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Mechanisms of cardiac collagen deposition in experimental models and human disease

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Abstract

The inappropriate deposition of extracellular matrix within the heart (termed cardiac fibrosis) is associated with nearly all types of heart disease, including ischemic, hypertensive, diabetic, and valvular. This alteration in the composition of the myocardium can physically limit cardiomyocyte contractility and relaxation, impede electrical conductivity, and hamper regional nutrient diffusion. Fibrosis can be grossly divided into 2 types, namely reparative (where collagen deposition replaces damaged myocardium) and reactive (where typically diffuse collagen deposition occurs without myocardial damage). Despite the widespread association of fibrosis with heart disease and general understanding of its negative impact on heart physiology, it is still not clear when collagen deposition becomes pathologic and translates into disease symptoms. In this review, we have summarized the current knowledge of cardiac fibrosis in human patients and experimental animal models, discussing the mechanisms that have been deduced from the latter in relation to the former. Because assessment of the extent of fibrosis is paramount both as a research tool to further understanding and as a clinical tool to assess patients, we have also summarized the current state of noninvasive/minimally invasive detection systems for cardiac fibrosis. Albeit not exhaustive, our aim is to provide an overview of the current understanding of cardiac fibrosis, both clinically and experimentally.

INTRODUCTION

The heart is a perpetually working muscle whose function is to pump oxygenated blood throughout the body in order to maintain the viability of all organs, including the heart itself. It is a complex organ that is made up of a variety of cells including cardiomyocytes, vascular smooth muscle cells, endothelial cells, macrophages, and others. Fibroblasts are among the most abundant cell types in the heart. While the exact percentage remains controversial, they have been reported to comprise as many as half of the cells in the heart in rodent species.[1] Although cardiac fibroblasts serve a variety of purposes, their main role is to generate and maintain a scaffold infrastructure that holds the heart together, transduces the shortening of individual cardiomyocytes into efficient muscle pump activity, and helps anchor in place other cardiac cells that regulate cardiomyocyte function.[2,3] Given the close association

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between the heart's fibrous tissue infrastructure and cardiomyocytes, which are the contractile units of the heart, changes in the properties of the fibrous mesh can adversely affect both the heart's pumping function and its filling properties. Not surprisingly, alterations in either the quality or quantity of the fibrous tissue infrastructure contribute substantially to the development and severity of heart failure.

REPARATIVE VS REACTIVE FIBROSIS

To provide the scaffold necessary to hold the heart together and coordinate its function as a pump, cardiac fibroblasts must ultimately produce a variety of provisional and structural proteins, the most important of which are the collagens, particularly collagen I (Col I) and collagen III (Col III). Collectively, these extracellular matrix (ECM) proteins define the fibrous meshwork of the heart. Abnormalities in the ECM can occur due to abnormal quantities of ECM proteins (both excesses and deficiencies), alterations in ECM quality (eg, changes in crosslinking), and changes in the proportion of the various individual components of the ECM (including both alterations in the proportion of noncollagen to collagen matrix components[4] and in the relative amounts of Col I to Col III). Regulation of the amount and composition of the ECM is a dynamic process involving both the production and degradation of collagen molecules. Cardiac fibrosis, which is a cause or companion of many cardiovascular diseases, occurs when there is an imbalance between these processes so that the production of ECM proteins exceeds their degradation.

There are 2 types of fibrosis in the heart.[5] Cardiac fibrosis that develops in response to a loss of cardiomyocytes is considered to be reparative fibrosis. This type of fibrosis is stimulated by myocyte necrosis and is an essential reparative response to injury and cell death. In the heart, perhaps the most prominent and relevant example of this is the generation of a replacement scar for a segment of myocardium that has undergone extensive cardiomyocyte death as a consequence of a myocardial infarction (MI). The timely formation of an adequate replacement scar in the infarct zone is a critical response and the failure for this to occur enhances the likelihood of post-MI myocardial rupture, a complication that is usually fatal. Replacement of devitalized bulging myocardium in the infarct zone by stiffer, less distensible fibrous tissue also limits post-MI dilatation. In controlling the increase in ventricular radius, deposition of a replacement scar helps to limit increases in wall stress in the chamber. As wall stress is an important stimulus for further maladaptive remodeling of the ventricle, expeditious formation of the replacement scar would also be expected to impact a patient's subsequent clinical course.

In contrast to replacement fibrosis, reactive fibrosis is the term used for the diffuse deposition of collagen throughout the myocardium. It occurs in the absence of cell death and can be stimulated by prolonged periods of stress or by exposure to profibrotic mediators. Imposition of a pressure load on the heart, as occurs with aortic stenosis or systemic hypertension,[6,7] increases wall stress in the left ventricle and has been shown to promote reactive fibrosis in the chamber. Activation of neurohormonal systems, both intracardiac and systemic, which give rise to increased levels of mediators that stimulate cardiac fibroblasts to produce ECM proteins (eg, angiotensin II (Ang II), aldosterone, catecholamines), also produces reactive fibrosis in the heart.[8,9] Furthermore, diseases or conditions that trigger an

inflammatory response, either systemically or locally, can cause reactive fibrosis to develop. As will be discussed subsequently, these include obesity, diabetes, metabolic syndrome, infections of the heart, drugs, and radiation. The pattern of ECM deposition in reactive fibrosis, while more diffuse than with reparative fibrosis, can vary. Depending on the stimulus, reactive fibrosis can develop in a relatively homogeneous pattern throughout the myocardium (interstitial fibrosis), while in other situations it may be more prominent in the tissue surrounding intracardiac blood vessels (ie, perivascular fibrosis). In systemic hypertension, there is a gradient of fibrosis in the left ventricle from the endocardial to epicardial surface, reflecting the gradient in wall stress seen in the chamber as a result of the increased pressure load.

