Essential Role of Smad3 in Infarct Healing and in the Pathogenesis of Cardiac Remodeling

Marcin Bujak, MD; Guofeng Ren, PhD; Hyuk Jung Kweon, MD; Marcin Dobaczewski, MD; Anilkumar Reddy, PhD; George Taffet, MD; Xiao-Fan Wang, PhD; Nikolaos G. Frangogiannis, MD

Background—Postinfarction cardiac repair is regulated through timely activation and repression of inflammatory pathways, followed by transition to fibrous tissue deposition and formation of a scar. The transforming growth factor-β/Smad3 pathway is activated in healing infarcts and may regulate cellular events critical for the inflammatory and the fibrotic responses.

Methods and Results—We examined the effects of Smad3 gene disruption on infarct healing and the pathogenesis of cardiac remodeling. In the absence of injury, Smad3-null hearts had comparable function to and similar morphology as wild-type hearts. Smad3-null animals had suppressed peak chemokine expression and decreased neutrophil recruitment in the infarcted myocardium but showed timely repression of inflammatory gene synthesis and resolution of the inflammatory infiltrate. Although myofibroblast density was higher in Smad3-null infarcts, interstitial deposition of collagen and tenascin-C in the remodeling myocardium was markedly reduced. Compared with wild-type animals, Smad3−/− mice exhibited decreased dilative remodeling and attenuated diastolic dysfunction; however, infarct size was comparable between groups. Transforming growth factor-β–mediated induction of procollagen type III and tenascin-C in isolated cardiac fibroblasts was dependent on Smad3, which suggests that decreased fibrotic remodeling in infarcted Smad3-null hearts may be due to abrogation of the profibrotic transforming growth factor-β responses.

Conclusions—Smad3 loss does not alter the time course of resolution of inflammation in healing infarcts, but it prevents interstitial fibrosis in the noninfarcted myocardium and attenuates cardiac remodeling. Thus, the Smad3 cascade may be a promising therapeutic target for the treatment of myocardial infarction. (Circulation. 2007;116: 2127-2138.)

Key Words: remodeling • infarction • inflammation • pathology

Infarct healing is intertwined with an inflammatory response that ultimately leads to formation of a scar. Release of proinflammatory mediators results in infiltration of the infarcted myocardium with neutrophils and macrophages that clear the wound from dead cells and matrix debris.1 However, optimal infarct healing requires timely activation of inhibitory signals that suppress chemokine and cytokine synthesis, resulting in resolution of the inflammatory infiltrate. Repression of inflammatory mediators is followed by infiltration of the infarcted myocardium with myofibroblasts and deposition of extracellular matrix (ECM) proteins, which leads to formation of a collagen-based scar. Inflammatory and fibrotic pathways are critically involved in the pathogenesis of ventricular remodeling, a complex process that results in dilation, hypertrophy, and enhanced sphericity of the ventricle and is associated with adverse prognosis.2,3 Although resolution of the postinfarction inflammatory response and activation of fibrotic pathways play a key role in infarct healing, the mechanisms that mediate transition from the inflammatory phase to fibrous tissue deposition remain unknown.

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Through its pleiotropic effects, transforming growth factor (TGF)-β is ideally suited as a key mediator in the transition from inflammation to fibrosis. TGF-β1 decreases leukocyte adhesion on the endothelium and inhibits neutrophil transendothelial migration.4 Furthermore, the actions of TGF-β on mature macrophages are predominantly suppressive, inhibiting proinflammatory cytokine and chemokine synthesis and decreasing reactive oxygen generation. The phenotype of the TGF-β1–null mouse suggests that the antiinflammatory actions of the molecule are critical for suppression of the inflammatory response and for regulation of tissue homeostasis.6 In addition, TGF-β plays a key role in the development of tissue fibrosis.7 TGF-β stimulation induces conversion of fibroblasts to myofibroblasts,8 enhances ECM protein synthe-
sis, and suppresses the activity of proteases that degrade ECM by inhibiting matrix metalloproteinase (MMP) expression and by inducing synthesis of protease inhibitors, such as plasminogen activator inhibitor (PAI)-1 and tissue inhibitors of metalloproteinases (TIMPs).9

