproduced herein (14). In 2 and 4 wk post-MI hearts, Smad 7 protein (43 kDa) expression was significantly decreased in the infarct scar compared with sham and viable (surviving) myocardium (Fig. 6). In the 8 wk post-MI group, immunoreactive Smad 7 staining was significantly decreased in both infarct scar and viable myocardium tissue slices, with a greater decrease in scar tissue compared with that in viable tissues (Fig. 6). The 25-kDa band specific for TGF-β1 was elevated in both infarct scar and neighboring viable tissue samples (the myocardium that is remote to the infarct scar) in 2 and 4 wk post-MI hearts (Fig. 7), in agreement with our previous findings (14). The increase in TGF-β1 expression at the scar site was greater than the trend observed in neighboring viable tissue. Western blot analysis of immunoreactive TβRI level revealed a decrease in the infarct scar in 2 wk post-MI tissue, whereas it was decreased in both the viable and infarct scar tissue in 4 wk tissue (Fig. 8). Cytosolic

Fig. 4. Smad 7 overexpression and collagen expression in cardiac fibroblasts. A: representative cultured primary cardiac fibroblasts stained for immunoreactive exogenous (transfected) Smad 7 protein and the identical field stained for collagen type I and for cellular nuclei (Hoechst 33342 staining). B: same as in A, but double stained for collagen type III. Arrows, identical cell stained for both Smad 7 and fibrillar collagen proteins. Original magnification, ×400.

Fig. 5. Smad 7 localization in the 8 wk post-myocardial infarction (MI) rat heart. Top: immunoreactive Smad 7 shown in a sham-operated control heart, viable tissue, and infarct scar tissue. Bottom: nuclear staining of each section as indicated. Magnification, ×400.
phosphorylated Smad 2 was decreased only in infarct scar tissue in 2 wk post-MI compared with sham-operated control samples, whereas its expression was attenuated in both scar and viable tissue samples in 4 wk post-MI hearts (Fig. 9). The attenuation of phosphorylated Smad 2 expression in infarct scar was also significant compared with viable tissue sample values. To further clarify whether transloca-
tion of phosphorylated Smad 2 exists in the infarcted heart, the expression of phosphorylated Smad 2 was assayed in 2 and 4 wk post-MI infarct scar by immunofluorescence analysis. Immunoreactive phosphorylated Smad 2 colocalized with cellular nuclei within the scar (Fig. 10, C and F), a trend that was not apparent in the bordering myocardium. Thus the translocation of phosphorylated Smad 2 from the cytoplasm to nuclei is variable in different regions of the infarcted heart, and this trend is highly apparent in the cells occupying the infarct scar.

Fig. 8. Western blot analysis of TGF-β1 type I receptor (TβRI) in 2 and 4 wk post-MI hearts. A: representative Western blots showing TβRI (53 kDa) protein in sham-operated control hearts (lane 1) and viable tissue (lane 2) as well as scar tissue (lane 3) from 2 and 4 wk post-MI left ventricular samples. B: membranes from experiments shown in A were stained with Coomassie blue to verify relatively even protein loading among lanes. C: histogramic representation of quantified data from multiple samples from groups shown in A (quantified by densitometric scanning). Data are means ± SE of 4–6 experiments. *P ≤ 0.05 vs. shams; †P ≤ 0.05 vs. viable tissue.

Fig. 9. Cytosolic phosphorylated R-Smad 2 in 2 and 4 wk post-MI hearts. A: representative Western blots showing phosphorylated Smad 2 (62 kDa) protein in sham-operated control hearts (lane 1) and viable tissue (lane 2) as well as scar tissue (lane 3) from 2 and 4 wk post-MI left ventricular samples. B: Western blots of β-tubulin in the same tissue samples showing relatively even protein loading among lanes. C: histogramic representation of quantified data from multiple samples from groups shown in A (quantified by densitometric scanning). Data are means ± SE of 4–6 experiments. *P ≤ 0.05 vs. shams; †P ≤ 0.05 vs. viable tissue.
DISCUSSION