The distinction between reparative and reactive fibrosis is important as these processes have different triggers, mechanisms, and consequences. However, distinct separation between them in the complex environment of human heart disease is not straightforward. For instance, while it is widely recognized that reparative fibrosis is responsible for the generation of replacement scar in the setting of an MI, it probably also plays a role in the development of the diffuse intracardiac fibrosis caused by microischemia in patients with small vessel disease.[10,11] The 2 forms of fibrosis also likely coexist in many patients. An example of this is in the post-MI heart, where in addition to the reparative fibrosis that develops to generate replacement scar, there is diffuse reactive fibrosis in segments of myocardium that are distant from the infarct zone.[12]

EFFECTS OF FIBROSIS ON THE HEART

Whereas reparative fibrosis serves an important role in maintaining the integrity of the heart after an MI, reactive fibrosis is clearly a less beneficial process. In the normal heart, cardiomyocytes are integrated into contractile units by a well-defined fibrous network. This fibrous mesh helps transduce the function of individual cells into an effective organ pump. Excess amounts of ECM can adversely affect contractile performance of the heart by disrupting normal electrical conduction pathways. The resulting conduction abnormalities seen on electrocardiograms (eg, bundle branch block patterns) alter the well-ordered coordination of mechanical activity that is needed for optimal cardiac function.[13] Excessive fibrous tissue in the heart also affects the transduction of force from individual cardiomyocytes into forceful and well-coordinated pump function.[14] The deposition of fibrous tissue around small nutrient blood vessels in the heart (ie, perivascular fibrosis) can further impair cardiac function by causing local areas of microischemia to develop. Cardiac fibrosis has been shown to play a major role in determining the level of myocardial stiffness in patients affected by heart failure with preserved ejection fraction (HFpEF).[15,16] Zile et al found that in biopsy specimens of human left ventricular myocardial tissue taken from patients with HFpEF, there were increases in collagen volume fraction of ~5-fold compared to that in patients with hypertension alone, due mainly to increases in insoluble collagen. They further showed a significant positive correlation between the level of collagen-dependent tissue stress and echocardiography-derived measures of diastolic dysfunction (late atrial diameter and estimated pulmonary artery wedge pressure).[17] Excess amounts of collagen in the ECM can also impact diastolic function by impairing elastic recoil of the myocardium as the myocytes relax. Deposition of ECM throughout the heart can predispose

to arrhythmias, both through reentry and other mechanisms. While there is a recognized association between interstitial fibrosis, ventricular arrhythmias, and sudden cardiac death, [18-21] fibrosis in the wall of the atria has been described as playing a role in the development of atrial fibrillation.[22-24]

CAUSES OF FIBROSIS IN THE HUMAN HEART

The loss of cardiomyocytes stimulates the development of reparative fibrosis in the heart. Coronary artery disease that leads to the development of an MI is the prototypic initiator of reparative fibrosis but other conditions that result in cell death can cause this to occur. Disease in small intramyocardial coronary arteries that causes areas of microischemia can result in a more diffuse distribution of reparative fibrosis. Cardiac contusion resulting in myocardial necrosis can stimulate the development of a replacement scar in areas where cardiomyocytes have been lost. Infection of the heart by viruses and other pathogens and toxic effects of absorbed or ingested agents (eg, alcohol) can also lead to cardiomyocyte loss and development of replacement fibrosis.

Reactive fibrosis is seen in the heart as individuals age[25,26] and it plays an important role in causing stiffening of the heart and development of HFpEF. This process is accentuated by conditions that increase pressure load on the heart. Thus, patients with systemic hypertension or aortic stenosis are prone to develop stiff left ventricles due to the deposition of interstitial fibrosis.[6,7] Reactive fibrosis in noninfarcted zones of the heart is also a key component in post-MI cardiac remodeling, and it is stimulated by a host of factors including global increases in left ventricular wall stress as well as neurohormonal and other inflammatory influences.[27] Diffuse cardiac fibrosis has been found in patients with diabetes, obesity, and metabolic syndrome.[28-30]

A variety of drugs that act as serotonergic receptor agonists including anorectics, antimigraine drugs, anti-parkinson drugs, and recreational drugs have been associated with myocardial and cardiac valvular fibrosis.[31,32] Carcinoid tumors of the gut that secrete large amounts of serotonin into the systemic venous circulation[33] and ingestion of foods with high serotonin content have been associated with fibrosis in the right side of the heart. Cardiac fibrosis has been shown to be stimulated by smoking and can develop as a result of passive second hand inhalation of cigarette smoke.[34] It has also been reported in patients with heart disease due to genetic mutations as well as in athletes.[20,35]

Cardiac fibrosis can be seen in cancer patients treated with a variety of chemotherapeutic agents such as anthracyclines/anthraquinones, cyclophosphamide, trastuzumab, and other monoclonal antibody-based tyrosine kinase inhibitors and antimetabolites.[36] It can also develop as a consequence of radiation therapy when there is exposure of the heart.

ANIMAL MODELS OF CARDIAC FIBROSIS AND MECHANISMS INVOLVED

Most, if not all, of the mechanisms of cardiac fibrosis have been elucidated through animal models and in vitro cell culture systems. A summary of some of the experimental animal models that have been used to represent the human condition are shown in Table I. Albeit

not exhaustive, some critical mechanistic details that have been garnered from these models are outlined below.

Transforming growth factor-β.

The pleiotropic transforming growth factor-β (TGF-β) family, consisting of TGF-β1, −β2, and −β3, possesses diverse functions within the body, and its stimulating effects on fibrosis (especially TGF-β1) are well known.[60,61] In fact, TGF-β1 has been called the master regulator of fibrosis. TGF-βs are expressed by many cell types, but they are secreted as inactive, latent forms. These latent forms consist of the homodimeric TGF-β subunits, each noncovalently bound to its N-terminal prodomain (termed TGF-β latency-associated protein [LAP]), and with the latter linked to latent TGF-β binding protein (LTBP, existing as 4 isoforms) by disulfide bonds (reviewed in[62]). The N-terminus of LTBP contains an ECM binding domain that anchors the inactive complex to the ECM. The homodimeric TGF-β cannot bind its receptor until it is released from this inactive complex. There are multiple mechanisms capable of activating latent TGF-β, including protease release (eg, matrix metalloproteinase (MMP)-2 and MMP-9[63]), thrombospondin-1 binding,[64] reactive oxygen species,[65] pH extremes that denature LAP,[66] integrin binding,[67] and mechanical separation of LTBP and LAP on stiff matrices.[68]

Binding of TGF-β1 to its cell-surface, type II receptor (TβRII) causes recruitment and transphosphorylation of the type I receptor (ALK5 and/or ALK1 in some cell types[69]). In the canonical pathway, this leads to Smad phosphorylation and activation (eg., Smad2 and Smad3 are activated by ALK5, while Smad1, Smad5, and Smad8 are activated by ALK1). These activated Smads then bind to Smad4, and the complex translocates into the nucleus to modify transcription. Inhibitory Smads (Smad6 and Smad7) can be upregulated subsequently to feedback negatively on the signaling pathway. In the noncanonical pathways, TβRII can activate other signaling pathways, such as those involving mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase activation of AKT, and activation of RhoA leading to stabilization of F-actin. (See Derynck and Zhang[70] for a more thorough review of TGF-β signaling.)

