Cardiomyocyte-Specific Transforming Growth Factor β Suppression Blocks Neutrophil Infiltration, Augments Multiple Cytoprotective Cascades, and Reduces Early Mortality After Myocardial Infarction

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Rationale: Wound healing after myocardial infarction involves a highly regulated inflammatory response that is initiated by the appearance of neutrophils to clear out dead cells and matrix debris. Neutrophil infiltration is controlled by multiple secreted factors, including the master regulator transforming growth factor β (TGFβ). Broad inhibition of TGFβ early postinfarction has worsened post–myocardial infarction remodeling; however, this signaling displays potent cell specificity, and targeted suppression particularly in the myocyte could be beneficial.

Objective: Our aims were to test the hypothesis that targeted suppression of myocyte TGFβ signaling ameliorates postinfarct remodeling and inflammatory modulation and to identify mechanisms by which this may be achieved.

Methods and Results: Mice with TGFβ receptor–coupled signaling genetically suppressed only in cardiac myocytes (conditional TGFβ receptor 1 or 2 knockout) displayed marked declines in neutrophil recruitment and accompanying metalloproteinase 9 activation after infarction and were protected against early-onset mortality due to wall rupture. This is a cell-specific effect, because broader inhibition of TGFβ signaling led to 100% early mortality due to rupture. Rather than by altering fibrosis or reducing the generation of proinflammatory cytokines/chemokines, myocyte-selective TGFβ inhibition augmented the synthesis of a constellation of highly protective cardiokines. These included thrombospondin 4 with associated endoplasmic reticulum stress responses, interleukin-33, follistatin-like 1, and growth and differentiation factor 15, which is an inhibitor of neutrophil integrin activation and tissue migration.

Conclusions: These data reveal a novel role of myocyte TGFβ signaling as a potent regulator of protective cardiokine and neutrophil-mediated infarct remodeling. (Circ Res. 2014;114:1246-1257.)

Key Words: heart rupture ■ inflammation ■ myocardial infarction ■ neutrophils ■ transforming growth factor beta

Coronary artery disease is the leading cause of death worldwide, and myocardial infarction (MI) is among its most severe manifestations.1 In the early post-MI period, mortality is due to arrhythmia, cardiogenic shock, and myocardial rupture, whereas ischemic heart failure with molecular and chamber remodeling impacts long-term prognosis. Early aggressive cardiac care including reperfusion therapy has improved outcomes. However, many infarcts remain nonreperfused,2 and the molecular and cellular events initiated within hours to days often dictate the ultimate consequences of the injury. This continues to drive the efforts to better understand early signaling events that control myocardial damage and repair.

Irreversible injury to cardiomyocytes develops shortly after the onset of ischemia and leads to cell death. Within hours, a rapidly induced, highly regulated inflammatory response consisting largely of neutrophils and monocyte/macrophages is initiated in the myocardium to remove dead cells and matrix debris.3–5 These first responder cells also secrete enzymes, free radicals, and cytokines that can induce matrix destabilization and infarct expansion.4,6 In humans, neutrophilia is associated with adverse chamber remodeling and propensity to cardiac rupture after infarction.7–9 The acute inflammatory response is followed by proliferation of vascular cells and matrix-depositing fibroblasts, formation of granulation tissue, and replacement of this tissue by a collagen-rich scar with
high tensile strength. This temporal sequence must be finely orchestrated to prevent excessive early ventricular remodeling.

Transforming growth factor β (TGFβ) is a master regulator of the inflammatory, proliferative, and fibrotic components of infarct healing, placing it in a strategic position to coordinate this temporal sequence. The expression of all 3 TGFβ isoforms is upregulated after ischemia. TGFβ ligand binds to TGFβ receptor 2 (TGFβR2) that in turn recruits and phosphorylates TGFβ receptor 1 (TGFβR1) to initiate SMAD-dependent transcriptional regulation by the classical pathway. TGFβR2 may also couple to alternative cascades such as members of mitogen-activated protein kinase pathways. Direct administration of TGFβ during the inflammatory phase reduces myocardial injury associated with the attenuation of proinflammatory cytokines such as tumor necrosis factor α (TNFα). Conversely, early inhibition of TGFβ increases post-MI mortality, exacerbates left ventricular dilation, and enhances cytokine synthesis. TGFβ induces chemotaxis and activation of circulating monocytes and polymorphonuclear cells, but has a predominantly suppressive effect on mature macrophages. TGFβ is also a potent inducer of myofibroblast formation and stimulates synthesis of extracellular matrix proteins. In addition to this pleomorphic regulation, TGFβ signaling varies depending on the cell type and the receptor (TGFβR1 versus TGFβR2) being modulated, impacting its net role in the heart under pathological stress.

In this study, we tested the hypothesis that myocyte TGFβ impacts cardiac stress signaling after MI in a manner that differs from that observed with global TGFβ suppression. Surviving myocytes in the peri-infarct zone adjoining the necrotic core are strategically positioned to signal to inflammatory, vascular, and other interstitial cells that migrate into the region. Using cell-selective gene knockdown strategies, we show that neutrophilia and myocardial neutrophil migration are suppressed and a constellation of protective cardiokines activated by blocking myocyte TGFβ signaling, preventing early mortality due to infarct rupture.

Methods

An extended Methods section is available in the Online Data Supplement.

Mouse Model of Myocyte-Targeted TGFβR Knockdown

Cardiomyocyte-targeted TGFβ receptor–knockout mouse were generated as described. Briefly, Myh6-MerCreMer (MCM) transgenic mice were crossed with TGFβR1fl/fl or TGFβR2fl/fl to derive Myh6-MCM+/−/TGFβR1fl/fl (TGFβR1−/−) or Myh6-MCM+/−/ TGFβR2fl/fl (TGFβR2−/−). To induce recombination, tamoxifen citrate (Sigma) was administered in soft diet (Bioserve) at 80 mg/kg per day for 10 days. Experiments were conducted 2 to 4 weeks after tamoxifen treatment when echocardiography confirmed normal baseline function. C57Bl/6j mice (Jackson Laboratories) were used in antibody studies. TGFβ neutralizing antibody (clone 1D11) and control Ab (clone 13C4) were obtained from Genzyme. Ab treatment was initiated at the time of left anterior descending artery ligation by intraperitoneal injection (10 mg/kg body weight, 3 times per week).