The members of the TGF-β superfamily transduce their signal from the membrane to the nucleus through transmembrane type I and type II serine/threonine receptors and their downstream effectors, the Smad proteins. In addition, some TGF-β responses are mediated via Smad-independent pathways. Activation of the Smad2/3 cascade plays an essential role in ECM protein gene expression10 and regulates fibrous tissue deposition in a variety of experimental models.11 Furthermore, a growing body of evidence suggests that Smad3 signaling is involved in TGF-β-mediated suppression of inflammatory mediator synthesis.12 Accordingly, we hypothesized that Smad3 may be a critical mediator of the cellular events associated with infarct healing. We found that Smad3 gene disruption does not alter the time course of resolution of the inflammatory infiltrate in healing infarcts. However, Smad3 is critically involved in ECM deposition in the infarcted heart. Smad3-null mice have decreased collagen deposition in the scar and in the noninfarcted remodeling myocardium and exhibit reduced dilative remodeling and attenuated diastolic dysfunction after myocardial infarction. Smad3 signaling is essential for the profibrotic effects of TGF-β on isolated cardiac fibroblasts.

Methods

Murine Model of Reperfused Myocardial Infarction

Smad3-null and wild-type (WT) mice used for infarction experiments were genotyped with polymerase chain reaction protocols.13 Female and male mice, 8 to 12 weeks of age, were anesthetized with sodium pentobarbital (60 μg/g IP). A closed-chest model of reperfused myocardial infarction was used.14 The left anterior descending coronary artery was occluded for 1 hour, then reperfused for 6 hours to 7 days. At the end of the experiment, the heart was excised, fixed in zinc formalin, and embedded in paraffin for histological studies or snap-frozen for RNA isolation. Sham animals were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 24-hour, 72-hour, and 7-day reperfusion protocols (8 animals/group). Mice used for RNA extraction underwent 6, 24, and 72 hours of reperfusion (8 animals/group). Additional animals were used for perfusion fixation after 7 days of reperfusion to assess remodeling-associated parameters.

Immunohistochemistry and Quantitative Histology

Histological sections were stained immunohistochemically with the following antibodies: anti-α-smooth muscle actin antibody (Sigma, St Louis, Mo), rat anti-mouse macrophage antibody Mac-2 (Cedarlane, Burlington, Ontario, Canada), rat anti-neutrophil antibody (Serotec, Raleigh, NC), rabbit anti-mouse tenascin-C (R&D Systems, Minneapolis, Minn), and rabbit anti-p-Smad2 antibody (Cell Signaling, Danvers, Mass). A peroxidase-based technique was used, and quantitative assessment of neutrophil, macrophage, and myofibroblast density was performed as described previously.15 The collagen network was identified with picrosirius red staining.15 The area of collagen staining in the infarcted area was assessed with ImagePro software and expressed as a percentage of the area of the infarct. To assess the extent of interstitial fibrosis in the remodeling noninfarcted myocardium, collagen-stained sections were scanned at 0.05-mm intervals from the border of the infarct, and the area of collagen staining was quantified for each interval and compared between WT and Smad3-null animals. The extent of tenascin-C deposition in the infarcted heart was assessed by measuring the tenascin-positive area of the remodeling myocardium, expressed as a ratio to the area of the infarct (tenascin:infarct ratio).

Echocardiography

Short-axis M-mode echocardiography was performed before instrumentation and after 7 days of reperfusion (WT n=7; Smad3−/− n=8) with an 8-MHz probe (Sequoia C256; Acuson, Mountain View, Calif). The following parameters were measured as indicators of function and remodeling: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and fractional shortening (FS=[LVEDD–LVESD]×100/LVEDD). Left ventricular mass (LVM) was assessed with the following formula: LVM=1.05(septal thickness+LVEDD+posterior wall thickness)−LVEDD16. The percent change in these parameters after infarction was assessed quantitatively with the following formulas: (1) ΔLVEDD=(LVEDD at 7 days−LVEDDpre)×100/LVEDDpre, ΔFS=(FSpre−FS at 7 days)×100/FSpre and (2) ΔLVM=(LVM at 7 days−LVMpre)×100/LVMpre, where “pre” indicates preinfarction.

Measurement of Left Ventricular Pressure

Left ventricular pressure was recorded in infarcted Smad3−/− (n=9) and WT (n=11) animals after 7 days of reperfusion and in sham controls (WT n=14; Smad3−/− n=4) with a modified Radi PressureWire catheter (Radi Medical Systems, Uppsala, Sweden) as described previously.17 Left ventricular end-diastolic pressure was compared between WT and Smad3-null animals.