TGF-β has pleiotropic effects throughout the body. Within the heart, it is known to stimulate cardiac fibroblasts to exhibit a profibrotic phenotype. These changes include myofibroblast conversion (see below) with resultant increases in collagen secretion,[71] decreases in collagen degradation,[72,73] and increases in synthesis of other profibrotic mediators (eg, see next section below). Regarding cardiomyocytes, TGF-β is known to induce a hypertrophic response.[74,75] The effects of TGF-β on endothelial cells are complex, inducing angiogenesis, angiostasis, or endothelial-to-mesenchymal transition (EndMT), depending on the conditions.[76,77] The TGF-β family has been implicated to some extent in all animal models of heart disease involving fibrosis. This includes MI,[78,79] pressure overload,[80] Ang II-induced cardiomyopathy,[81] and diabetic cardiomyopathy.[82] With respect to MI, although TGF-β is considered to negatively affect cardiomyocyte physiology and promote fibrosis, it has been shown to be cardioprotective in ischemia/reperfusion injury.[83,84] It is likely that such differences are due to low levels of TGF-β being necessary for proper tissue homeostasis, while high levels lead to cardiomyopathy and fibrosis, as has been postulated

previously by other investigators.[60] In addition, signaling differences between cell types can affect outcome,[85] and latent vs active TGF-β forms are frequently not distinguished, thus confounding interpretation. Interestingly, both p38α MAPK[86] and Smad3[87] signaling have been implicated in myofibroblast conversion in fibrotic animal hearts, suggesting the involvement of both noncanonical and canonical TGF-β signaling.

Examples of other mediators of cardiac fibrosis.

Angiotensin II.—Acting mainly via its type I receptor (AT1R), Ang II is known to induce profibrotic responses from cardiac fibroblasts, including increased ECM synthesis.[88-91] At least some, if not all of these effects are believed to occur indirectly via expression of TGF-β1[81,92,93] and/or transient receptor potential cation channel subfamily C member 6 (TRPC6), the latter of which activates calcineurin/nuclear factor of activated T-cells (NFAT) signaling.[94]

Endothelin-1.—Via endothelin receptor type A, endothelin-1 (ET-1) has been shown to increase the collagen production of cultured human cardiac fibroblasts.[95] ET-1 has been implicated in the cardiac fibrosis observed with aging,[96] streptozotocin-induced experimental diabetes,[97] and Ang II infusion.[98] ET-1 has also been shown to be mitogenic to cultured neonatal rat cardiac fibroblasts, a process that was dependent on the production of intracellular reactive oxygen species.[99]

Connective tissue growth factor.—Connective tissue growth factor (CTGF, also called CCN2) expression is associated with fibrosis in human heart failure patients[100] and experimental animal models.[101] CTGF expression can be induced by stimulation of cardiac fibroblasts and cardiomyocytes with TGF-β and its upregulation has been implicated in the profibrotic responses to TGF-β.[102] In 2015, Accornero et al published a rather thorough investigation of the effects of CTGF on cardiac fibrosis using multiple mouse models and concluded that CTGF was of minimal importance.[103] However, more recently, Ang II-induced cardiac fibrosis was shown to be dependent on the autocrine production of CTGF from fibroblasts, but not myocytes,[104] and intraperitoneal injection of an anti-CTGF monoclonal antibody was able to improve post-MI left ventricular remodeling, including remote-site interstitial fibrosis, in a mouse model.[105] The reasons for these discrepancies are unknown, but certainly deserve more study.

Catecholamines.—Chronic adrenergic stimulation of the heart can lead to myocyte hypertrophy and cardiac fibrosis. Mouse and human cardiac fibroblasts are known to express β2-adrenergic receptors,[106,107] which typically activate adenylate cyclase with the resulting cyclic AMP being inhibitory to profibrotic fibroblast activity.[108] However, chronic β2-adrenergic receptor stimulation leads to G protein-coupled receptor kinase 2 (GRK2)-β-arrestin-dependent uncoupling of β-adrenergic signaling, which enhances the profibrotic phenotype. This β-arrestin-dependent process also appears to be active in fibrotic, diseased hearts.[109,110]

Growth on stiff matrices.—Growth on stiff substrates (eg, tissue culture plastic) is well known to activate fibroblasts. In addition to the activation of AT1R[111] and TGF-β, the latter

necessitating the involvement of integrins,[68] Rho-dependent formation of F-actin stress fibers on stiff matrices dissociates monomeric G-actin-myocardin-related transcription factor-A (MRTF-A) complexes, allowing MRTF-A to remain in the nucleus. Nuclear MRTF-A can then act in concert with serum response factor and/or TGF-β-activated Smads to upregulate the transcription of profibrotic markers, such as α-smooth muscle actin (α-SMA) and collagen genes.[112-114]

Source of collagen-secreting cells.

The cell types that have been implicated as responsible for collagen secretion in the diseased heart are numerous, and include resident interstitial fibroblasts (and myofibroblasts), cells derived from EndMT (or epithelial-to-mesenchymal transition (EMT)), inflammatory cells (see below), glioma-associated oncogene (Gli)+ pericytes, and infiltrating fibrocytes (typically circulating CD34+, CD45+, Col I+ bone marrow progenitor cells, but see Pilling et al[115] for more selective markers). Fibrocytes were shown to be involved in fibrosis of ischemia/reperfusion cardiomyopathy in mice,[116] and ablation of pericytes ameliorated cardiac fibrosis induced by ascending aortic constriction in mice.[117] EndMT has been implicated in pressure overload-induced (transverse aortic constriction [TAC]) cardiac fibrosis in mice, as assessed by *Tie1Cre* and *FSP1-GFP* cell lineage tracking.[118] However, Tie1, which was previously thought to be endothelial-specific, was shown to be expressed also by subsets of hematopoietic cells,[119,120] and FSP1, which was believed to be fibroblast-specific, was subsequently shown to be expressed in hematopoietic, endothelial, and vascular smooth muscle cells.[121] Indeed, a thorough analysis of COL1A1-expressing cells after aortic banding in mice, avoiding conclusions based on Tie1 and FSP1, did not find a contribution of EndMT to fibrosis.[122] In addition, more recent reports in mice using in-depth lineage tracing have found that myofibroblasts in injured hearts and COL1A1-expressing fibroblasts in infarcted hearts are derived from transcription factor 21+ (Tcf21+) tissue-resident fibroblasts[123] and resident fibroblasts of epicardial origin,[124] respectively. Although all lineage tracing experiments have limitations, these latter 2 studies used models that are considered to be among the best currently available. This strongly suggests that most, if not all, collagen-producing cells in diseased hearts arise from resident fibroblasts. However, in the latter report,[124] fibrocytes were noted on the epicardial surface of the hearts near the ligation suture, indicating that other cell types could be involved in certain remodeling situations.

Inflammatory cell infiltration.