Cellular Distribution and Smad 7 Function

TGF-$\beta_1$ signal transduction relies upon the rapid TβRI-mediated phosphorylation of R-Smads followed by R-Smad/Co-Smad dimerization and translocation to the cellular nucleus. I-Smads function to dampen this signal by a negative feedback loop in response to the same stimulus (TGF-$\beta_1$), and this modulation is optimized by appropriate subcellular distribution (29). Quiescent or nonphosphorylated R-Smad 2 and R-Smad 3 are predominantly localized in the cytoplasm (26, 33). However, it is in the nucleus where the Smad complex binds to target genes as the endpoint of the TGF-$\beta_1$ signal, and thus the net R-Smad signal to the nucleus is an important determinant of TGF-$\beta_1$ function (24, 47). It is known that Smad 7 regulates TGF-$\beta_1$ ligand-initiated signaling by competing with the receptor-regulated Smads for receptor-based phosphorylation and activation and that this function takes place in the cytoplasm (16). Endogenous I-Smad 7 is not constitutively expressed but appears to be rapidly induced by TGF-$\beta_1$ in several cell types including different fibroblast cell lines and dermal primary fibroblasts (7, 25). However, the TGF-$\beta_1$ induction pattern and subcellular distribution of I-Smad 7 have not been investigated in primary cardiac fibroblasts. Also, little information is available regarding I-Smad 7 function in cardiac fibroblasts, which in turn contributes exclusively to deposition of cardiac matrix proteins (10, 44).

In the present study, we characterized the basal expression of I-Smad 7 in primary cardiac fibroblasts and demonstrated that its expression is transiently induced by TGF-$\beta_1$ stimulation. Furthermore, we found that endogenous I-Smad 7 expression is localized to the cytoplasm in the absence and presence of TGF-$\beta_1$. This result suggests that, although TGF-$\beta_1$ transiently activates I-Smad 7 protein, it does not mediate a significant shift in the subcellular localization of I-Smad 7 in these cells. An earlier report has shown that endogenous I-Smad 7 in dermal primary fibroblasts was localized in the nuclei in the absence or presence of TGF-$\beta_1$; this response is not consistent with the putative regulatory role of I-Smad 7 (30). Nevertheless, this apparent difference in subcellular distribution of I-Smad 7 in primary cells may reflect tissue specificity in cellular responses. In an effort to further characterize TGF-$\beta_1$ ligand-induced intracellular trafficking of I-Smad 7, COS-7 cells were transiently transfected with Flag-labeled I-Smad 7. We observed that ectopically
expressed Smad 7 was localized mainly in the nuclei in quiescent cells and also noted marked rapid translocation of immunoreactive I-Smad 7 to the cytoplasm with the onset of TGF-β1 treatment (30 min). This response was biphasic insofar as immunodetectable I-Smad 7 was noted to predominantly localize within the inter-nuclear space 2 h after the onset of TGF-β1 treatment. On the basis of these data, we suggest that I-Smad 7 activation by TGF-β1 (e.g., presence in the cytoplasm) in these cells is transient. Previous reports that address ectopically expressed I-Smad 7 have shown that recombinant I-Smad 7 is localized in the cytoplasm in mink lung epithelial cells (48) or in the nuclei in COS-1 cells (18). Together, these data also support the supposition that a cell-specific difference exists for the distribution of I-Smad 7. In the present study, the export of Flag-Smad 7 from the nucleus to cytoplasm in COS-7 cells stimulated with TGF-β1 was not consistent with the primary cardiac fibroblast results. This disparity might be related to the differences between immortalized and primary cells and/or the behavior of endogenous versus recombinant exogenous protein. Nevertheless, both sets of results demonstrated that relatively rapid Smad 7 activation and/or expression in the cytoplasm is responsive to TGF-β1 in primary and immortalized fibroblasts.