Perfusion Fixation and Assessment of Ventricular Volumes

For assessment of postinfarction remodeling, infarcted hearts after 7 days of reperfusion were used for perfusion fixation (n=12 for WT and n=10 for Smad3-null animals) as described previously.15 Sham animals (WT n=7; Smad3−/− n=5) were used as controls. The size of the infarct was expressed as a percentage of the left ventricular volume.

RNA Extraction and Ribonuclease Protection Assay

mRNA expression of the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, monocyte chemoattractant protein (MCP)-1, and interferon-γ-inducible protein (IP)-10, as well as of the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, TGF-β1, -β2, and -β3, MMP-2, -3, -8, and -9, and TIMP-1, -2, -3, and -4, was assessed in the infarcted heart with a ribonuclease protection assay kit (RiboQuant; Pharmingen, San Diego, Calif).

Protein Extraction and Western Blotting

Protein was isolated from whole hearts (sham and 24- and 72-hour reperfusion; n=5 per group). Western blotting with rabbit anti-Smad2, anti-phosphorylated-Smad2 (anti-p-Smad2), anti-p-Smad1/5 (Cell Signaling), and goat anti-Smad7 antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was performed as described previously.18

Isolation and Stimulation of Murine Cardiac Fibroblasts

Fibroblasts were isolated from murine WT or Smad3-null hearts, cultured as described previously,19 and stimulated with recombinant TGF-β1 (100 ng/L; R&D Systems) for 4 to 16 hours. At the end of the experiment, total RNA or protein was isolated from the fibroblasts. MMP-2, -3, -8, and -9 and TIMP-1, -2, -3, and -4 mRNA expression was assessed with a ribonuclease protection assay. Protein expression of procollagen type III and tenasin-C was examined by Western blotting with a goat anti-procollagen III antibody (Santa Cruz) and a rabbit anti-tenasin-C antibody (Chemicon, Temecula, Calif).
Statistical Analysis
Statistical analysis was performed with ANOVA followed by t test corrected for multiple comparisons (Student-Newman-Keuls). Paired t test was used to compare echocardiographic parameters before myocardial infarction and after 7 days of reperfusion. Data were expressed as mean±SEM. Statistical significance was set at 0.05.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Activation of the Smad2/3 Pathway in the Border Zone of Murine Myocardial Infarcts
Western blotting experiments demonstrated that infarcted mouse hearts had increased p-Smad2 expression after 24 to 72 hours of reperfusion (Figure 1A), whereas total Smad2 levels were comparable between groups. The p-Smad2:Smad2 ratio was increased after 24 to 72 hours of reperfusion, which indicates activation of the Smad2/3 pathway. p-Smad2 expression was increased in the infarcted myocardium after 72 hours of reperfusion. The p-Smad2:Smad2 ratio was increased after 24 to 72 hours of reperfusion, which indicates activation of the Smad2/3 pathway. p-Smad2 expression was decreased in the infarcted myocardium after 72 hours of reperfusion. The p-Smad2:Smad2 ratio was increased after 24 to 72 hours of reperfusion, which indicates activation of the Smad2/3 pathway. p-Smad2 expression was decreased in the infarcted myocardium after 72 hours of reperfusion. The p-Smad2:Smad2 ratio was increased after 24 to 72 hours of reperfusion, which indicates activation of the Smad2/3 pathway. p-Smad2 expression was decreased in the infarcted myocardium after 72 hours of reperfusion. The p-Smad2:Smad2 ratio was increased after 24 to 72 hours of reperfusion, which indicates activation of the Smad2/3 pathway.

Mortality in Smad3-Null and WT Animals Undergoing Reperfused Infarction Protocols
Although Smad3 knockouts and WT animals had comparable mortality rates during experimental infarction (Smad3/−/− 14.9% versus WT 15.6%, P=NS), histologically confirmed cardiac rupture was observed only in WT animals (incidence of rupture: Smad3/−/− 0% versus WT 7.8%; P=0.14).