Inflammatory cells are known to be involved in cardiac fibrosis that is associated with MI. These cells can be resident tissue macrophages[125] as well as infiltrating inflammatory cells, such as neutrophils, monocytes, and macrophages.[126] This inflammation can be broadly divided into 2 phases: the initial proinflammatory phase involved in inflammatory cell recruitment and removal of dead tissue, and the reparative phase involved in tissue healing.[127] Disruption of either of these phases (by augmentation or inhibition) can be detrimental, leading to excessive fibrosis, chamber dilatation, or even infarct rupture. Inflammation has also been shown to be involved in cardiac fibrosis induced by other pathological states. For example, CXCR2-expressing monocytes, macrophages, and neutrophils were involved in the cardiac remodeling (including fibrosis) observed in Ang II-treated (1μg/kg/min)

hypertensive mice.[128] Using lineage tracking, Ivey et al recently showed that resident fibroblast proliferation and inflammatory cell increases both peaked at one week, regardless of fibrotic insult (ie, TAC, isoproterenol injection, or coronary artery ligation), suggesting a connection between them.[129] We have previously shown that inflammatory cytokines, such as tumor necrosis factor-α, can upregulate AT1R,[130,131] making cultured rat cardiac fibroblasts more responsive to Ang II stimulation,[89] so the potential for crosstalk between cell types exists. Overall, inflammatory cells are certainly involved in cardiac fibrosis that is associated with myocardial damage (ie, reparative fibrosis). Their influence on reactive fibrosis is still uncertain. However, immunoinflammatory dysfunction has been implicated in the increased reactive cardiac fibrosis and diastolic dysfunction associated with aging in mice.[132]

Activated fibroblasts and myofibroblasts.

Fibroblasts in the normal adult heart are considered quiescent, although as noted recently by Mouton et al, they are not truly quiescent, expressing genes necessary for ECM homeostasis.[133] Fibroblasts in diseased hearts become "activated" to various degrees, a term that has broad meaning. Experimental MI is one of the best understood models of cardiac fibrosis because it produces overt, well-defined collagen deposition. Using this model, both Fu et al[134] and Mouton et al[133] have drawn similar conclusions in that "activation" of cardiac fibroblasts involves proliferation/migration that precedes, and overlaps with, ECM production and maturation. However, the latter investigators noted that proinflammatory genes were upregulated at earlier time points (ie, <3 days), while the former group reported that infarct-resident cells at later time points (ie, 2—4 weeks) gained a unique phenotype, which the investigators termed "matrifibrocyte," to help maintain scar integrity. These "matrifibrocytes" had lost α-SMA expression, while retaining elevated COL1A1 and COL3A1 expression. How fibroblasts "activate" in other models of cardiac fibrosis, especially those involving reactive fibrosis, is less clear, but ultimately it is the accumulation of ECM proteins that is critical.

Overall, myofibroblasts are considered to be strongly activated fibroblasts and the major collagen producers in many fibrotic tissues. Indeed, myofibroblasts have been found in many animal models of cardiac fibrosis, including coronary artery ligation,[86,129,135] TAC,[87] and isoproterenol injection.[129] By definition, these cells have acquired the capacity to synthesize the contractile α-SMA. However, not all Col I-expressing cells are positive for α-SMA in fibrotic heart tissue, estimated at 89% 5 days after permanent coronary artery ligation,[129] 61% after 7 days of isoproterenol,[129] and 15% at both 7 and 28 days after TAC.[122] There could be many reasons for this lack of overlap. For example, fibroblasts can convert to protomyofibroblasts, which have actin stress fibers, enhanced collagen expression, and extra domain A (EDA)-fibronectin expression, but they do not express α-SMA.[136] Platelet-derived growth factor is one factor that has been shown to initiate the conversion of fibroblasts to protomyofibroblasts[137]; active TGF-β is then necessary to convert protomyofibroblasts to myofibroblasts.[68,136] In addition, as indicated with the "matrifibrocyte" experiments above, a myofibroblast could downregulate α-SMA expression while still maintaining elevated collagen secretion. Therefore, fibroblasts that have been activated to increase collagen synthesis do not necessarily need to be myofibroblasts, and a

mix of cell phenotypes could be responsible for collagen secretion in fibrotic regions of the heart, depending on the animal model or human disease state. A simplified summary of fibroblast activation, taking into consideration some of the mechanisms mentioned above, is shown in Fig 1.

MicroRNAs.

MicroRNAs (miRNAs) have also been implicated in affecting cardiac fibrosis. For example, miR-21 has been shown to promote TGF-β1-mediated fibroblast to myofibroblast transition in rat cardiac fibroblasts[138] and potentially to regulate fibrosis at the MI zone by its expression in cardiac macrophages.[139] miR-22 has been shown to be upregulated in the border zone of MI mice and to increase fibroblast functions via downregulating caveolin-3 expression in cultured neonatal rat cardiac fibroblasts.[140] miR-130a was shown to be upregulated in the hearts of Ang II-infused mice and its upregulation promoted profibrotic gene expression and myofibroblast transformation, possibly by targeting peroxisome proliferator-activated receptor-γ (PPARγ).[141] miR-155 was shown to be involved in cultured fibroblast activation and Ang II-induced cardiac fibrosis in mice; the investigators concluded that miR-155-dependent downregulation of suppressor of cytokine signaling 1, which augmented TGF-β signaling, was responsible.[142] Zhao et al demonstrated that paracrine transfer of miR-328 from cardiomyocytes to cardiac fibroblasts could activate the TGF-β pathway and increase fibrosis in mice.[143] In addition, miR-133a downregulation was associated with fibrosis after TAC in mice, possibly increasing serum response factor, CTGF, and COL1A1 expression.[43] It seems that any miRNA that can target critical factors during fibroblast activation has the potential to influence fibrotic outcomes.

Other considerations.

Fibroblast activation plays a major role in cardiac fibrosis, but other processes can certainly modulate outcomes. Lysyl oxidase (LOX)-mediated collagen crosslinking, a normal process to strengthen collagen fibrils and fibers, can lead to excessive stiffening of fibrotic tissue, impeding cardiac function.[144,145] Tissue transglutaminase, another secreted enzyme that can crosslink collagen albeit differently than LOX, has been associated with diastolic dysfunction in the hearts of mice subjected to TAC. These effects were reportedly due to both enzymatic and non-enzymatic functions of tissue transglutaminase.[146] Our group has previously shown that cardiac fibroblasts display a different expression pattern of endoplasmic reticulum-localized, single-stranded procollagen-modifying enzymes when they are ascorbate-starved vs when they are ascorbate-replete.[147] Given that ascorbate may be limiting in ischemic and/or oxidative environments, this could have implications for their functioning *in vivo,* although the complexities of this system make study and predictions difficult.