Smad 7 and the Expression of Collagen in Cardiac Fibroblasts

TGF-β1 is a major stimulus for tissue fibrosis by enhancing the synthesis of collagens and other matrix components as well as regulating fibroblast differentiation, proliferation, and apoptosis (22, 28). The basis for TGF-β1 regulation of collagen synthesis has developed rapidly since earlier studies identified the Smad family (37). Both R-Smads and Co-Smad 4 are necessary for the transcriptional activation of collagen genes (6, 42). As an inhibitory Smad protein, Smad 7 was found to reduce the basal activity of a2(I) procollagen gene (COLIA2) in a transient transfection experiment in skin fibroblasts (7). A recent in vivo study showed that adenoviral Smad 7 gene overexpression in lung tissue was associated with a diminution in lung fibrosis induced by bleomycin, whereby suppression of type I procollagen mRNA and reduced hydroxyproline content in treated mice were demonstrated (32). However, the functional relationship between Smad 7 and collagen gene expression in cardiac fibroblasts is still undefined.

In the heart, fibrillar collagens types I and III are the major components of the cardiac ECM (43), and it is known that changes in the amount and distribution of fibrillar collagens have adverse effects on the functions of the heart (8). I-Smad 7 transfection of primary fibroblasts was associated with decreased protein expression level of collagen type I/III compared with untransfected fibroblasts. These results indicate that ectopically expressed I-Smad 7 could efficiently attenuate the normal induction of expression of collagen genes in primary cardiac fibroblasts. However, it remains unclear whether this blockade is TGF-β dependent, and a putative direct effect of I-Smad 7 on fibrillar collagen synthesis in cardiac fibroblasts remains to be elucidated.

Smad 7 Expression and TGF-β1 Activation in MI Hearts

Myocardial fibrosis is defined as a significant rise in collagen concentration or collagen volume fraction above normal values (43). In the early stages of cardiac remodeling after MI, cardiac fibrosis occurs within the infarct site to promote normal wound healing; however, ongoing fibrosis in this region and in surviving LV tissue remote to the infarct site and right ventricles will eventually lead to abnormal myocardial stiffness and ultimately to ventricular dysfunction (45). In the experimental rat model of MI, the time points of 2, 4, and 8 wk after induction of infarction has been characterized as early stage of infarct with ongoing healing of the infarct scar, early stage MI with healed scar, with modest fibrosis of remnant fibrosis and overt cardiac fibrosis stages, respectively (11). The 8-wk time point is marked by significant alteration of LV geometry and remodeling (9). TGF-β1 is a powerful stimulus for myocardial remodeling of both myocyte and non-myocyte cardiac components, and continuous TGF-β1 activation is associated with promotion of pathological hypertrophy and myocardial fibrosis (3). It has been shown that the expression of TGF-β1 is increased in the infarct zone in the rat MI model (36), and this increase precedes the elevation of ECM proteins (41). With the use of the rat MI model, we noted elevated expression of TGF-β1 in scar and viable tissue both in 2 and 4 wk post-MI hearts, with a greater increase in TGF-β1 protein at the site of infarction compared with viable tissue. Previous work from our laboratory (13) showed similar TGF-β1 expression patterns in 8 wk post-MI rat hearts. Although increased expression of TGF-β1 does not necessarily equate to increased activation of this cytokine, these data show that TGF-β1 is expressed at elevated levels in the infarcted heart at the early stage of MI and remains overexpressed through to the chronic phase of MI.

As the downstream effectors of TGF-β signaling, Smad proteins have been implicated in MI and in the subsequent cardiac remodeling process. Smad 2, Smad 3, and Smad 4 have been shown to be significantly increased in the border and scar tissue (13), whereas phosphorylated R-Smad 2 is decreased in the cytosolic fraction of border and scar tissue in 8 wk post-MI hearts (14). In bleomycin-induced lung fibrosis mice, I-Smad 7 has been shown to prevent TGF-β1-mediated fibrosis (32). In the present study, we addressed the role of inhibitory I-Smad 7 in myocardial fibrosis and noted decreased expression of Smad 7 both in viable tissue and scar tissue in animals with overt heart failure, with a greater decrease in the infarct scar than in remote remnant tissues. In 2 and 4 wk post-MI hearts, decreased expression of I-Smad 7 was only detectable in the infarct scar itself. Because the infarct scar is populated mainly by fibroblasts and myofibro-
blasts at 4 wk and beyond, and their main function is to mediate matrix deposition, these results support the regulatory role of I-Smad 7 in myocardial fibrosis formation.