Smad3-Null Mice Had Decreased Neutrophil Density in the Infarcted Myocardium and Exhibited Timely Resolution of the Inflammatory Infiltrate
In the absence of injury, Smad3-null hearts had normal histological characteristics and did not show evidence of...
spontaneous inflammation. After reperfused infarction, both WT and Smad3-null mice exhibited infiltration of the infarcted myocardium with neutrophils and macrophages and showed extensive replacement of dead cardiomyocytes with granulation tissue after 72 hours of reperfusion. Compared with WT mice, Smad3-null animals showed decreased neutrophil density in the infarcted myocardium after 24 (P<0.05) and 72 (P<0.01; Figure 2) hours of reperfusion. Macrophage density in the infarcted myocardium was comparable between Smad3-null and WT mice after 24 hours of reperfusion and was only slightly higher in Smad3-null mice after 72 hours of reperfusion (P<0.05; Figure 3). Both Smad3-null and WT animals showed clearance of the neutrophilic infiltrate and significant reduction in macrophage density after 7 days of reperfusion, which suggests that Smad3 deficiency does not alter the time course of resolution of the inflammatory process in healing infarcts (Figures 2 and 3).

Smad3-Null Mice Had Decreased Peak Chemokine mRNA Expression and Exhibited Timely Repression of Chemokine mRNA Synthesis After 24 to 72 Hours of Reperfusion

Mouse infarcts showed marked chemokine induction, which peaked after 6 hours of reperfusion, followed by repression of chemokine mRNA expression after 24 to 72 hours. Compared with WT animals, Smad3-null mice had decreased peak mRNA expression of the chemokines MCP-1, MIP-1α, MIP-1β, MIP-2, and IP-10 in the infarcted heart. Both Smad3 knockout and WT mice exhibited timely repression of chemokine mRNA expression in the infarct after 24 to 72 hours of reperfusion (Figure 4).

Cytokine mRNA Expression in Infarcted Smad3-Null Hearts

Reperfused mouse infarcts exhibited a marked but transient induction of proinflammatory cytokine mRNA, which peaked

Figure 2. Smad3-null mice exhibited decreased neutrophil infiltration of the infarct and showed timely resolution of the inflammatory infiltrate. Neutrophils were identified in infarcted WT (A through C) and Smad3 knockout (KO) hearts (D through F) after 24 hours (A, D), 72 hours (B, E), and 7 days of reperfusion (C, F). G. Neutrophil density was significantly lower in Smad3-null infarcts after 24 and 72 hours of reperfusion (*P<0.05, **P<0.01). Both WT and Smad3-null animals had timely resolution of the neutrophilic infiltrate after 7 days of reperfusion (scale bar=80 μm).
after 6 hours of reperfusion. Smad3 knockout mice had slightly lower peak TNF-α mRNA levels but comparable IL-1β and IL-6 expression in the infarcted heart (Figures 5A through 5C). Smad3 deficiency did not alter the time course of repression of the cytokine response after 24 to 72 hours of reperfusion.

Smad3-Null Mice Had Increased Myofibroblast Density in the Healing Infarct

After 72 hours of reperfusion, mouse infarcts were infiltrated with myofibroblasts, identified as spindle-shaped α-smooth muscle actin–expressing cells. Compared with WT animals, Smad3 knockout mice had significantly increased myofibroblast density in the infarcted myocardium after 72 hours of reperfusion (Figures 5D through 5F).

Smad3-Null Mice Exhibited Reduced Fibrotic Remodeling of the Infarcted Ventricle

Infarct healing resulted in replacement of dead cardiomyocytes with a collagen-based scar and was associated with fibrotic interstitial remodeling of the noninfarcted myocardium. Myocardial areas neighboring the scar exhibited more intense interstitial remodeling, showing a high collagen content (Figure 6D) and extensive deposition of tenascin-C (Figure 6G), a matricellular protein induced in remodeling tissues. In infarcted WT hearts, interstitial collagen content in the noninfarcted remodeling myocardium was highest in areas that neighbored the infarct and progressively decreased in myocardial areas remote from the border of the infarct (Figure 6D and 6F). Compared with WT animals, Smad3-null mice showed reduced collagen content in the scar after 7 days of reperfusion (Figure 6C) and exhibited markedly attenuated collagen deposition in the noninfarcted remodeling myocardium (Figure 6D through 6F). Deposition of tenascin-C was noted in myocardial areas adjacent to the infarct and served as a marker of interstitial remodeling. The area of tenascin-C staining was significantly smaller in infarcted Smad3-null mice, which suggests that Smad3 gene disruption resulted in attenuation of the remodeling process (Figure 6G through 6I).
Smad3-Deficient Animals Showed Reduced Dilative Remodeling After Myocardial Infarction

