Getting to the Heart of the Matter: New Insights Into Cardiac Fibrosis

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Abstract: Fibrotic diseases are a significant global burden for which there are limited treatment options. The effector cells of fibrosis are activated fibroblasts called myofibroblasts, a highly contractile cell type characterized by the appearance of α-smooth muscle actin stress fibers. The underlying mechanism behind myofibroblast differentiation and persistence has been under much investigation and is known to involve a complex signaling network involving transforming growth factor-β, endothelin-1, angiotensin II, CCN2 (connective tissue growth factor), and platelet-derived growth factor. This review addresses the contribution of these signaling molecules to cardiac fibrosis. (Circ Res. 2015;116:1269-1276. DOI: 10.1161/CIRCRESAHA.116.305381.)

Key Words: cicatrix • endothelins • fibrosis • platelet-derived growth factor

Cardiac fibrosis, characterized by the excessive production and deposition of scar tissue, is often a result of conditions such as hypertension and diabetes mellitus. The cells ultimately responsible for the development of scar tissue are mesenchymal cells resident within connective tissue called myofibroblasts, which possess the highly contractile protein α-smooth muscle actin (α-SMA). In myofibroblasts, α-SMA is assembled into stress fibers that can remodel the surrounding extracellular matrix (ECM) because they are connected to ECM through specialized cell surface structures called focal adhesions. In the diseased heart, cardiomyocytes are lost to necrotic cell death, and myofibroblasts are activated to initiate a reparative fibrosis. The adult mammalian heart has negligible regenerative capacity, and thus, normal cardiac repair, for example, postinfarction, is dependent on the clearance of dead cells and on the formation of a scar tissue to help preserve heart integrity. Conversely, a hyperactive repair program has been hypothesized to cause pathological fibrosis. A complex interaction among a network of growth factors/cytokines and hormones is responsible for initiating and maintaining fibrotic responses in vivo (Figure 1). In particular, angiotensin II (Ang-II), endothelin-1 (ET-1), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) appear to work together to induce activation of resident interstitial fibroblasts, promote persistence of myofibroblasts and induce the expression of a wide variety of ECM components, including collagen type I. The ultimate origin of myofibroblasts is unclear, but it could result from a variety of processes including growth factor–mediated differentiation of resident mesenchymal cells, recruitment of pericyte-like progenitor cells, or by epithelial–mesenchymal transition. Recently, it has become increasingly appreciated that the cellular microenvironment also plays a critical role in promoting pathological responses to these growth factors/cytokines and hormones. For example, the role of elevated mechanical loading/tension and elevated proadhesive signaling, promoted by matricellular proteins, such as CCN2 (formerly referred to as connective tissue growth factor), seems to be extremely important. Matricellular proteins are nonstructural ECM components that modulate growth factor responses to aging, mechanical loading, and regeneration, and hence, they promote angiogenesis, inflammation, tissue repair, and fibrosis. As a result of this complexity, the appropriate intervention points to base rational antifibrotic therapies remain unclear.

Transforming Growth Factor-β

There are 3 TGF-β ligands, TGF-β1, TGF-β2, and TGF-β3. Of these, TGF-β1 has been the most studied. TGF-β1 expression is upregulated in both normal tissue repair and pathological fibrosis. TGF-β1 is initially produced as a secreted latent form, which is proteolytically activated in a fashion that involves integrin-mediated ECM contraction (Figure 2). Once activated, TGF-β binds to TGF-β type I and TGF-β type II receptors. The TGF-β type I receptor is a kinase, and in the case of fibroblasts, it is termed activin-linked kinase (ALK) 5. ALK5 phosphorylates Smad2 and 3; phosphorylated Smad2/3 bind to Smad4, translocate into the nucleus, and activate gene transcription (Figure 2). In addition to this so-called canonical pathway, TGF-β activates noncanonical signaling pathways (eg, mitogen-activated protein kinase pathway) that appear to modify gene expression in a promoter-specific fashion.