In noninfarcted sham-operated hearts, I-Smad 7 protein is expressed at basal level and may be linked to baseline inhibition of effect on the basal TGF-β1 signal. In the infarcted heart, it is possible that elevated expression of TGF-β1 in the scar and viable tissue might suppress the activation of I-Smad 7 in the post-MI heart. Although TGF-β1 was shown to induce the expression of Smad 7 in fibroblasts in this study, this induction was transient and therefore indicated the existence of a negative feedback loop within the pathway. Thus the chronic presence of relatively high levels of TGF-β1 from 2 to 8 wk may result in decreased expression of I-Smad 7 in viable tissue after 8 wks. This effect could play a role in contributing to cardiac fibrosis and heart failure, and we suggest that Smad 7 responses are not confined to TGF-β1 signaling per se. Nevertheless, other factors may complement or antagonize I-Smad 7 function. For example, other members of the TGF-β superfamily may have effects on the expression and function of Smad 7 (1). Outside the classic TGF-β signaling pathway, interferon-γ is known to augment I-Smad 7 expression with attenuated nuclear accumulation of R-Smads (40), and it is possible that other growth factors, cytokines, or hormones may also regulate I-Smad 7 function and/or expression by unknown mechanisms. The full scope of the interplay of TGF-β1, TGF-β superfamily members, and novel ligands is unclear in this regard and requires further study.

I-Smad 7 associates with activated TGF-β1 receptors and interferes with the activation of R-Smad 2 by competing for receptor interaction and phosphorylation (16, 32); therefore, we sought to define the changes in TpRI and phosphorylated R-Smad 2 in 2 and 4 wk post-MI hearts. We found that TpRI expression was decreased in the infarct scar at 2 wk and decreased in both viable and scar tissue in the 4 wk post-MI heart compared with samples from age-matched sham hearts. The mechanism that subserves this trend is unclear. We speculate that the decreased expression of TpRI is linked in a negative-regulatory capacity to increased levels of TGF-β1 in the infarcted heart. Decreased receptor levels may be a response to continuous or prolonged TGF-β1 stimulation and may play an important role in maintaining a balanced TGF-β1 signal. TGF-β receptor downregulation was noted by Centrorella and colleagues (5) in experiments wherein plated primary osteoblasts were stimulated with exogenous TGF-β1. Amplification of signal via the remaining ligand-activated TpRI via repeated phosphorylation of R-Smad 2 proteins on Smad anchor for receptor activation (SARA) docking proteins (for a review, see Ref. 28) may explain the continued operation of this pathway. Our previous data show that total Smad 2 (unphosphorylated and phosphorylated Smad 2) is increased in the total protein (i.e., cytosolic protein plus nuclear protein) isolated from infarct scar and viable tissue compared with sham tissue (14). Together with the reduction of phosphorylated Smad 2 in the cytosolic fraction in infarct scar at 2 wk, noted as well as in scar and viable tissues at 4 wk, we suggest that decreased expression of phosphorylated Smad 2 may be due to the translocation from the cytoplasm to nucleus. This conclusion is also supported by our immunofluorescence analysis of 2- and 4-wk infarct scars showing that phosphorylated Smad 2 was localized primarily to the cellular nuclei. Taken together, these results suggest increased translocation of phosphorylated R-Smad 2 from the cytoplasm to nuclei in infarct tissues, and this trend may be due to a loss of the inhibitory effect of I-Smad 7 on R-Smad 2 activation and is associated with elevation of cardiac fibroblast function.

In summary, we demonstrated that transient over-driven expression of exogenous I-Smad 7 in the primary cardiac fibroblasts is sufficient to reduce the expression of fibrillar collagen genes in these cells. We provide evidence that decreased expression of I-Smad 7 may contribute to the development of cardiac fibrosis in the post-MI heart. Whether I-Smad 7 is a potential molecular target in therapeutic strategies to attenuate post-MI cardiac fibrosis requires further investigation.

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