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Mesenchymal Stem Cell-derived Inflammatory Fibroblasts Mediate Interstitial Fibrosis in the Aging Heart

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Abstract

Pathologic fibrosis in the *aging* mouse heart is associated with dysregulated resident mesenchymal stem cells (MSC) arising from reduced stemness and aberrant differentiation into dysfunctional inflammatory fibroblasts. Fibroblasts derived from *aging* MSC secrete higher levels of 1) collagen type 1 (Col1) that directly contributes to fibrosis, 2) monocyte chemoattractant protein-1 (MCP-1) that attracts leukocytes from the blood and 3) interleukin-6 (IL-6) that facilitates transition of monocytes into myeloid fibroblasts. The transcriptional activation of these proteins is controlled via the farnesyltransferase (FTase)-Ras-Erk pathway. The intrinsic change in the MSC phenotype acquired by advanced age is specific for the heart since MSC originating from bone wall (BW-MSC) or fibroblasts derived from them were free of these defects. The potential therapeutic interventions other than clinically approved strategies based on findings presented in this review are discussed as well.

Keywords

MSC; inflammatory fibroblast; myeloid fibroblasts; RasGrf1; aging; fibrocyte

1. Introduction

In elderly patients [1] and *aging* mice [2] progressive deposition of extracellular matrix proteins (ECM) in the heart causes a diffuse interstitial fibrosis that leads to elevated passive stiffness. Increased passive stiffness produces incomplete relaxation during early diastolic filling that induces exercise intolerance and predisposes to development of heart failure. Heart failure with preserved ejection fraction but impaired diastolic function is prevalent in

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older individuals and markedly increases the risk of mortality [3]. Available treatments that have been developed specifically for systolic heart failure have failed to demonstrate efficacy in patients with preserved ejection fraction and diastolic dysfunction [4–6]. Increased fibrosis has been also associated with both atrial [7] and ventricular [8] arrhythmias and experimental treatments targeting fibrosis have been shown to be beneficial in lowering arrhythmia inducibility [9].

Cardiac fibroblasts, via control of ECM protein synthesis and its degradation, maintain the myocardial structure [10]. In the heart, ECM consists predominantly of collagen type I (Col1), and (to a much smaller degree), collagen type III [11], fibronectin [12, 13], laminin [13], and elastic fibers [14]. EMC synthesis is tightly regulated and any disturbance may have serious consequences; in the normal healthy heart collagen content is low [15] but its synthesis is upregulated in response to various stimuli such as mechanical stretch [16], ischemia [17], pressure overload [13] or aging [15]. It has been also demonstrated that paracrine factors such as angiotensin II [17, 18], endothelin 1 [19], transforming growth factor beta (TGF- β) [20] and platelet derived growth factor (PDGF) [21, 22] increase expression of collagens.

Fibroblasts can respond to stimuli via matrix remodeling by increasing expression of ECM proteins or the expression of factors that modulate matrix such as metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) [23]. They may also proliferate, migrate, mature into contractile myofibroblasts and express various cytokines and chemokines when activated (as reviewed by Porter [24]).

In the aging heart resident mesenchymal stem cells (MSC) are dysregulated and differentiate into dysfunctional fibroblasts that chronically secrete collagens [25] and cytokines and favor ongoing inflammation [26]. We have recently proposed a mechanism by which these inflammatory mesenchymal fibroblasts may attract leukocytes from blood and facilitate their transition into myeloid fibroblasts [26, 27]. This article will review the abnormalities associated with immuno-dysregulation in the aging heart – in particular, the source of defects in MSC and mesenchymal fibroblasts that contribute to adverse remodeling.

The definition of “aged” varies with human populations and mouse strains. However, “older persons” have been defined as 65 years of age according to World Health Organization. In C57BL/6 mice, gene expression studies were based on 16 month-old mice as “young” and 24 month-old mice as “aging” [28], although we have seen fibrosis beginning at 14 months of age in this strain [2]. In examples of other studies, young mice were used between the ages of 2 to 6 months, and aging mice between 23 and 27 months of age [29, 30]. It is clear that aging is a continuum, and so the choice historically has been to compare young fully adult mice (4–6 months of age) with aging mice that are not yet debilitated.