Animal model limitations.

Although much information has been garnered from experimental models of cardiac fibrosis, there are still limitations to extrapolating the conclusions derived from these models to human patients. In addition to questions of species variations as well as physiological discrepancies due to heart size differences, the models themselves can have limitations. For example, the frequently used TAC model of pressure overload may be more similar to aortic

coarctation in adults than the more common aortic stenosis or hypertension that it is presumed to represent. Where experimental models are most limiting is arguably in reflecting human reactive fibrosis. Diffuse interstitial fibrosis could develop over many years in humans, remaining subclinical throughout most of that time. Such passage of time is difficult to mimic in animal models due to time and budget constraints as well as species longevity. It is imperative in this situation to gain a better understanding of how fibrosis progresses in human patients and how those changes correlate to symptoms.

Various methods have been used to assess myocardial fibrosis in humans. The gold standard is direct evaluation of myocardial tissue by endomyocardial biopsy and histopathologic assessment.[148] This method is invasive, carries risks to patients, and has the additional drawback of potential sampling errors.[149] The use of noninvasive and/or minimally invasive techniques for monitoring cardiac fibrosis in humans would be greatly beneficial both as a research tool to further understanding and as a clinical tool to assess patients. Although the technologies are still arguably in their infancy, the last sections of this review will deal with the current state of these critical detection systems, namely serum cardiovascular fibrosis markers and imaging.

CARDIOVASCULAR FIBROSIS MARKERS IN HUMANS

Biomarkers of cardiovascular fibrosis.

The most recent American Heart Association/American College of Cardiology/Heart Failure Society of America guidelines for the management of heart failure give a class IIb recommendation for measurement of biomarkers of myocardial fibrosis for additive risk stratification in patients with either acute decompensated or chronic heart failure.[150] Specifically, the document notes that biomarkers of myocardial fibrosis, including soluble suppression of tumorigenicity 2 (ST2) receptor, galectin-3 (Gal-3), and highly sensitive cardiac troponin (hsTn) are predictive of outcomes in heart failure patients, and are additive to the established natriuretic peptides in their prognostic value. The adoption of markers of fibrosis into the guidelines reflects the findings of a large body of translational and clinical studies, while also acknowledging that large-scale, prospective studies validating their clinical utility are still lacking. Below is a concise overview of these 3 clinically relevant serum markers and some preclinical markers of cardiac fibrosis.

Galectin-3.

Gal-3 is a β-galactoside-binding lectin and a matricellular protein with important roles in cell adhesion, inflammation, and tissue fibrosis.[151] Gal-3 is expressed by fibroblasts and inflammatory cells, including activated macrophages, and is involved in myofibroblast activation via the TGF-β signaling pathway.[152-154] It is also linked to collagen production, macrophage infiltration, and cardiac hypertrophy.[153] Upregulation of Gal-3 has been demonstrated in animal models of myocardial,[155] vascular,[156] renal,[157] and hepatic[154] fibrosis.

Gal-3 expression is low in normal myocardium in humans, but is upregulated in various pathologic conditions.[158] In a rat model of hypertensive heart failure, Gal-3 expression was

increased at an early stage of hypertrophy, before clinical heart failure, specifically in the rats that eventually developed heart failure.[153] In addition, infusion of Gal-3 into the pericardial space of normal rats induced fibrosis and heart failure,[153] while inhibition of Gal-3 may protect against fibrosis, adverse cardiac remodeling, and the development of heart failure.[155,159]

Some analyses have suggested that circulating levels of Gal-3 may not correlate with human myocardial levels of Gal-3 and tissue fibrosis,[160] while other clinical studies have suggested a link. In patients with giant coronary aneurysms due to Kawasaki disease, both circulating Gal-3 levels and myocardial expression of Gal-3 (in densely fibrotic areas of the myocardium and arterial media) are elevated.[161] Regardless of its association with tissue fibrosis, higher circulating Gal-3 levels are associated with increased risk of death or readmission for heart failure in patients with either acute or chronic heart failure.[162] Higher Gal-3 levels are also associated with incident heart failure risk and mortality among individuals with acute coronary syndrome,[163] and among apparently healthy community-dwelling individuals.[164-166] The clinical uptake of Gal-3 testing is still low; however, Gal-3 remains an important research tool for evaluating links between myocardial dysfunction and fibrosis and inflammation.

Soluble ST2.

ST2 is a member of the interleukin (IL)-1 receptor-like family of proteins. It is expressed as both a transmembrane form (ST2L) and a soluble receptor (sST2). Expression of both isoforms of ST2 is upregulated in cardiomyocytes and fibroblasts in response to mechanical stress. The ligand for ST2 is IL-33, which is also upregulated in response to mechanical stress. The IL-33/ST2L interaction triggers a cascade via NF-κB that protects against inflammation, myocardial fibrosis, and cardiac hypertrophy. However, sST2 acts as a decoy receptor; when sST2, which lacks transmembrane and intracellular components, binds to IL-33, it does not initiate the beneficial signaling cascade.[167] Thus, high levels of sST2 are associated with increased tissue fibrosis and organ dysfunction.[168]

In animal models, blocking IL-33/ST2L interactions results in excess tissue fibrosis and myocardial hypertrophy after exposure to TAC-induced cardiac strain. In contrast, treatment with recombinant IL-33 reduced hypertrophy and fibrosis and improved survival after TAC in wild type but not ST2 null mice.[167]

In acute and chronic heart failure, and in individuals at risk for heart failure, increased sST2 levels are associated with a worse prognosis.[168] A recent meta-analysis with patient-level data showed that this prognostic ability is independent of natriuretic peptide and hsTn levels.[169] Unlike Gal-3, sST2 is gaining some traction in clinical use as several clinical cohorts have provided some degree of validation for its prognostic use, albeit primarily with retrospective analyses. Like Gal-3, the specific clinical response warranted by an elevated sST2 level remains to be elucidated and confirmed with prospective trials.

hsTn as an indirect indicator.