Infarction Model

Mice were anesthetized (etomidate 15 mg/kg intraperitoneally, isoflurane 2% to 4% by ventilator, buprenorphine 0.1 mg/kg subcutaneously 6–8h), intubated and ventilated, left anterior descending artery was ligated by a percutaneous approach, and visualized with a stereomicroscope. Coronary ligation was performed using a 7-0 suture placed caudal to the left atrial appendage. Successful ligation was confirmed by blanching of the myocardium and by ECG monitoring. The wound was closed in layers using 6-0 suture. Mice were closely monitored after surgery and analgesia provided as needed (buprenorphine). The protocol was approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee.

Isolated Ventricular Cardiomyocyte Culture Studies

Neonatal rat cardiac ventricular myocytes were isolated from 1- to 3-day-old Sprague–Dawley rats as described and maintained in 10% fetal bovine serum containing culture media after isolation. For RNA interference, ON-TARGETplus SMARTpool reagent against rat TGFβR1 was purchased from Dharmacon. ON-TARGETplus nontargeting pool was used as control. Twenty-four hours after plating, cells were transfected with 25 nmol/L siRNA using DharmaFECT 1 reagent following the manufacturer’s protocol in OptiMEM media (Gibco). Twenty-four hours before cytokine stimulation, cells were serum-starved. TNFα (50 ng/mL), interleukin (IL)-1β (10 ng/mL; both R&D Systems), or TGFβ1 (5 ng/mL; Sigma) were used for cytokine stimulation. For hypoxia studies, cells were exposed to a nitrogen atmosphere containing 5% CO2 and 1% O2 in modular incubator chambers (Billups-Rothenberg). PC3 cells transfected with hypoxia-responsive elements driving the expression of GFP and transcript expression of Slc2a1 (GLUT1) served to confirm efficient hypoxia. RNA was harvested 24 hours after cytokine stimulation or exposure to hypoxia.

Statistical Analyses

Data are presented as mean±SEM. Group comparisons were performed by 1- or 2-way ANOVA, nonpaired 2-tailed Student t test, Kruskal–Wallis ANOVA, or Mann–Whitney U test as appropriate. Bonferroni correction was used unless indicated otherwise. Survival was compared using log-rank test. P values ≤0.05 were considered significant. Sample sizes and individual statistical results are provided in figures.

Results

Myocyte TGFβ Inhibition Prevents Death Due to Wall Rupture After Myocardial Infarction

We initially studied mice with both cardiomyocyte selective TGFβR1 and TGFβR2 knockdown. After infarction, littermate controls exhibited ~40% mortality that occurred generally in the first 10 days. By contrast, both TGFβR2−/− and TGFβR1−/− mice were protected (1 death in 17 mice; P<0.05; Figure 1A). When control mice were administered a pan-cell targeting TGFβ−neutralizing antibody, all died by day 5, whereas mice receiving control antibody displayed the anticipated 40% mortality (Figure 1B). Thus,
post-MI protection was unique to myocyte TGFβ targeting. Postinfarction mortality was due not to arrhythmia, hemodynamic disparities, or differences in wall thickness or infarct size, but rather to myocardial rupture accompanying chamber dilation (Figure 1D–F; Online Figure IA–ID). Though genotype had a major impact on early mortality, the temporal evolution of left ventricular dysfunction and dilation after MI was similar among the groups (Figure 1C and 1D). Six-week

Figure 1. Myocyte transforming growth factor β (TGFβ) inhibition reduces early mortality after myocardial infarction (MI). Survival curves for cardiomyocyte TGFβR1/

C

D

E

F

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(terminal) heart and lung weight, remote fibrosis, and fetal gene expression (Nppa, Nppb, and Myh7) were also similar (Online Figure IIA–IID). Given the similar phenotypes for myocyte TGFβRI and TGFβRII deletion models, we focused our subsequent analyses to TGFβRIcKD animals.

Mortality disparities began at day 3. At this time, all 3 TGFβ isoforms were equally upregulated in the peri-infarction zone of controls and TGFβRIcKD mice (Figure 2A). Canonical TGFβ signaling reflected by Smad2/3 phosphorylation was activated in the peri-infarct zone (Figure 2B and 2C), whereas noncanonical signaling indexed by TGFβ-activated kinase 1 or mitogen-activated kinases was not (Figure 2D and 2E). These noncanonical kinases were not differentially changed in TGFβRIcKD hearts, with the exception of C-Jun N-terminal kinase that displayed reduced phosphorylation. Confocal immunofluorescence revealed Smad3 activation in both cardiac myocytes and interstitial cells in infarcted control hearts, but only in interstitial cells in TGFβRIcKD (Figure 2F). Thus, TGFβRI gene deletion in myocytes did not alter ligand expression or total myocardial Smad2/3 activation, but did suppress canonical TGFβ signaling in these cells.

**Myocyte TGFβRI Deletion Attenuates Matrix Metalloproteinase 9 Activity Without Impacting Collagen Deposition**

Acute infarct remodeling and vulnerability to wall thinning and rupture represents a balance between matrix degradation linked to matrix metalloproteinase (MMP) activation and fibrous tissue formation. At day 3 post-MI, when mortality differences began appearing, fibrosis was not yet present (Figure 3A). Collagen gene expression was increased but similar between groups (Figure 3B). However, the expression of Mmp9 rose nearly 30-fold post-MI, and this was significantly blunted in TGFβRIcKD myocardium (Figure 3C). The MMP inhibitor TIMP1 was similarly elevated in both groups (Figure 3F), thereby lowering the Mmp9/Timp1 ratio (Figure 3G). Changes in expression were paralleled by MMP9 gelatinolytic activity (Figure 3D).