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As reviewed previously,²⁻⁵ TGF-β promotes ECM deposition by (1) upregulating ECM and tissue inhibitors of matrix metalloproteinase (TIMP) gene expression and (2) suppressing matrix metalloproteinase (MMP) gene expression. In cell adhesion and integrin-dependent fashion, TGF-β causes fibroblasts to differentiate into myofibroblasts.¹⁰ Dermal wounds treated with anti-TGF-β strategies (eg, antibodies or antisense oligonucleotides) show reduced ECM and scar tissue deposition.¹¹,¹² Similarly, Smad3-deficient mice display accelerated wound healing, decreased granulation tissue formation, and reduced inflammation.¹³,¹⁴ Fibroblasts derived from Smad3-deficient mice are relatively resistant to the ability of TGF-β to induce collagen and other ECM genes.¹⁵,¹⁶

The canonical TGF-β pathway contributes to cardiac fibrosis. In the border zone of healing infarcts, the TGF-β/Smad3 pathway is activated and induces remodeling.¹⁷,¹⁸ In cardiac fibrosis and remodeling, TGF-β is initially derived from immune cells and is subsequently produced by myofibroblasts (Figure 3).¹⁹

In cultured cardiac fibroblasts, the ability of TGF-β to induce procollagen type III and tenasin-C depends on Smad3, and Smad3-deficient hearts are relatively resistant to interstitial fibrosis.⁷ TGF-β1 inhibition of cardiac fibroblast proliferation requires Smad3.¹⁸ Moreover, TGF-β1 induction of collagen lattice contraction and α-SMA expression also requires Smad3.¹⁹ Finally, TGF-β1 prevents cardiac fibroblast apoptosis in response to infarction through Smad3 and also extracellular signal–regulated kinases 1/2 and Akt.²⁰ Intriguingly, in cardiac fibrosis observed postmyocardial infarction, decreased miR-24 expression exists in hypertrophic hearts; in cardiac fibroblasts, TGF-β increases miR-24 expression, whereas overexpression of miR-24 reduces TGF-β secretion and Smad2/3 phosphorylation.²¹ The likely target of miR-24 is furin, a protease that is implicated in processing of latent TGF-β.²¹

ALK5 inhibitors have been developed as potential antiﬁbrotic agents. In a rat coronary artery ligation model of myocardial infarction, the ALK5 inhibitor GW 788388 decreased TGF-β activity and reduced both systolic dysfunction and left ventricular (LV) remodeling.²² Similarly, the ALK5 inhibitor SM16 attenuated pressure load–induced fibrosis in vivo and TGF-β–induced Collα2 and lysyl oxidase expression in vitro.²³ However, although SM16 treatment improved diastolic function and cardiac output, this compound also caused a significant increase in inﬂamatory heart valve lesions.²³ Neutralizing anti-TGF-β antibodies, when used in a model of experimental myocardial fibrosis, decreased fibroblast activation and collagen mRNA expression, but did not appreciably affect myocyte hypertrophy, blood pressure, and systolic function.²⁴ In a related yet separate study, neutralizing anti-TGF-β antibodies decreased collagen production and increased matrix metalloproteinase expression but worsened vascular remodeling and resulted in increased mortality.²⁵ Collectively, these data obtained using both ALK5 inhibitors and anti-TGF-β antibodies provide support for the idea that targeting the canonical TGF-β signal pathway might not be viable clinically.