Although cardiac troponin is known primarily as a marker of myocyte injury and necrosis, myocyte necrosis ultimately leads to replacement fibrosis and several lines of evidence have

shown an association between cardiac troponin and myocardial fibrosis. Troponin is a regulatory protein within the contractile apparatus of striated muscles, which helps modulate calcium-mediated actin-myosin interactions. Two of the 3 subunits of the troponin complex, T and I, have cardiac isoforms that are distinct from the skeletal isoforms. Thus, measurement of either cTnT or cTnI is highly specific for cardiomyocyte injury.[170]

Cardiac troponin is released from cells in the setting of cardiomyocyte necrosis and cell membrane degradation, but low levels of release are also seen in the absence of cell necrosis. Although the precise mechanisms of these elevations in the absence of necrosis remain unclear, inflammatory factors leading to degradation and fragmentation of troponin combined with increased membrane permeability may be one explanation.[171]

With the evolution of hsTn assays that can now detect circulating cardiac troponin in the majority of healthy individuals, it has become clear that even within the “normal” range, higher hsTn is associated with incident heart failure and mortality.[162,172] In a large multiethnic cohort of individuals initially free of cardiovascular disease, baseline levels of hsTnT were associated with longitudinal changes in left ventricular structure that were consistent with adverse remodeling. In addition, hsTn levels were associated with replacement myocardial fibrosis, as imaged by late gadolinium enhancement (LGE) on cardiac magnetic resonance imaging.[173] The pattern of enhancement was not typical of an ischemic pattern. hsTn levels have also been associated with fibrosis in patients with severe aortic stenosis[174] and with hypertrophic cardiomyopathy.[175,176]

Clinically, the use of hsTn is now entrenched in medicine for diagnosis of MI as well as prognosis in acute coronary syndromes and in heart failure.[177] Its association with fibrosis in subclinical presentations of disease is a more recent discovery, and whether the identification of this association may lead to early targeted preventative therapies remains an area of interest.

Preclinical markers of collagen metabolism.

While not currently in clinical use, a large number of markers associated with collagen synthesis and degradation have been evaluated for an association with myocardial fibrosis.[178] HFpEF is characterized by increased interstitial deposition and crosslinking of Col I, with a small increase in the Col I/Col III ratio. Three classes of proteins reflecting the metabolism of collagen have received significant attention, in particular MMPs, tissue inhibitors of metalloproteinases (TIMPs), and procollagen terminal peptides/telopeptides (markers of collagen turnover). These markers have been associated with various cardiovascular disease risk factors,[179-181] left ventricular structural changes (TIMP-1),[182] left ventricular remodeling after MI (type I collagen C-terminal telopeptide [CITP]),[183] hypertension-induced HFpEF and heart failure with reduced ejection fraction (HFrEF) (MMP-1:TIMP-1 ratio),[184] and incident cardiovascular events and mortality (TIMP-1 and N-terminal peptide of procollagen type III).[185]

Despite these associations, it is unknown whether any of these markers will come to have significant clinical impact, but studies are beginning to evaluate this possibility. Recently, the CITP:MMP-1 ratio, a marker inversely associated with the degree of myocardial collagen

crosslinking, was studied as an effect modifier for response to spironolactone. Among 381 patients with stable NYHA class II or III HFpEF who were randomized to daily spironolactone or placebo, a low CITP: MMP-1 ratio (suggesting a high degree of myocardial collagen crosslinking) identified a population who were resistant to the beneficial effects of spironolactone on left ventricular diastolic dysfunction as assessed by E/e′.[186] In contrast, subjects with a high CITP:MMP-1 ratio showed improvement in diastolic function with spironolactone treatment. This study suggests that biomarker phenotyping of patients will assist with the search for therapeutic options in HFpEF, and that the degree of fibrosis is a critical factor to consider.

Other markers associated with regulation of collagen turnover, including TGF-β1,[60] growth differentiation factor-15, osteopontin,[187] and others,[178] have been associated with myocardial fibrosis in animal models and preliminary studies, but they lack clear and consistent clinical applications.

DETECTION OF MYOCARDIAL FIBROSIS IN HUMAN PATIENTS BY IMAGING

Echocardiography.

Echocardiography is perhaps the oldest noninvasive imaging technique for assessment of myocardial fibrosis. The altered acoustic impedance by fibrosis can be quantified by backscatter techniques[188] and has been validated by direct biopsy in patients with aortic stenosis and dilated cardiomyopathy.[189,190] This method is reliable and widely available, but limited by image quality, and hence is currently typically not used. More recently, strain imaging by speckle tracking has been used in a variety of diseases in which left ventricular functional abnormalities may be present. Strain provides a local measure of left ventricular function, which may be adversely affected by fibrosis. However, because strain imaging is only functional, it does not provide a direct assessment of the tissue. In patients with hypertrophic cardiomyopathy, it has been demonstrated that a reduction in strain is associated with an increase in fibrosis as determined by quantification of delayed gadolinium enhancement with magnetic resonance imaging (MRI), but this has not been directly validated against biopsy.[191]

Positron emission tomography.

Utilizing positron emission tomography with ^{15}O-labeled water and carbon monoxide (C^{15}O) allows quantification of perfusion and can indirectly assess fibrosis through a perfusable tissue index. This index assesses the amount of myocardium perfused by water, with fibrotic myocardium exchanging water less rapidly. This method has been compared to patients with known dilated cardiomyopathy and presumed fibrosis, but has not been validated by direct biopsy. A reduction in perfusable tissue index is considered representative of a higher degree of fibrosis.[192,193]

Cardiac MRI.

For defining cardiac anatomy, cardiac MRI (also abbreviated as CMR) provides high levels of accuracy and has been shown to have significant utility in noninvasive characterization of

myocardial tissue and etiologies for heart failure.[194] LGE is one of the most widely utilized modalities for assessment of focal myocardial scar and fibrosis. LGE imaging is able to depict differences in signal intensity in T1-weighted images obtained approximately 10 minutes after the administration of a gadolinium-based contrast agent. In fibrotic tissue, T1 recovery times are shortened due to higher concentrations of gadolinium in the extracellular space while in normal myocardium gadolinium washes out more quickly. As a result of this difference, an inversion time can be chosen so that normal myocardium appears dark and fibrotic myocardial tissue appears bright on inversion recovery images.[195] This technique has been well validated and correlated to the severity and extent of myocardial fibrosis.[196]

There is a great deal of clinical experience with LGE and it is widely available on clinical cardiac MRI systems. However, this technique has several important limitations. LGE only provides a relative signal that measures fibrosis compared to other regions of myocardium. Hence, it is most useful when there are regions of myocardium that are known or expected to be normal and importantly, it is less useful in detecting myocardial fibrosis that is diffuse (ie, reactive fibrosis). Moreover, there is a lack of correlation with collagen volume assessed by biopsy samples in diffuse fibrosis.[194] LGE is also not specific for fibrosis as increased signal can be seen in regions with edematous or inflamed tissue.[197] Finally, LGE is largely a qualitative process with evaluation performed by visual assessment of images obtained (although tools do exist for quantitative characterization of LGE). This results in inter- and intraobserver variability and challenges in comparing results to follow-up examinations or between individual patients.[198]