**Figure 2. Expression and activation patterns of members of the transforming growth factor β (TGFβ) signaling pathway. A. Tgfb1-3 isoform expression is upregulated at d 3 post–myocardial infarction (MI; infarct and border zone) without differences between genotypes; *$P<0.05$ vs respective sham; Tgfb1: borderline significance ($P=0.064$) vs respective sham; n=5 per group. B and C, SMAD2 (Ser465/467) and SMAD3 (lower band) phosphorylation is increased after MI; *$P<0.001$ vs sham; n=3–5. D and E, Phosphorylation status of the mitogen-activated protein kinase family members ERK1/2 (Thr202/Tyr204) and p38 (Thr180/Tyr182) is not altered. C-Jun N-terminal kinase phosphorylation (Thr183/Tyr185) is decreased in TGFβRIcKD infarcts, and TGFβ-activated kinase 1 phosphorylation (Thr187) is reduced in both TGFβRIcKD and TGFβRIcKD infarcts; *$P<0.05$ vs TGFβRIcKD MI; n=3–5; α-Tubulin serves as loading control; 1-way or Kruskal–Wallis ANOVA. F, pSMAD3 is absent in the remote area and present in myocytes (arrowheads) and interstitial cells (arrows) in the border zone of control MIs. Intersitial cell pSMAD3 is still present in TGFβRIcKD hearts. DAPI indicates 4′,6-diamidino-2-phenylindole; p/t, ratio of phosphorylated to total protein; and WGA, wheat germ agglutinin.
MMP2 expression and activity were unchanged (Figure 3C–3E). Given the reports that myocytes can be a source of MMP9,23,24 we examined Mmp gene expression and gelatinolytic activity in isolated cells as well. Both Mmp2 and Mmp9 expression rose markedly but similarly in control and TGFβR1cKD myocytes, and gelatinolytic activity was only modestly altered, indicating that other cell types likely regulated total myocardial MMP activity (Online Figure IIIA–IIIC). This was confirmed by confocal immunofluorescence showing MMP9 to be largely present in the interstitium (Online Figure IIID), colocalizing with neutrophils in blood vessels and the perivascular region (Ly6G staining; Figure 3H).

Myocyte TGFβ Inhibition Markedly Reduces Neutrophil Recruitment to the Heart

Neutrophils are a major source of activated MMPs;25 therefore, we examined whether their myocardial recruitment was altered in TGFβR1<sup>KD</sup> hearts. At 24 hours, when neutrophils typically peak in the infarct zone,3 their amount was dramatically reduced in TGFβR1<sup>KD</sup> hearts versus controls (Figure 4A and 4B). Gene expression of the neutrophil chemokine receptor CXCR2 declined correspondingly (Figure 4C). Because neutrophils and other leukocytes home to the heart from the peripheral circulation and lymphatic tissues, we tested if myocyte TGFβR1 signaling also impacted this mobilization. Blood obtained at 24 hours after infarction showed neutrophilia in controls but not in TGFβR1<sup>KD</sup> mice (Figure 4D). Myocardial macrophages also play a prominent role in infarct remodeling,23 and although blood monocyte levels declined in TGFβR1<sup>KD</sup> MI mice compared with controls (Online Figure IVD), myocardial recruitment was similar in both hearts (Online Figure IVA–IVC). Because neutrophil homing can be triggered by cytokine or chemokine expression, we next examined whether these factors were differentially expressed in TGFβR1<sup>KD</sup> hearts. We found only a modest decline in TNFα, whereas other factors such as IL-1β, IL-6, and neutrophil chemokines CXCL1-3 markedly increased post-MI in both control and TGFβR1<sup>KD</sup> hearts (Figure 4E).
Reticulum Stress Response

Thrombospondin 4–Coupled Endoplasmic R1cKD mice at both gene (Figure 5C) and protein (Figure 5B) levels. Thbs4 was recently shown to be cardiac-protective when enhanced in myocytes, by stimulation of endoplasmic reticular (ER) stress responses.30 We found Thbs4 mainly expressed within cardiomyocytes at the border zone (Figure 5C), and multiple proteins involved with ER stress (activating transcription factor 6A, binding immunoglobulin protein, arginine-rich mutated in early stage tumors [ARMET], protein disulfide isomerase, calreticulin) were upregulated in TGFβR1<sup>−/−</sup> post-MI myocardium (Figure 6A and 6B). A smaller but still significant increase in the expression of Thbs4 and many ER stress proteins was also observed in sham-operated TGFβR1<sup>−/−</sup> hearts and cardiomyocytes (Figures 5 and 6; Online Figure V), indicating this pathway was primed before MI.

Myocyte TGFβ Inhibition Induces the Expression of Multiple Protective Cardiokines

IL-33 is an alarmin, a factor released after tissue injury, that acts as an endogenous danger signal mediating inflammatory responses.28 It is a ligand for the ST2 receptor and protective against several forms of heart diseases.30–32 In infarcted control hearts, Il13 expression rose slightly >2-fold, but in TGFβR1cKD hearts, Blood neutrophilia to higher protein levels as well (Figure 7B and 7C). Gene expression levels of ST2 membrane and soluble receptors were unaltered by TGFβR1 deletion (Online Figure VI). The cellular source of IL-33 in infarcted hearts appeared to be predominantly perivascular interstitial cells (Figure 7D).