Postinfarction ventricular remodeling in WT and Smad3-null animals was assessed with quantitative morphometry and echocardiography. In the absence of injury, WT and Smad3-null hearts had comparable chamber dimensions and left ventricular mass (Table 1). After 7 days of reperfusion, WT animals showed a marked increase in LVEDD (Figure 7F; Table 1) and left ventricular end-diastolic volume (Figure 7C; Table 1), which reflects dilative remodeling of the infarcted ventricle. However, Smad3-null animals had decreased peak mRNA expression of the chemokines MIP-1α (A), MIP-1β (B), MIP-2 (C), IP-10 (D), and MCP-1 (E) in the infarcted heart (*P<0.05, **P<0.01, ***P<0.001). Both WT and Smad3-null animals showed timely repression of chemokine synthesis after 72 hours of reperfusion. Ratio to L32 indicates ratio to housekeeping gene L32.

Smad3-Deficient Animals Showed Reduced Dilative Remodeling After Myocardial Infarction

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Smad3-null animals exhibited attenuated ventricular dilation after myocardial infarction, showing significantly lower LVEDD (Figure 7F) and left ventricular end-diastolic volume (Figure 7C) than their WT littermates; infarct size was not different between groups (Figure 7G). In contrast, infarcted WT and Smad3-null (E) mice, D, WT mice exhibited significant interstitial fibrosis in the remodeling myocardium neighboring the infarct (arrows). The extent of collagen deposition decreased progressively in myocardial areas remote from the infarct (arrowheads). E, Smad3-null mice had markedly attenuated interstitial fibrosis in the noninfarcted myocardium. F, Quantitative analysis demonstrated that Smad3-null mice (red) had significantly lower collagen deposition than WT animals (black) in viable myocardial areas located 0.25, 0.5, and 0.75 mm from the infarct border zone (**P<0.01). G and H, Immunohistochemical staining identified remodeling myocardial areas that exhibited interstitial deposition of the matricellular protein tenascin-C (arrows) in WT (G) and Smad3-null (H) animals. The extent of tenascin-C deposition (measured by the tenascin:infarct area [T/I]) was significantly lower in Smad3-null mice (***P<0.001).

**Figure 6.** Smad3-null mice exhibited reduced fibrotic remodeling of the infarcted heart. The collagen network was stained in WT (A) and Smad3-null (B) infarcts after 7 days of reperfusion (scale bar=100 μm). The scar contained large amounts of collagen (arrows), whereas the neighboring viable myocardium exhibited extensive interstitial fibrosis (arrowheads). Smad3-null mice (KO) had lower collagen deposition in the infarct than did WT animals (C). D through F, Assessment of interstitial fibrosis in the remodeling noninfarct heart in WT (D) and Smad3-null (E) mice. D, WT mice exhibited significant interstitial fibrosis in the remodeling myocardium neighboring the infarct (arrows). The extent of collagen deposition decreased progressively in myocardial areas remote from the infarct (arrowheads). E, Smad3-null mice had markedly attenuated interstitial fibrosis in the noninfarcted myocardium. F, Quantitative analysis demonstrated that Smad3-null mice (red) had significantly lower collagen deposition than WT animals (black) in viable myocardial areas located 0.25, 0.5, and 0.75 mm from the infarct border zone (**P<0.01). G and H, Immunohistochemical staining identified remodeling myocardial areas that exhibited interstitial deposition of the matricellular protein tenascin-C (arrows) in WT (G) and Smad3-null (H) animals. The extent of tenascin-C deposition (measured by the tenascin:infarct area [T/I]) was significantly lower in Smad3-null mice (***P<0.001).

Smad3-null animals exhibited attenuated ventricular dilation after myocardial infarction, showing significantly lower LVEDD (Figure 7F) and left ventricular end-diastolic volume (Figure 7C) than their WT littermates; infarct size was not different between groups (Figure 7G). In contrast, infarcted WT and Smad3−/− hearts showed comparable left ventricular mass (Table 1), which suggests that Smad3 deficiency does not prevent postinfarction ventricular hypertrophy.