Quantitative evaluation of myocardial fibrosis can be assessed by MRI with T1 mapping techniques. Various sequences have been developed to directly measure the longitudinal relaxation time, which measures how quickly proton spins within myocardial tissue re-equilibrate due to interactions with the surrounding tissue after being excited with a radiofrequency pulse. Areas of fibrosis cause T1 shortening compared to that of normal myocardium and the results can be displayed using parametric pixel illustration maps of relaxation times (Fig 2).[199,200] This method can acquire a set of interpretable images with a single breath hold acquisition; it is reproducible and with reportedly high levels of intraobserver agreement.[201] T1 mapping has been validated by comparisons with biopsies in patients with cardiomyopathy, valvular disease (aortic stenosis, aortic regurgitation, and mitral regurgitation), and myocarditis. Postcontrast enhanced T1 mapping in combination with native (noncontrast) T1 mapping and the hematocrit level allows calculation of the extracellular volume (ECV) fraction. As elevated quantities of ECV are associated with increased fibrosis and collagen deposition, this is helpful in further quantifying the degree of fibrosis.[202] Measurements of ECV have been validated with histologic analyses of biopsy specimens for several different diseases.[203]

T1 mapping in specific disease states.

T1 mapping has been shown to have prognostic utility in a number of diseases. In patients with dilated cardiomyopathy, native T1 values and ECV have been shown to be predictive of composite all-cause mortality and heart failure events.[205] Elevated native T1 mapping values have also been shown to be an independent predictor of adverse outcomes in patients with

moderate or severe aortic stenosis.[206] Surveillance with T1 mapping in adult cancer patients 3 years after treatment with anthracycline-based chemotherapies showed elevated proportions of ECV when adjusted for other known risk factors. The longterm implications of elevations in ECV on left ventricular function in this patient population are not yet known.[207] The relationship between cardiovascular risk and fibrosis in men and women has also been investigated using T1 mapping. There is evidence that a greater degree of fibrosis is associated with higher cardiovascular disease risk scores in men. However, this finding was not seen in women, and the exact role that T1 mapping may play in risk assessment is still unclear.[208]

CONCLUSIONS AND PERSPECTIVES

We have reviewed the current understanding of cardiac fibrosis in human disease, experimental models of cardiac fibrosis, and the mechanisms involved, as well as the current techniques available to assess cardiac fibrosis in patients. Many gaps in knowledge still remain, with the greatest deficiencies existing in the jump from animal models to human patients and the development of techniques to adequately assess fibrotic regions without biopsy. Myocardial fibrosis is almost universally seen in all forms of heart disease and the degree of cardiac dysfunction is generally proportional to the degree of ECM deposition.[209] However, in later stages of heart disease and/or when collagen deposition becomes symptomatic, the resulting fibrosis may no longer be reversible.[210,211] With continued understanding, the hope one day is to be able to intervene and halt or slow aberrant ECM deposition before symptoms appear and this point of no return is reached.

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Abbreviations:

Ang II	angiotensin II
AT1R	angiotensin II type 1 receptor
CITP	type I collagen C-terminal telopeptide
CMR	cardiac MRI
Col I	collagen I
Col III	collagen III
COL1A1	collagen type I alpha 1 chain
COL3A1	collagen type III alpha 1 chain
CTGF	connective tissue growth factor

ECM	extracellular matrix
ECV	extracellular volume
EDA	extra domain A
EMT	epithelial-to-mesenchymal transition
EndMT	endothelial-to-mesenchymal transition
ET-1	endothelin-1
Gal-3	galectin-3
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
hsTn	highly sensitive cardiac troponin
IL	interleukin
LAP	TGF-β latency associated protein
LGE	late gadolinium enhancement
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
miRNA	microRNA
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MRTF-A	myocardin-related transcription factor-A
ST2	suppression of tumorigenicity 2
ST2L	transmembrane ST2
sST2	soluble ST2
TAC	transverse aortic constriction
Tcf21	transcription factor 21
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
α-SMA	α-smooth muscle actin

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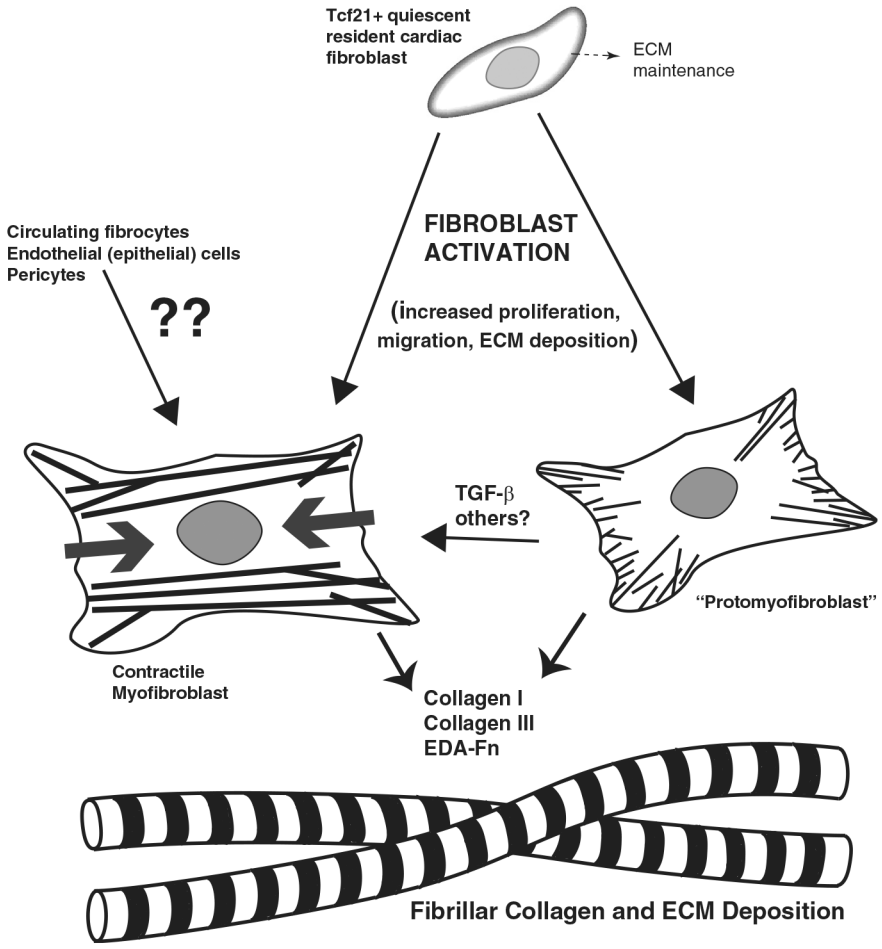

Fig 1.