The marked rise in IL-33 and Thbs4/ER stress responses suggested that the removal of canonical myocyte TGFβ signaling might impact the synthesis of other protective cardiokines as well. Growth and differentiation factor 15 (GDF-15) is a TGFβ-related cytokine expressed and secreted by cardiac myocytes that rises after MI and is induced by the transcription factor follistatin-like 1 (FSTL1).33,34 The deletion of GDF-15 enhances neutrophil recruitment to the infarct and increases myocardial rupture, whereas the administration of recombinant protein achieves the opposite.35 The net disparity in GDF-15 expression was quantitatively similar to that found

**TGFβR1<sup>−/−</sup> Hearts Exhibit Enhanced Thrombospondin 4–Coupled Endoplasmic Reticulum Stress Response**

The failure to suppress proinflammatory mediators despite reduced neutrophil migration suggested that the activation of alternative anti-inflammatory and cytoprotective pathways may be involved. One candidate was thrombospondin because mice lacking thrombospondin 1 show expanded macrophage and fibroblast activity in the border zone with destabilized infarcts,26 and mice lacking thrombospondin 2 demonstrate spontaneous left ventricular rupture after angiotensin infusion.27 Thbs1 expression did rise after MI in controls but also similarly in TGFβR1<sup>−/−</sup> hearts; Thbs2 was unaltered. However, thrombospondin 4 (Thbs4) expression rose much more in TGFβR1<sup>−/−</sup> mice at both gene (Figure 5A) and protein (Figure 5B) levels. Thbs4 was recently shown to be cardiac-protective when enhanced in myocytes, by stimulation of endoplasmic reticular (ER) stress responses.30 We found Thbs4 mainly expressed within cardiomyocytes at the border zone (Figure 5C), and multiple proteins involved with ER stress (activating transcription factor 6A, binding immunoglobulin protein, arginine-rich mutated in early stage tumors [ARMET], protein disulfide isomerase, calreticulin) were upregulated in TGFβR1<sup>−/−</sup> post-MI myocardium (Figure 6A and 6B). A smaller but still significant increase in the expression of Thbs4 and many ER stress proteins was also observed in sham-operated TGFβR1<sup>−/−</sup> hearts and cardiomyocytes (Figures 5 and 6; Online Figure V), indicating this pathway was primed before MI.

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between GDF-15+/− and wild-type mice, the latter displaying 50% lower mortality due to myocardial rupture. GDF-15 interacts directly with neutrophil β2 integrins to limit their activation and extravascular migration into tissue. As expected, gene expression of both FSTL1 and GDF-15 increased after MI in controls, but both rose significantly more in TGFβR1cKD hearts (Figure 7E and 7F). Effective immunostaining for GDF-15 was precluded by the poor sensitivity of existing antibodies; however, we were able to identify strong FSTL1 immunofluorescence in both infarct myocytes and interstitial cells (Figure 7G).

The collective presence of various protective cardiokines was accompanied by a reduction in cell death. Apoptotic cells were identified by immunostaining for cleaved caspase-3 and were almost exclusively located in the border zone region after MI and consisted of both interstitial cells and cardiomyocytes (Figure 8A; Online Figure VII). Cleaved caspase-3 levels were significantly lower in TGFβR1cKD hearts (Figure 8B).

**Cytokine- or Hypoxia-Stimulated GDF-15 Regulation Is Inversely Controlled by TGFβ**

The integrative complexity of multiple cell types signaling via various cytokines and cardiokines raised the question of whether the changes could be directly attributed to myocyte TGFβR downregulation. To test this, we exposed rat neonatal myocytes to 2 simulated aspects of infarction insult, cytokines (IL-1β and TNFα) or hypoxia. Cytokine stimulation induced GDF-15 gene expression, and the coadministration of TGFβ1 ligand attenuated this response. This suppressive effect of TGFβ1 on cytokine-induced GDF-15 expression was lost when TGFβR1 was silenced (Figure 8C).

In the case of hypoxia, we observed a downregulation of GDF-15 in cultured cardiomyocytes, and this was prevented by TGFβR1 silencing (Figure 8D).

FSTL1 and Thbs4 gene expression was also examined in these cells, but here, regulation by TGFβ1 stimulation and TGFβR1 silencing led to effects opposite to those observed.
Discussion

The discovery that early neutrophil activation, myocardial migration, and consequent infarct destabilization and rupture are orchestrated by cardiomyocyte TGFβ signaling is surprising, given that the wound healing process is generally considered the domain of inflammatory and matrix-depositing cells. We hypothesized that this finding was amplified after myocardial infarction (MI), given that the wound healing process is generally considered to be dominated by inflammation, matrix degradation, and infarct expansion. This was attributed to the role of TGFβ in suppressing inflammation via neutrophils, myofibroblast activation, and extracellular matrix generation. Here we show that the cell type being modulated plays a critical role, and that canonical TGFβ signaling in the myocyte contributes to the opposite, that is, neutrophil migration and suppressing cardiokines. Selective inhibition of this pathway is protective.

Shortly after the onset of irreversible ischemic damage, myocytes and other cells export cytokines and chemokines that initiate the immune response. As they die, cells also release internal contents including DNA that are highly proinflammatory (damage-associated molecular patterns) along with the appearance of immune cells that secrete cytokines and chemokines themselves creates feed-forward amplification of inflammation, matrix degradation, and infarct expansion. During this early period, the deposition of new matrix and collagen fibers is yet to occur, and the myocardial wall is particularly vulnerable to systolic stress–induced expansion and rupture. The propensity of myocardial wall rupture has been linked to neutrophil infiltration and the activity of matrix metalloproteinases. Later, inflammation recedes and the heart transitions to form a more stable collagen-rich scar. How does myocyte TGFβ inhibition regulate this sequence? The expression of major proinflammatory cytokines and neutrophil chemokines was comparable between experimental groups. Furthermore, although the suppression of canonical TGFβ signaling in myocytes may have protected some myocytes from programmed cell death, functional decline post-MI was generally similar, suggesting that the primary mechanism of cardioprotection was different. Another possibility was that replacement fibrosis in the infarct zone and hence tensile strength was enhanced by myocyte TGFβR1 deletion; however, collagen deposition was negligible at this time and collagen gene expression elevated to comparable levels between genotypes. Rather than these mechanisms, we propose that the blockade of myocyte TGFβ canonical signaling resulted in the coordinated activation of a secreted protein cascade that included multiple protective cardiokines.