**Smad3 Gene Disruption Protected the Heart From the Development of Diastolic Dysfunction After Infarction**

In the absence of injury, WT and Smad3-null hearts had normal left ventricular function (Table 1). Seven days after infarction, WT mice showed a marked decrease in FS that indicated systolic dysfunction (Table 1). FS in Smad3-null animals also decreased 7 days after infarction; however, the difference in comparison with preocclusion values was not statistically significant (Table 1). WT and Smad3-null animals had comparable FS 7 days after myocardial infarction. To compare diastolic function in infarcted Smad3-null and WT hearts, we performed invasive hemodynamic assessment of the left ventricular end-diastolic pressure. In the absence of infarction, left ventricular end-diastolic pressure was comparable between WT and Smad3-null hearts (Figure 7J). Seven days after myocardial infarction, WT mice showed a significant increase in left ventricular end-diastolic pressure that
indicated diastolic dysfunction of the infarcted ventricle. Infarcted Smad3-null mice exhibited significantly lower left ventricular end-diastolic pressure than WT animals ($P<0.05$), which suggests that Smad3 gene disruption attenuated diastolic dysfunction after infarction (Figure 7H through 7J).

**Effects of Smad3 Deficiency on MMP/TIMP Expression Profile in the Infarcted Heart**

Because MMPs critically regulate postinfarction remodeling, we examined the effect of Smad3 gene disruption on the MMP and TIMP expression profile in the infarcted heart. Infarcted WT and Smad3-null hearts showed comparable MMP-9, MMP-8, TIMP-1, and TIMP-4 mRNA expression (Table 2). However, Smad3-null mice had somewhat lower MMP-2, MMP-3, and TIMP-3 mRNA levels in the infarcted heart than WT animals after 24 hours of reperfusion. After 72 hours of reperfusion, MMP-2 mRNA expression was lower in infarcted Smad3-null hearts, whereas MMP-3 levels were slightly higher than in WT infarcts (Table 2).

**Smad3 Critically Regulates Profibrotic TGF-β Responses in Isolated Cardiac Fibroblasts**

Fibroblasts isolated from WT hearts showed robust upregulation of procollagen III (Figure 8A and 8B) and tenascin-C (Figure 8D and 8E) on stimulation with TGF-β1. In contrast, TGF-β1 did not induce procollagen III (Figure 8A and 8C) or tenascin-C (Figure 8D and 8F) expression in Smad3-null cardiac fibroblasts, which suggests that TGF-β-mediated ECM protein synthesis is dependent on Smad3 signaling. In addition, TGF-β1 stimulation markedly induced TIMP-1 and TIMP-2 mRNA synthesis in WT cardiac fibroblasts (Figure 8G and 8H) without affecting MMP-2, -3, -8, or -9 or TIMP-3 or TIMP-4 expression (not shown). In contrast, Smad3-null cardiac fibroblasts did not show TIMP-1 or TIMP-2 upregulation on stimulation with TGF-β1 (Figure 8G and 8H), which indicates that Smad3 signaling is essential for the effects of TGF-β on cardiac fibroblast TIMP expression.

**Discussion**

We present the first evidence that Smad3 signaling is critically involved in myocardial infarct healing and plays an important role in the pathogenesis of cardiac remodeling. We demonstrated that Smad3 signaling is not essential for resolution of the postinfarction inflammatory response but that it critically regulates fibrotic remodeling of the infarcted ventricle. In addition, we showed that the profibrotic actions of TGF-β on cardiac fibroblasts are mediated by Smad3. Our findings have important implications in defining the role of TGF-β/Smad3 signaling in the infarcted myocardium.

TGF-β is a pleiotropic and multifunctional cytokine, known to exert diverse and often contradictory cellular effects on all cell types that may be involved in infarct healing. TGF-β-mediated actions are not only dependent on the cell type but also on its stage of differentiation and on the cytokine milieu. Myocardial infarction is associated with marked upregulation of TGF-β isoforms, induction of TGF-β activators, and increased expression of the downstream effectors of TGF-β signaling. Upregulation of TGF-β in the infarct border zone indicates local activation of the TGF-β pathway (Figure 1). Several studies have suggested a role for TGF-β in healing myocardial infarcts. Experiments using anti–TGF-β gene therapy through transfection with the extracellular domain of TGFRII suggested distinct time-dependent effects of TGF-β inhibition on the inflammatory and fibrotic response after myocardial infarction. Early TGF-β inhibition resulted in increased mortality, enhanced neutrophil infiltration, and increased proinflammatory cytokine and chemokine gene expression in the infarcted heart, which suggests an important role for TGF-β signaling in resolution of inflammation and repression of cytokine and chemokine synthesis. In contrast, late TGF-β inhibition reduced fibrous tissue deposition, which attenuated the adverse remodeling of the infarcted ventricle. The diverse actions of TGF-β in the infarcted myocardium may be mediated via activation of Smad-dependent or -independent pathways.