Instead of blocking canonical TGF-β signaling, a better alternative might be to block noncanonical signaling pathways. For example, TGF-β activates TAK-β–activated kinase (TAK) 1, a mitogen-activated protein kinase kinase kinase (Figure 2). Supporting the hypothesis that TAK1 contributes to cardiac fibrosis, it has been shown that TAK1 is activated in cardiomyocytes after pressure overload generated by aortic constriction and that cardiac-specific overexpression of activated TAK1 in mice causes cardiac hypertrophy and heart failure.²⁶ In addition, dominant negative TAK1 inhibits TGF-β–induced hypertrophic events in mouse cardiomyocytes and fibroblasts, including ECM production.²⁷ One of the targets of TAK1 is p38; p38 is activated in heart failure evolving in response to sustained hemodynamic overload and contributes to cardiac fibrosis (Figure 2).²⁸⁻²⁹ Another noncanonical pathway may involve the generation of reactive oxygen species. Matrix remodeling by TGF-β is amplified by increases in oxidative stress; TGF-β induces myofibroblast differentiation in cardiac fibroblasts via NADPH oxidase (NOX) 4 (Figure 2).³⁰ In myxomatous mitral valve disease in humans, elevated oxidative stress is observed associated with increases in NOX2.
and 4. Treatment of cardiac fibroblasts with small interfering RNA against NOX4 suppresses the expression of TGF-β target genes, including fibronectin, collagen I, α-SMA, and CCN2, indicating that NOX4 was involved in TGF-β-induced myofibroblast differentiation. As discussed above, TGF-β activates noncanonical signaling pathways, including the mitogen-activated protein kinase members c-Jun N-terminal kinase and p38. As c-Jun N-terminal kinase and p38 are redox sensitive and can be activated by reactive oxygen species in the cytoplasm, it is possible that reactive oxygen species may activate these pathways and augment the TGF-β signaling response (Figure 2). Collectively, these data suggest that targeting TAK1 or NOX4 downstream of TGF-β might be viable antifibrotic approaches.

**Angiotensin II**
The levels of Ang-II, an oligopeptide that causes vasoconstriction and increased blood pressure, are elevated in fibrotic hearts. In rats with myocardial fibrosis, Ang-II is both expressed and activated by macrophages and myofibroblasts (Figure 3). In cardiac myocytes and fibroblasts, Ang-II induces expression of TGF-β1 through the angiotensin type 1 receptor, and, in vivo, TGF-β is required for Ang-II to cause both cardiac hypertrophy and fibrosis (Figure 1). Indeed, in cardiac fibroblasts, Ang-II induces expression of collagen through TGF-β/Smad3 and extracellular signal–regulated kinase by an interleukin 6 (IL-6)–dependent mechanism (Figure 3). The ability of Ang-II to induce IL-6 expression also seems to involve nuclear factor κB activation. Supporting the idea that IL-6 is important for the fibrogenic function of Ang-II is a recent publication showing that IL-6 knockout mice, although not displaying altered development of Ang-II high salt–induced hypertension and cardiac hypertropy, were resistant to Ang-II–induced cardiac dysfunction, myocardial inflammation, and fibrosis.
Another mechanism underlying the fibrotic ability of Ang-II may involve miR-29b. Loss of miR-29b occurs in cardiac fibrosis; in vitro knockdown of miR-29b enhances whereas overexpression of miR-29b inhibits Ang-II–induced collagen type I and α-SMA expression. The basis of this observation may be that miR-29b targets a sequence within the TGF-β1 coding region (Figure 2). Ang-II suppresses both miR-29b and miR-133a in vivo; mutation of miR-133a binding sites in the 3′-UTR of CollA1 mRNA abolished miR-133a–mediated repression of reporter gene activity, showing that CollA1 is a bona fide target of miR-133a. Collectively, these data are consistent with the hypothesis that Ang-II is upstream of TGF-β and IL-6 in driving cardiac fibrosis.

Angiotensin receptor inhibitors, such as losartan, are effective in reducing cardiac fibrosis in both animals and humans. Losartan inhibits endothelial-to-mesenchymal transformation in mitral valve endothelial cells by blocking TGF-β–induced phosphorylation of extracellular signal–regulated kinase (Figure 2). Moreover, losartan attenuates obesity-induced metabolic and cardiovascular changes. Importantly, in a small study, losartan was shown to diminish progression of myocardial hypertrophy and fibrosis in patients with nonobstructive hypertrophic cardiomyopathy. These divergent results could occur because of the bioavailability or stability of the drugs thus far developed (ie, they arose because of compound class effects) or to the differences between acute and chronic disease models (ie, they arose because of the indications examined). Thus, it is unclear at present whether ET-1 and its receptors represent viable antifibrotic therapeutic targets.