The notion of a cardiac secretome is not new; well-known examples include natriuretic peptides, angiogenins II, or cytokines such as IL-6, TNFα, and TGFβ. However, recent studies have identified a broader set of cardiokines that play regulatory roles in cardiac remodeling. Intriguingly, we found that many of these, including IL-33, ARMET, FSTL1, and GDF-15, were enhanced in TGFβR1 knockout post-MI heart. IL-33 was unaltered. Furthermore, cell-selective TGFβ inhibition was required to observe this benefit, whereas early nonselective TGFβ antagonism was detrimental. The precise pathways involved differed from those invoked by pressure overload, where we found myocyte noncanonical signaling to be dominant and protective. In the post MI setting, classical TGFβ cascades were involved.

Previous studies investigated noncell-specific TGFβ inhibition in the setting of MI either by using neutralizing antibody against TGFβ, competitively inhibiting TGFβ signaling using gene transfer of soluble TGFβR2 to skeletal muscle, or using a small-molecule TGFβR1 inhibitor. All demonstrated detrimental effects when global TGFβ inhibition was initiated at the onset of ischemia. This was attributed to the role of TGFβ in suppressing inflammation via neutrophils, myofibroblast activation, and extracellular matrix generation. Here we show that the cell type being modulated plays a critical role, and that canonical TGFβ signaling in the myocyte contributes to the opposite, that is, neutrophil migration and suppressing cardiokines. Selective inhibition of this pathway is protective.

Figure 6. Myocyte transforming growth factor β (TGFβ) inhibition generates an enhanced endoplasmic reticulum (ER) stress response. A and B, ER stress response markers are increased in TGFβR1 knockout hearts at baseline, and this difference is amplified after myocardial infarction (MI). A, immunoblot; B, quantification; **P<0.05 vs TGFβR1 knockout sham; *P<0.05 vs TGFβR1 knockout MI; n=3–6; Atf6α-N indicates activating transcription factor 6α (nuclear form); ARMET, arginine-rich mutated in early stage tumors (also known as MANF); BIP, binding immunoglobulin protein (also known as HspA5 or GRP78); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and PDI, protein disulfide isomerase. Kruskal–Wallis (ATF6α-N, BIP) or 2-way ANOVA.

in vivo. TNFα and IL-1β cytokine stimulation lowered the expression of both genes, and hypoxia reduced Fst1 expression, and this was not reversed by TGFβR1 silencing (Online Figure VIII). Conversely, TGFβ ligand stimulation induced the expression of both Fst1 and Thbs4. Thus, for these proteins, the net changes in vivo depended on other factors not mimicked by the in vitro model or are the consequence of integrative multicell-type crosstalk.

cytokines were unaltered. Furthermore, cell-selective TGFβ inhibition was required to observe this benefit, whereas early nonselective TGFβ antagonism was detrimental. The precise pathways involved differed from those invoked by pressure overload, where we found myocyte noncanonical signaling to be dominant and protective. In the post MI setting, classical TGFβ cascades were involved.
among the most enhanced, and although its inflammatory regulation varies with the disease, in the heart it protects against pathological remodeling after infarction, myocyte hypoxia, and pressure overload. Intriguingly, IL-33 was predominantly located in nonmyocyte cells in the border zone. This demonstrates that myocyte TGFβR1-dependent

Figure 7. Myocyte transforming growth factor β (TGFβ) inhibition induces the expression of interleukin-33 (IL-33), Follistatin-like 1 (FSTL1), and growth and differentiation factor-15 (GDF-15). A to C, Myocyte TGFβ inhibition specifically and markedly induces gene (A) and protein (B) expression of the cardioprotective interleukin-33 (C; immunoblot; B: quantification), bar shading code is shown to the right of panel F. D, IL-33 immunofluorescence is predominantly located in interstitial cells; scale bar, 50 μm. E and F, Fstl1 and Gdf15 expression levels are increased in TGFβR1cKD myocardial infarction (MI); *P<0.01 vs respective sham; #P<0.05 vs TGFβR1fl/fl MI; n=4–8. G, FSTL1 immunofluorescence is located in interstitial cells and infarct myocytes. DAPI indicates 4',6-diamidino-2-phenylindole; TnT, troponin T; and WGA, wheat germ agglutinin; scale bar, 50 μm. Kruskal-Wallis or 1-way (B) ANOVA.

Figure 8. Myocyte transforming growth factor β (TGFβ) inhibition reduces apoptosis post–myocardial infarction (MI), and TGFβ1 modulates the expression of growth and differentiation factor-15 (GDF15) on cytokine stimulation or hypoxia in isolated cardiomyocytes. A, Apoptotic cells (cleaved caspase-3) reside almost exclusively in the infarct border zone and consist of myocyte and nonmyocyte cells. B, The amount of apoptosis after MI is reduced by myocyte TGFβ inhibition (bottom: immunoblot; top: quantification); *P<0.05 vs sham; #P<0.05 vs TGFβR1fl/fl MI; n=3–5. C, TGFβ1 (5 ng/mL) by itself does not alter Gdf15 expression but attenuates tumor necrosis factor α (50 ng/mL), and interleukin-1β (IL-1β; 10 ng/mL) induced upregulation of Gdf15 expression. This effect is lost when TGFβR1 is silenced; n=4–9 per group; 24-h stimulation. D, Hypoxia reduces transcript expression of GDF15. Silencing of TGFβR1 prevents downregulation of GDF15 with hypoxia; *P<0.01 vs normoxic control; n=6–9 per group; 24 h of hypoxia. 1-way (B) or Kruskal-Wallis ANOVA (C, D, within same color bars); *by unpaired t test.
signaling is not confined to the myocyte itself, but that intercellular crosstalk originating from the myocyte can ultimately drive IL-33 expression in nonmyocyte cells. Protection against ischemic injury by IL-33 has also been linked to C-Jun N-terminal kinase inhibition and reduced apoptosis, and we observed reduced C-Jun N-terminal kinase phosphorylation in TGFβR1−/− animals.