Simplified pictorial representation of cardiac fibroblast activation. Actin stress fibers are depicted in the protomyofibroblast as thin lines and contractile α-smooth muscle actin-containing fibers are depicted in the myofibroblast as thick lines (with thick gray arrows denoting contractile tension). Fibroblast activation in the heart involves a complex mix of pathways, but the most important result is the increased deposition of ECM. EDA-Fn, extra domain A fibronectin; ??, questionable contribution, limited contribution, or contribution only in certain contexts.

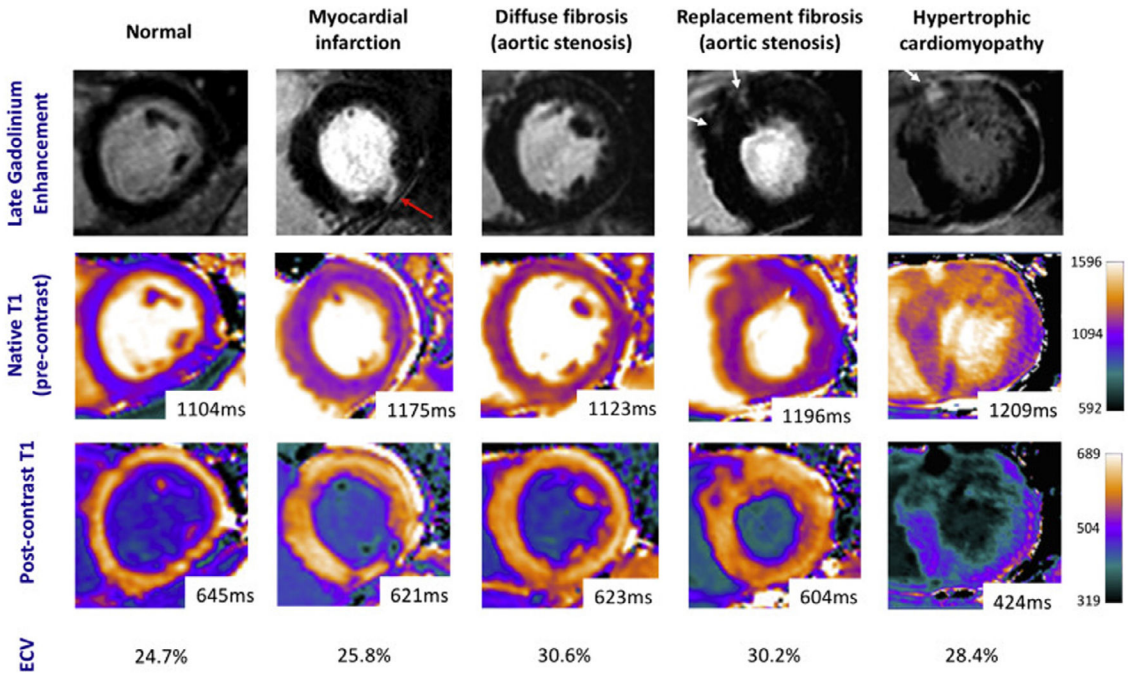

Fig 2.

Example cardiac magnetic resonance images in common cardiac conditions showing late gadolinium enhancement, native (precontrast) and postcontrast T1 mapping, and calculated extracellular volume (ECV) fractions. Red arrows identify areas of subendocardial delayed enhancement. White arrows identify midwall delayed enhancement. Reproduced, with permission, from Everett et al.[204] (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table I.

Animal models of cardiac fibrosis

Human condition	Animal model	Cardiac fibrosis characteristics	Reference
Ischemia	Permanent occlusion of coronary artery *	• Replacement scar at infarct site • Variable fibrosis at peri-infarct region and at remote sites • Typical time for scar maturation is 1–2 mo • Degree of severity depends on positioning of ligating suture	37,38
	Temporary occlusion of coronary artery (ischemia/reperfusion) *	• Similar outcome as with permanent occlusion, but severity depends on timing of reperfusion	39,40
Hypertension or pressure overload	*Macaca fascicularis* model of progressive hypertension up to 88 wk	• Minor changes in cardiac collagen content, but obvious structural alterations in collagen fibril sizes and arrangement • Eventual appearance of necrotic myocytes and replacement fibrosis	41
	Transverse aortic constriction (TAC) in mice and rats *	• Increased interstitial and periarteriolar collagen deposition starting early (3 d) and persisting through decompensation • Not studied as thoroughly in rats	42-45
	Spontaneous hypertensive rats (SHR) given 8% salt for 5 wk	• Increased interstitial fibrosis mediated by renin-angiotensin system	46
	L-NAME † administration in drinking water	• Increased interstitial fibrosis up to 7 wk in mice and rats	47,48
Diabetes	Western diet in Sprague Dawley rats for 18 wk	• ~50% increase in interstitial fibrosis by Sirius Red/Fast Green staining	49
	Type I diabetes induced by streptozotocin injection *	• Significantly enhanced fibrotic foci associated with apoptotic myocytes up to 6 mo • Possible involvement of microvascular and metabolic dysfunction	50,51
Drug/chemical	Chronic ethanol consumption (4%; up to 14 wk) in mice	• ~4-fold increase in LV collagen content (picrosirius red staining) after 14 wk • Myofibroblast increases observed	52
	Doxorubicin-induced cardiac fibrosis in rats	• ~2-fold increase in LV collagen volume, at least partially due to activity of substance P and direct effects on cardiac fibroblasts	53
	Butyrylcholinesterase knockout mice receiving intraperitoneal cocaine (20 mg/kg) daily for 7 d	• Perivascular fibrosis in heterozygotic and null mice	54
Other	5-oxoprolinase (OPLAH) null mice	• ~4 × increase in LV fibrosis at 14–20 wk of age believed to be due to oxidative stress	55

Human condition	Animal model	Cardiac fibrosis characteristics	Reference
	Rats with 2-mo aldosterone treatment and 1% NaCl/0.3% KCl drinking water	• Biventricular fibrosis (perivascular and interstitial) seemingly independent of hypertension	56
	Mouse obesity/diabetes model (homozygous leptin receptor inactivation; *db/db* mice) at 6–12 mo of age[‡]	• Increased perimysial/endomysial/periadventitial fibrosis associated with increased collagen synthesis but not myofibroblast conversion • Sex differences were noted	57
	Cryoinjury of myocardium in adult and neonatal mice	• Replacement fibrosis at site of injury	58
	Angiotensin II infusion in rats and mice	• Increased perivascular and interstitial fibrosis in RV and LV • In shorter time frames (eg, 14 d) appears to be independent of hypertension	59

[*] This model has been used extensively in the literature.

[†] L-NAME, L-N[G]-nitroarginine methyl ester, a broad inhibitor of nitric oxide synthases; LV, left ventricle; RV, right ventricle.

[‡] This also could be considered a diabetes model.

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