**Endothelin-1**

ET-1, cleaved to a physiologically active peptide by endothelin-converting enzyme, is the significant endothelin isoform in humans and possesses powerful promitogenic and vasoconstrictive activities. ET-1 activates the endothelin-A (ETA) and the endothelin-B (ETB) receptors (Figure 1) and is predominantly produced by endothelial cells, but can be produced by other cell types, such as macrophages, cardiomyocytes, and fibroblasts (Figure 3). Through the ETA receptor, ET-1 decreases DNA synthesis and increases collagen production in cardiac fibroblasts. In isolated valves, ET-1 increases compliance of aortic cells but does not effect cells on the ventricular side of the valves, consistent with the observation that cells on the aortic side possess elevated levels of actin protein. Cultured vascular SMCs; furthermore, MRTF-A silencing attenuates ET-1–induced synthesis and release of proinflammatory mediators, including IL-6 and monocyte chemotactic protein-1. Thus, ET-1 and MRTF-A are likely to work in a feed-forward manner to promote inflammation and fibrogenic activity.

Initial reports in humans showed that short-term administration of bosentan to patients with severe heart failure resulted in hemodynamic and cardiac benefits. However, a series of randomized controlled clinical trials examining the effects of ET receptor antagonists on coronary artery disease and heart failure have not generated positive results; in many studies, endothelin receptor antagonism resulted in harmful effects, generally attributable to enhanced fluid retention. For example, the ETA antagonist darusentan, in the EARTH (Endothelin A Receptor antagonist Trial in Heart failure) study, or enrasentan (a dual ET$_A$/ET$_B$ antagonist) did not show any favorable effects, despite improved hemodynamics. These divergent results could occur because of the bioavailability or stability of the drugs thus far developed (ie, they arose because of compound class effects) or to the differences between acute and chronic disease models (ie, they arose because of the indications examined). Thus, it is unclear at present whether ET-1 and its receptors represent viable antifibrotic therapeutic targets.

**CCN2**

Matricellular proteins are highly spatiotemporally regulated nonstructural components of the ECM that, in a context-dependent fashion, modulate cellular responses to signaling molecules, such as cytokines and ECM components. Formerly referred to as connective tissue growth factor, CCN2, a member of the CCN family of matricellular proteins, is upregulated in fibroblasts in response to TGF-β and is overexpressed in fibrotic disease, including cardiac fibrosis including those caused by myocardial infarction, diabetes mellitus, tumors, and hypertrophy (Figure 3). In animals exposed to Ang-II, myocardial CCN2 mRNA peaked at 6 hours, whereas TGF-β peaked at 3 days compared with saline control. CCN2 expression occurred before fibrocyte migration (1 day) into the myocardium or ECM deposition (3 days). CCN2 protein expression was observed localized to resident cells by day 3 of Ang-II exposure. Ang-II, when exposed to cultured cardiomyocytes and microvascular endothelial cells, caused an increase in CCN2 expression that was blocked by an anti-TGF-β–neutralizing antibody (Figure 3). Indeed, in atrial fibroblasts, Ang-II increases CCN2 expression via mitogen-activated protein kinases/TGF-β1/tumor necrosis factor receptor–associated factor 6 pathway. Intriguingly, in uninjured hearts, CCN2 is specifically localized to endothelial cells. In several studies, it has been shown that CCN2...
promotes the fibrogenic activity of TGF-β, likely through promoting adhesive signaling (Figure 1).2,78

CCN2 both in vitro and in vivo causes hypertrophy of rat cardiomyocytes.79,80 Expression of CCN2 mRNA is induced by both high glucose and palmitate in H9c2 cells and in mouse neonatal cardiomyocyte primary cultures.81 Small interfering RNAs against CCN2 reduced the abilities of high glucose and palmitate to induce hypertrophy and apoptosis.31 These CCN2 effects were shown to rely on the activity of the tyrosine kinase A receptor that has previously been implicated in CCN2-dependent signaling (Figure 1).81 CCN2 silencing using small interfering RNA of activated primary cardiac fibroblasts resulted in strongly reduced expression of stretch-induced chemokines (Ccl2, Cc17, and Cc18), matrix metalloproteinases (MMP2 and MMP9), ECM (Col3a1), and a cell-to-cell contact protein (Cx43).32 Ang-II–induced expression of hypertrophic marker genes or collagen was not affected by treatment with anti-CCN2 monoclonal antibodies, whereas anti-CCN2 monoclonal antibodies caused resistance to adverse LV remodeling and LV dysfunction in hearts resulting from pressure overload caused by thoracic aortic constriction.83 Conversely, when a thoracic aortic constriction model was used, anti-CCN2 antibodies reduced both collagen levels and hypertrophic marker gene expression, reduced cardiomyocyte crosssectional area and LV dilatation, and preserved LV systolic function.83 Collectively, these results indicate that, in cardiac fibrosis, strategies targeting CCN2 might be considered.