GDF-15, also known as macrophage inhibitory cytokine 1, is a member of the TGFβ superfamily. It is expressed in infarct myocytes and upregulated in infarcted mouse or human hearts. When overexpressed in myocytes, GDF-15 acts as an antihypertrophic factor coupled to the activation of SMAD2/3. Here, we show this signaling in reverse, in that by selectively blocking SMAD2/3 signaling, GDF-15 expression rises; thus, a negative feedback loop seems present. Kempf et al showed that GDF-15 suppresses neutrophil migration by interfering with β2 integrin activation and consequently with neutrophil adhesion and endothelial transmigration. Mice deficient in GDF-15 have increased cardiac neutrophil recruitment after MI associated with wall rupture, whereas enhancing GDF-15 levels is protective. Thus, the augmentation of GDF-15 would be highly consistent with the antiangioprotective effects seen in early infarct remodeling. GDF-15 knockdown has also been reported to affect monocytes and macrophages, although in the present analysis of early infarct remodeling, the dominant influence seems to be on neutrophils. FSTL1 is a potent inducer of GDF-15 promoter activity and gene expression and protective in cardiac pressure overload and ischemia/reperfusion1, and was prominently expressed in myocytes after infarction and further regulated in TGFβR1−/− hearts.

One potential way to integrate the collection of protective cardiokines is through a Thbs4-coupled ER stress response. One ER stress factor, ARMET (also known as mesencephalic astrocyte-derived neurotrophic factor), was differentially enhanced in the TGFβR1-knockdown model, is induced by ischemia, and its secretion reduces tissue damage. However, the analysis of gene arrays previously obtained in hearts overexpressing Thbs4 in cardiac myocytes did not identify IL-33, FSTL1, or GDF-15 expression changes. This indicates that the collective changes are unlikely coupled to Thbs4–ER stress alone, but to other TGFβ receptor–mediated transcriptional regulation. The finding that in isolated myocytes, the Thbs4 response to TGFβ stimulation was opposite that seen in vivo further supports the importance of complex cell-cell and/or cell-matrix interactions. This may in part involve the role of Thbs4 as a matrix–myocyte mechanosignaling molecule in addition to its role in ER stress. The difficulty in simulating the in vivo environment in a culture dish makes it hard to dissect out these specific influences. Importantly, however, disruption of myocyte TGFβR signaling is sufficient to profoundly alter these multicellular factors and ultimately impact inflammatory and remodeling responses.

A limitation of this study is that the signaling cascade critically responsible for the effects observed in myocyte TGFβR knockdown remains uncertain. Although GDF-15 did seem to be regulated in a consistent direction in the in vitro myocyte models, other cytokines were not, but these reductionist systems do not mimic the complex in vivo environment of an infarction. Because there are no current small-molecule inhibitors of the molecules identified, more complex, dual conditional genetic deletion models would be needed, and this is presently difficult. The present work required studies in mice to enable genetic modulations, but we recognize that such models may not translate to findings in humans, particularly in regard to inflammation. Lastly, the behavior in reperfused infarcts may differ from that in the permanent left anterior descending artery occlusion model. However, ischemia/reperfusion in mice has its own limitations and is less likely to induce wall thinning and rupture phenotype that we studied. Furthermore, a substantial number of MIs remain nonreperfused.

In conclusion, we demonstrate that myocyte TGFβ is a master regulator of acute neutrophil inflammatory response in ischemic hearts and controls the myocardial expression of multiple protective factors, each revealed to confer cytoprotection or diminish neutrophil recruitment. This supports a cohesive regulatory pathway, highlights the importance of cell-specific targeting of the TGFβ pathway, and reveals why noncell-selective therapies may have failed in the past. The identification of TGFβ-coordinated pathway(s) that can centrally regulate these multiple cardiokine mediators may ultimately lead to a potential therapeutic approach to suppress maladaptive remodeling in the heart.

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Disclosures
None.

References


What Is Known?

- Transforming growth factor β (TGFβ) is a ubiquitously expressed cytokine that regulates development, fibrosis, growth, and inflammation.
- In the heart, myocyte and nonmyocyte cells express TGFβ ligand and its cognate receptors TGFβR1 and TGFβR2.
- Global TGFβ inhibition during acute myocardial infarction (MI) remodeling is detrimental.

What New Information Does This Article Contribute?

- Targeted disruption of TGFβ signaling in the cardiomyocyte protects from left ventricular wall rupture and improves survival after MI by reducing neutrophil recruitment and myocardial matrix metalloproteinase (MMP9) expression and activity.
- Thrombospondin 4 and associated endoplasmic reticulum (ER) stress response markers are enhanced by cardiomyocyte TGFβ inhibition.
- Protective cardiokines, including the neutrophil integrin activation inhibitor GDF-15 and interleukin-33, are enhanced in TGFβR1-knockdown hearts.

TGFβ is a prominent regulator of inflammatory, hypertrophic, and fibrotic processes in myocardial remodeling caused by various forms of cardiac stress and disease. Cardiac myocytes as well as interstitial cells in vessels and the extracellular matrix crosstalk via TGFβ, and their selective contributions are critical to the net stress response. Here, we show that targeted disruption of TGFβ receptor signaling only in cardiomyocytes improves survival after MI by preventing ventricular wall rupture, whereas nonselective TGFβ inhibition promotes wall rupture and death. This protection involves the suppression of inflammatory cell recruitment to the myocardium, specifically neutrophils, and is associated with reduced MMP9 activity. We also observed enhancement of the myocyte ER stress response and an induction of multiple cardioprotective secreted factors (cardiokines) from both myocyte and nonmyocyte cells. This positions myocyte TGFβ upstream of a multicellular protective signaling cascade that potently impacts the inflammatory response and subsequent infarct remodeling. It also highlights the importance of TGFβ-dependent intercellular communication and the need for therapeutic modulators of this signaling that are either cell-specific or are more selective in targeting downstream effectors.
Cardiomyocyte-Specific Transforming Growth Factor β Suppression Blocks Neutrophil Infiltration, Augments Multiple Cytoprotective Cascades, and Reduces Early Mortality After Myocardial Infarction