A growing body of evidence suggests involvement of the TGF-β/Smad3 pathway in both induction and resolution of the inflammatory response. Smad3-null animals showed decreased local infiltration of monocytes in skin excisional wounds and had reduced cutaneous inflammation after exposure to ionizing radiation. Furthermore, Smad3−/− monocytes exhibited a blunted chemotactic response to TGF-β. On the other hand, recent investigations support a role for Smad3 in mediating the antiinflammatory actions of TGF-β. The ability of TGF-β1 to inhibit cytokine-mediated MCP-1 ex-
pression in macrophages was dependent on Smad3 signaling. Consistent with this impaired response, cardiac allografts in Smad3-null mice developed accelerated intimal hyperplasia accompanied by increased infiltration of adventitial macrophages expressing MCP-1. The present data demonstrated that Smad3 gene disruption results in decreased neutrophil infiltration in the infarcted myocardium, accompanied by reduced peak chemokine and cytokine expression. In contrast, the time course of macrophage recruitment in the healing infarct was not affected. TGF-β1 is one of the most...
potent described chemoattractants for neutrophils; Smad3 signaling may play an important role in TGF-β-induced neutrophil recruitment. On the other hand, Smad3-null animals exhibited timely repression of chemokine and cytokine synthesis, followed by clearance of the leukocytic infiltrate, which suggests that the Smad3 pathway does not play an essential role in TGF-β–mediated resolution of the inflammatory response after infarction.

Smad3 was essential for fibrous tissue deposition in the infarcted myocardium. Smad3-null animals exhibited decreased deposition of collagen in the infarct and in the noninfarcted remodeling myocardium (Figure 6). Furthermore, Smad3 loss was associated with reduced deposition of tenascin-C, which indicates attenuated interstitial remodeling. These findings are consistent with the established role of Smad3 signaling in tissue fibrosis: Smad3-null mice are resistant to the development of TGF-β–mediated pulmonary fibrosis, and loss of Smad3 reduces scarring in skin exposed to ionizing radiation. The antifibrotic effects of Smad3 deficiency were not due to reduced fibroblast infiltration into the infarct or to perturbed myofibroblast differentiation. In fact, myofibroblast density in the infarcted myocardium was significantly higher in Smad3-null infarcts (Figure 5D through 5F), a finding possibly due to the partial resistance of Smad3-null fibroblasts to the antiproliferative actions of TGF-β. However, TGF-β–mediated induction of procollagen type III and tenascin-C in cardiac fibroblasts was Smad3-dependent (Figure 8), which suggests that decreased fibrotic remodeling in infarcted Smad3-null hearts may be due to abrogation of the profibrotic TGF-β responses.

Remodeling of the infarcted heart results in dilation, hypertrophy, and changes of the ventricular geometry and is a predictor of adverse outcome in patients with myocardial infarction. TGF-β appears to be a crucial regulator of cardiac remodeling through its direct and potent actions in cardiomyocyte hypertrophy and cardiac ECM metabolism. TGF-β inhibition during the proliferative phase of healing resulted in attenuated left ventricular remodeling, decreasing cardiomyocyte hypertrophy and reducing interstitial fibrosis in the noninfarcted ventricle. Anti–TGF-β therapy also had a significant effect on the geometry of the infarct, shortening and thickening the infarcted segment without affecting the absolute size of the infarct. The effects of TGF-β in the pathogenesis of remodeling appear to be mediated in part through Smad3 signaling. Smad3 loss resulted in significant attenuation of ventricular dilation but did not abrogate hypertrophic remodeling after myocardial infarction (Figure 7; Table 1). Decreased ventricular dilation in Smad3-null mice is not due to a reduction in infarct size but is associated with reduced fibrotic remodeling of the viable areas in the infarcted heart (Figure 6). Deposition of ECM proteins in the remodeling noninfarcted myocardium appears to be a key cellular event in the pathogenesis of adverse remodeling and is dependent on Smad3 signaling. Furthermore, reduction of interstitial fibrosis also prevented development of diastolic dysfunction in Smad3-null mice (Figure 7H through 7J).