Platelet-Derived Growth Factor
PDGF consists of a complex family of homodimeric or heterodimeric growth factors, including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD. These collectively signal through 2 PDGF receptors, α and β.34,45 After myocardial infarction, PDGF-A and D are significantly increased in endothelial cells, macrophages, and myofibroblasts.86 Enhanced PDGF-A, PDGF-D, and PDGF receptors are coincident with angiogenesis and inflammatory and fibrogenic responses in the infarcted myocardium, suggesting their regulation on cardiac repair.86 Transgenic mice overexpressing the active core domain of PDGF-D in the heart displayed enhanced proliferation of both cardiac interstitial fibroblasts and arterial vascular smooth muscle cells resulting in cardiac fibrosis followed by dilated cardiomyopathy and, subsequently, cardiac failure.87 In vitro, PDGF-D significantly enhanced TGF-β1 synthesis, which was eliminated by TGF-β blockade with small interfering RNA; moreover, the stimulatory role of PDGF-D on fibroblast proliferation and collagen synthesis was abolished by TGF-β blockade.88 In infarcted heart, the perivasculature and mononuclear-like cells show activation of the PDGF signaling pathway.89 This pattern is interesting given the potential involvement of pericyte activation as source of activated myofibroblasts in fibrosis.2,23 Injection of a neutralizing PDGF receptor α–specific antibody attenuated atrial fibrosis in pressure-overloaded hearts, whereas PDGF-AA stimulated both atrial fibrosis and fibrillation in normal hearts.90 In infarcted heart, TGF-β1, TIMP1 and 2, and type I collagen mRNA were all significantly increased in a PDGF receptor–dependent fashion; moreover, the ventricular dysfunction present in infarcted hearts and was mildly improved in the presence of the PDGF/c-Abi inhibitor imatinib.91 Similarly, PDGF-A, PDGF-C, or PDGF-D, when introduced into the heart using adenovirus-mediated delivery, significantly upregulated TGF-β1 mRNA expression and also accelerated cardiac fibrosis and arteriosclerosis.92 Collectively, these data indicate that PDGF may promote fibrosis by elevating TGF-β levels. These results strongly suggest that PGDF may be a good target for antifibrotic therapy in the heart; however, given the complexity of the molecules involved with the PDGF signaling pathway, developing antifibrotic agents against this pathway may be difficult.

Future Prospects and Conclusions
In the context of the heart, excessive scarring can cause increases in tissue stiffness, cardiomyocyte atrophy, arrhythmia, and hypoxia. Abundant data suggest that a complex interaction involving TGF-β, ET-1, Ang-II, and PDGF causes fibrogenesis. Because of the fact that these proteins, notably TGF-β, play important roles in other biological processes, including homeostasis and normal repair, broad targeting of these pathways may be problematic. It is interesting to note that perfinidone, a broad anti-inflammatory effect, has an effect blocking TGF-β and Ang-II–induced fibrosis.93-95 Clinical trials examining appropriate end points of cardiac fibrosis (eg, levels of serum procollagen type I carboxyterminal peptide or CCN2) are warranted; however, development of more specific agents is likely to be of benefit to minimize any potential side effects. Thus, recent work elucidating the signaling mechanisms underlying fibrotic signaling pathways is useful. Of these, TAK1, NOX, or MRTF-A may be of note, and further efforts elucidating the role of these effectors in fibrosis is warranted. Moreover, accumulating evidence that the tissue microenvironment plays a key role in fibrogenesis suggests that the matricellular protein CCN2 may be a key common mediator of fibrosis. Additional detailed studies examining the contribution of CCN2 and related matricellular proteins in cardiac fibrogenesis are required.

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