Peter P. Rainer, Scarlett Hao, Davy Vanhoutte, Dong Ik Lee, Norimichi Koitabashi, Jeffery D. Molkentin and David A. Kass

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Supplemental Material

Supplementary Methods

Mouse model of myocyte-targeted TGFbetaR-knockdown

Cardiomyocyte-targeted TGFβ receptor knockout mice were generated as described\(^1\). Briefly, Myh6-MerCreMer\(^{+/+}\) (MCM) transgenic mice (B6/129-TG Myh5-cre/Esr1Jmk/J; #005650, Jackson Labs) were crossed with C57Bl/6 to generate Myh6-MCM\(^{+/−}\)/no-flox animals. These were crossed with TGFbetaR1\(^{fl/fl}\) or TGFbetaR2\(^{fl/fl}\) (on C57Bl/6 background) to derive Myh6-MCM\(^{+/−}\)/TGFbetaR1\(^{fl/fl}\) (TGFbetaR1\(^{cKD}\)) or Myh6-MCM\(^{+/−}\)/TGFbetaR2\(^{fl/fl}\) (TGFbetaR2\(^{cKD}\)). Genotype was confirmed with primers to Cre and TGFbetaR2 or TGFbetaR1 floxed alleles. To induce recombination, tamoxifen citrate (Sigma) was administered in soft diet (Bioserve, MA) at 80mg/kg/day for 10 days. Confirmation of gene knockdown and protein reduction has been reported\(^1\)-\(^2\). Experiments were conducted 2-4 weeks after tamoxifen treatment when echocardiography confirmed normal baseline function. C57Bl/6J mice (Jackson Labs) were used in antibody studies. TGFβ neutralizing antibody (clone 1D11) and control Ab (clone 13C4) were obtained from Genzyme, MA. Ab treatment was initiated at the time of LAD-ligation by intraperitoneal injection (10mg/kg BW, 3x/wk).

Blood pressure and telemetry

Blood pressure was measured in conscious mice using an XBP1000 non-invasive tail blood pressure system (Kent Scientific Corporation, Torrington, CT, USA). After mice were acclimated to the apparatus, 20 measurements per animal were averaged to assess blood pressure. Electrocardiogram-telemetry was performed in n=5 mice using implantable sensors and remote acquisition hardware (Data Sciences International, MN).

Echocardiography

Trans-thoracic echocardiography was performed in conscious mice using a 13 MHz transducer (Acuson Sequoia C256; Siemens). M-mode LV dimensions were averaged from 3-5 beats at physiologic heart rates and fractional shortening and LV mass were calculated. Echocardiography and analyses was performed blinded to the experimental group.
Molecular analyses

RNA was extracted using TRIzol reagent (Invitrogen) from myocardium or isolated cardiomyocytes according to manufacturer’s instructions and reverse transcribed. Messenger RNA was analyzed by qRT-PCR on Applied Biosystems Prism 7900HT or BioRad CFX machines with Taqman (Applied Biosystems) or custom SYBR green primers. Primer sequences are provided in Table S1. The specificity of the SYBR green assays was confirmed by melting curve analysis. Mouse PCR array PAMM-035 (SA Biosciences) was used to screen for TGFβ/BMP signaling.

Western blot was performed using standard techniques. Tissue was lysed with RIPA buffer and subjected to SDS-PAGE using Nupage gels (Invitrogen) and transferred to nitrocellulose membranes. Antibodies to Smad2 (Ser465/467), ERK1/2 (Thr202/Tyr204), JNK (Thr183/Tyr185), p38 (Thr180/Tyr182), TAK1 (Thr187), αTubulin, cleaved caspase 3 (17/19kDa), Thbs4, cleaved Atf6a (50kDa), BIP, Calreticulin, ARMET, PDI, Gapdh were obtained from Cell Signaling Technology, Santa Cruz, Fitzgerald or Abcam and fluorescence labeled secondary antibodies were obtained from Licor. Membranes were scanned on an infrared imaging system (Odyssey, Licor) and quantification of band intensity performed using Odyssey Application Software 3.0. Some membranes (Thbs4 and ER stress markers) were developed using AP-conjugated secondary antibodies (Santa Cruz) and visualized using chemifluorescence with subsequent densitometric analysis using NIH Image J software. MMP2 and MMP9 gelatinolytic activity was assessed by subjecting tissue or cell lysates to SDS-electrophoresis in 0.1% gelatin containing zymogram gels (Novex, Life Sciences). Renatured and developed gels were stained and scanned on the Odyssey imaging system.

Histology/Immunostaining

Myocardium was fixed with 4% paraformaldehyde, paraffin embedded and sectioned into 4 μm slices. Masson’s trichrome staining was used to visualize fibrosis. Quantification of fibrosis content was performed in 4-6 regions of the remote area. Immunostaining: Slides were deparaffinized and subjected to heat-mediated antigen retrieval. Mac-3 (clone M3/84, BD Pharmingen), Ly6G (clone NIMP-R14, Abcam), cleaved caspase 3 (Cell Signaling Technology) primary antibodies were used followed by HRP-based staining and DAB revelation. Positive staining was quantified by pixel count, signal intensity, and number of positive cells per area using Aperio Image Scope software in parallel sections of the infarct and border zone. Immunofluorescence for SMAD3 (S423/425, Millipore), MMP9 (Abcam), Thbs4 (Santa Cruz), IL-33, FSTL1 (R&D Systems) was performed using AlexaFluor 488 conjugated secondary antibodies.
or tyramide signal amplification kit (Invitrogen) following the manufacturer’s protocol. Counterstain was performed using DAPI (Invitrogen), WGA (AlexaFluor 647, Invitrogen), and mouse sarcomeric-actinin (Sigma), Troponin-T (Thermo Scientific), or Laminin (Sigma-Aldrich) antibodies. Image acquisition was performed on a Zeiss LSM510-META laser scanning or a Nikon A1 confocal microscope.