MMPs have an established role in cardiac remodeling, and TGF-β promotes matrix deposition through upregulation of TIMPs, inhibiting the matrix-degrading activity of MMPs. Accordingly, we examined the effects of Smad3 deficiency on MMP and TIMP expression in the infarcted heart. Although our experiments indicated that TGF-β–mediated TIMP-1 and TIMP-2 upregulation in isolated cardiac fibroblasts is Smad3-dependent (Figure 8), these in vitro effects of the TGF-β/Smad3 pathway appeared not to play an important role in infarct healing. Infarcted Smad3-null and WT hearts had comparable TIMP-1 and TIMP-2 mRNA expression (Table 2). It appears that regulation of MMP and TIMP in the infarcted myocardium may not primarily involve the fibroblasts or may be dictated by Smad3-independent actions.

Smad3 signaling plays an essential role in the pathogenesis of postinfarction remodeling by mediating ECM deposition in the infarcted and remodeling myocardium. In contrast, the effects of TGF-β on resolution of the postinfarction inflammatory response are not dependent on Smad3. These findings suggest that the Smad3 cascade may provide a unique therapeutic opportunity in the treatment of myocardial infarction: Smad3 inhibition is likely to attenuate fibrotic remodeling without affecting the time course of clearance of the inflammatory infiltrate. Small-molecule inhibitors of Smad3 or overexpression of Smad7 may be of interest as therapeutic strategies to suppress Smad3 signaling in myocardial infarction.

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Disclosures

None.

References


Figure 8. Smad3 regulated the profibrotic actions of TGF-$\beta_1$ in isolated cardiac fibroblasts. A, TGF-$\beta_1$ stimulation induced procollagen III (pCol III) synthesis by WT but not Smad3-/- cardiac fibroblasts. C indicates control. B, Quantitative analysis showed marked induction of procollagen III expression by WT cardiac fibroblasts after 4 to 16 hours of stimulation with TGF-$\beta_1$ ($P<0.05$). C, In contrast, fibroblasts isolated from Smad3-null hearts showed no procollagen III upregulation on TGF-$\beta_1$ stimulation. D, TGF-$\beta_1$ stimulation induced tenasin-C (TN-C) synthesis by WT but not Smad3-/- cardiac fibroblasts. C indicates control. E, WT fibroblasts showed marked increase of tenasin-C synthesis after 16 hours of stimulation with TGF-$\beta_1$ ($P<0.05$). F, In contrast, TGF-$\beta_1$ did not induce tenasin-C expression by Smad3-null fibroblasts. G and H, TGF-$\beta_1$ induced TIMP-1 and TIMP-2 mRNA synthesis in WT but not in Smad3-/- fibroblasts ($**P<0.01$).

**CLINICAL PERSPECTIVE**

Postinfarction cardiac repair is regulated through timely activation and repression of inflammatory pathways, followed by transition to fibrous tissue deposition and formation of a scar. Transforming growth factor-beta exerts potent anti-inflammatory and profibrotic actions and may be ideally suited to serve as the “master switch” for the transition from inflammation to fibrosis in the healing heart. Transforming growth factor-beta signals through intracellular effectors, the Smads, or through Smad-independent pathways. Activation of the Smad3 pathway plays an essential role in extracellular matrix protein expression, regulates fibrous tissue deposition in several experimental models, and may mediate some of the anti-inflammatory effects of transforming growth factor-beta. We present the first evidence that Smad3 signaling is critically involved in healing myocardial infarction and plays an important role in the pathogenesis of cardiac remodeling. We demonstrate that Smad3 signaling is not essential for resolution of the postinfarction inflammatory response, but it critically regulates fibrotic remodeling of the infarcted ventricle. Smad3 loss prevents interstitial fibrosis in the noninfarcted remodeling myocardium and attenuates cardiac dilation and diastolic dysfunction. Profibrotic actions of transforming growth factor-beta on isolated cardiac fibroblasts are mediated, at least in part, via Smad3. These findings suggest that the Smad3 cascade may provide a unique therapeutic opportunity in the treatment of myocardial infarction: Smad3 inhibition is likely to attenuate fibrotic remodeling without affecting the time course of clearance of the inflammatory infiltrate. Small-molecule inhibitors of Smad3 or overexpression of Smad7 may be interesting therapeutic strategies to suppress Smad3 signaling in myocardial infarction.
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Marcin Bujak, Guofeng Ren, Hyuk Jung Kweon, Marcin Dobaczewski, Anilkumar Reddy, George Taffet, Xiao-Fan Wang and Nikolaos G. Frangogiannis

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