References (Methods)

Online Table 1

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References (Primer Sequences)

Online Figure I Differences in early mortality after myocardial infarction are caused by left ventricular wall rupture. (A) Developed systolic and diastolic blood pressures decreased to the same extent in both genotypes post-MI and heart rate did not differ [*p<0.05 vs. sham, n=4-6]. (B) The posterior left ventricular wall had slightly hypertrophied 24 hours post-MI and the infarcted anterior left ventricular wall thinned without differences between genotypes [PWEDT: posterior wall end-diastolic thickness, IVSED: interventricular septum end diastolic thickness, *p<0.05 vs. sham, n=7-11]. (C) No life-threatening arrhythmias were observed post-MI [n=5]. (D) Necropsy confirmed left ventricular wall rupture as the cause of death [left: gross pathology with blood clot at rupture site (arrow, arrowhead: LAD-ligation site), right: hematoxylin-eosin stained section demonstrates the presence of a thrombus at the rupture site (arrowheads). (E) TGFbetaR1<sup>kld</sup> animals demonstrated the same degree of dysfunction, dilation, and hypertrophy as TGFbetaR2<sup>kld</sup> animals [*p<0.05 vs. sham, #p<0.01 vs. sham, n=4-7]. 2-way (A, B), 1-way (E: FS%) or Kruskal-Wallis (E: others) ANOVA followed by Bonferroni or Holm-Sidak (A) correction.
Myocyte TGFβ inhibition does not alter fibrosis, hypertrophy, or fetal gene expression patterns in chronically remodeled ischemic hearts. **(A, B)** Fibrosis in the remote area at 6 weeks post-MI is mild [(A) Masson’s Trichrome stain, (B) quantification [n=3-7]. **(C)** Heart and lung weight normalized to tibia length (TL) does not differ between genotypes [*p<0.05 vs. sham, n=6-8]. **(D)** The fetal gene expression and heart failure markers *Nppa* (A-type natriuretic peptide), *Nppb* (B-type natriuretic peptide), and *Myh7* (β-myosin heavy chain) were induced after MI but did not differ between genotypes [p<0.05 vs. sham, n=8-9]. Kruskal-Wallis ANOVA.
Online Figure III Matrix-metalloproteinase expression and activity in isolated myocytes and tissue localization. (A) The expression of Mmp2, Mmp9 and Timp1 in cardiomyocytes parallels tissue expression patterns but the changes are attenuated. (B) Differences in MMP9 gelatinolytic activity are mild [(B) zymogram, (C) quantification [*p<0.05 vs. sham, #p<0.05 vs. TGFbetaR1^{fl/fl} MI, n=3]. (D) MMP9 immunofluorescence is predominantly localized in the interstitium [WGA: wheat germ agglutinin, DAPI: 4',6-diamidino-2-phenylindole, TnT: troponin T; scale bar: 10µM]. Kruskal-Wallis ANOVA.
Online Figure IV Myocyte TGFβ inhibition does not impact monocyte/macrophage recruitment to the infarcted heart. (A, B, C) Myocardial macrophage (Mφ) infiltration assessed by immunostaining for the macrophage marker Mac-3 ([B] scale bar: 1mm (overview) and 200µM (border zone and infarct zone), quantification (C), n=3-6) and (C) presence of Mac-2 (Lgals3) transcripts was not impacted by myocyte TGFβ inhibition post-MI [*p<0.001 vs. respective sham, n=5-6]. (D) Monocyte blood levels were reduced 24 hours post-MI in TGFbetaR1^{ckD} MI [n=4-7 per group, * p<0.05 vs. TGFbetaR1^{fl/fl} sham, #p<0.05 vs. TGFbetaR1^{fl/fl} MI]. Kruskal-Wallis ANOVA.
Online Figure V Endoplasmic reticulum stress response markers are up-regulated in TGFbetaR1\textsuperscript{cKD} sham mice. (A, B) The endoplasmic reticulum stress response markers thrombospondin 4 (Thbs4), activating transcription factor 6 alpha (nuclear form, ATF6\textalpha-N), immunoglobulin heavy chain binding protein (BIP, also known as Hspa5 or GRP78), arginine-rich mutated in early stage tumors (ARMET), protein disulfide isomerase (PDI), and calreticulin are induced in myocardial tissue [*p<0.05, n=5 per group], and (C) isolated cardiomyocytes by myocyte TGFβ inhibition in control conditions. Unpaired t-test.
Online Figure VI Transcript expression levels of the membrane-bound and soluble IL-33 receptors (lST2 and sST2) are not altered by TGFbetaR1^CKD [n=4-8]. Kruskal-Wallis ANOVA.
**Online Figure VII** Myocyte TGFβ inhibition reduces apoptosis post-MI. **(A)** Apoptotic cells (cleaved caspase-3) reside almost exclusively in the infarct border zone [low magnification, scale bar: 200µM] and **(B)** consist of myocyte and non-myocyte cells [higher magnification, scale bar: 50µM].
Online Figure VIII TGFβ1 modulation of Fstl1 or Thbs4 expression in cultured cardiomyocytes in response to cytokine stimulation (A, B) or hypoxia (D, E). Stimulation with TNFα [50 ng/ml] and IL-1β [10 ng/ml] reduces (A) Fstl1 and (B) Thbs4 expression [n=8-12 per group]. TGFβ1 ligand stimulation induces expression of Fstl1 and Thbs4. (D) Hypoxia reduces Fstl1 expression and (E) blunts TGFβ1 mediated Thbs4 expression changes [n=9-12, *p<0.05 vs. control (first bar)]. (C) Il6 expression serves as a positive control for cytokine stimulation [n=4 per group]. (F) HRE-driven GFP fluorescence in PC3 cells [scale bar: 200 µM] and (G) Slc2a1 (GLUT1) expression in myocytes serve as a positive control for hypoxia [n=3 per group]. Kruskal-Wallis (A, B), 1-way (C), or 2-way ANOVA (D, E empty bars), unpaired t-test (G).