2. Cellular and molecular mechanisms contributing to fibrosis in the *aging* heart

2.1. Dysfunctional MSC in the aging heart

We have found that pathologic interstitial fibrosis in the aging mouse heart is associated with the aberrant function of cardiac resident MSC [25, 26, 31]. These cardiac resident MSC are characterized by reduced expression of Nanog, Oct4 and Sox2, which results in the escape from a primitive state and drives their differentiation into activated Col1 expressing mesenchymal fibroblasts [25]. The age-dependent reduction of the undifferentiated state in cardiac MSC at least in part arises from their diminished expression of TGF- β receptor I (T β RI) [27]. Pharmacological inhibition of T β RI in MSC derived from young hearts results in a phenotype that resembles *aging* MSC, with diminished Nanog expression [27]. This decline of TGF- β responsiveness has been recognized by others as a factor that contributes to the loss of stemness [32]. The altered phenotype does not affect the ability of *aging* MSC to differentiate into chondrocytes or osteoblasts, but surprisingly, we found that MSC derived from *aging* hearts favor the adipocytic lineage. In fact, they are at a preadipocytic state as defined by highly upregulated expression of an early preadipocytic marker, delta-like 1 homolog (Dlk-1), even when cultured in medium supporting only non-committed cells [31]. Furthermore, treatment of MSC derived from the *aging* heart with a relatively low concentration of insulin in an *in vitro* adipogenic differentiation assay resulted in a higher number of fully committed mature adipocytes in comparison to MSC derived from young hearts [31]. The reason for the observed skewing towards the adipocytic lineage may be potentially explained by a reduced TGF- β responsiveness, as demonstrated by Choy et al. TGF- β activated Smad3 proteins repress C/EBP dependent transcriptional activation of genes critical for adipogenesis [33]. Because of the defect in the TGF- β signaling pathway, C/EBP is not a limiting factor in transcriptional activation of genes involved in lipid synthesis. Interestingly, chronic exposure to pathophysiological levels of insulin caused a reduction of Nanog expression in MSC derived from young hearts in *in vitro* experiments [25] suggesting that elevated circulating levels of insulin (as often seen in old [34] or obese patients [35] or *aging* [36] or obese rodents [37]) may promote the reduction of stemness and encourage premature MSC differentiation that triggers the depletion of the existing stem cell pool as suggested by others [38].

The unusual adipocytic lineage choice of *aging* cardiac MSC maybe analogous to studies in the mouse skeletal muscle, in which Lin^{neg}Sca1⁺CD34⁺PDGFRa⁺ cells have been identified as bipotential fibro/adipocyte progenitors [39]. Their differentiation is dictated by the environment and often after injury, especially in *aging* animals, myoblasts are replaced by a mix of fibroblasts and adipocytes [40, 41]. Because, as shown by others [42], cardiac mesenchymal fibroblasts are derived from Sca1⁺PDGFRa⁺ progenitors, that may suggest that a similar dysregulatory mechanism in progenitor cells leads to the above mentioned abnormalities in both the aging heart and skeletal muscle. In the embryonic mouse heart PDGFRa⁺ progenitors are initially located in proepicardium and epicardium and some of these cells persist into adulthood [42]. A recent study by Yamaguchi and colleagues indicates that mesenchymal transformation can give rise to adipose tissue in the mouse heart

[43]. In the aging human heart the increased number of adipocytes that are localized in the epicardium may suggest that they originate from PDGFRa⁺ progenitors [44, 45].

We recently also have demonstrated that cardiac MSC derived from the *aging* mouse heart acquire a proinflammatory phenotype and express higher levels of monocyte chemoattractant protein-1 (MCP-1) [27] and interleukin-6 (IL-6) [26]. Again, it has been demonstrated that the TGF- β pathway regulates MCP-1 expression levels; TGF- β -activated Smad3 proteins sequester the AP-1 complex (that is necessary for MCP-1 transcriptional control) [46] and prevent AP-1 binding to its cognate site on the MCP-1 promoter. Therefore an increased TGF- β expression usually correlates with MCP-1 downregulation when the TGF- β pathway is operational. Although IL-6 seems not to be negatively controlled by TGF- β , the transcriptional regulation of both cytokines involves the Ras pathway that is discussed below.

2.2. Inflammatory mesenchymal fibroblasts

The defects that were found in MSC are maintained in their progeny cells, mesenchymal fibroblasts [27]. We have discovered that mesenchymal fibroblasts originated from *aging* hearts change their phenotype into an inflammatory state and express higher levels of several cytokines, MCP-1 and IL-6 among them [26]. We have demonstrated that the reason for elevated expression of MCP-1 and IL-6 transcription correlates with an upregulated activity of the farnesyltransferase (FTase)-Ras-Erk pathway. The importance of FTase in this pathway relates to a role in Ras posttranslational modification (such as prenylation) that is essential for proper localization into a plasma membrane and its subsequent activation. Therefore agents that interfere with the biosynthesis of the farnesyl chain (such as statins) or activity of FTase reduce the activity of Ras. We have found upregulated transcription and activation of FTase in fibroblasts derived from *aging* MSC [25]. Ras activity is further controlled by two groups of proteins that facilitate the exchange of GDT into GTP (Ras activation) and the reverse (Ras inactivation) that keeps Ras signaling in balance [47]. We discovered that one of the Ras activators, Ras protein-specific guanine nucleotide releasing factor-1 (RasGrf1), was upregulated in *aging* MSC and mesenchymal fibroblasts derived from them. Silencing RasGrf1 resulted in reduced IL-6 expression. The Ras pathway that leads to IL-6 was also sensitive to Erk and FTase suppression [26] (Fig.1). While the elevated expression of cytokines in mesenchymal fibroblasts derived from old hearts were assessed in in vitro experiments, an elevated number of IL-6⁺DDR2⁺ cells (DDR2 is discoidin domain receptor 2, a collagen receptor) was documented in the aging heart tissue as well [23]. Although there is no true cardiac fibroblast-specific marker, the use of DDR2 is our best approximation of these CD45^{neg} (non-hematopoietic) cells as mostly fibroblasts. The coincidence of their IL-6 production with that of fibroblasts grown in vitro provides evidence that fibroblasts are likely to be among the resident mesenchymal cells that produce IL-6 in vivo [26].

The presence of inflammatory fibroblasts seems not to be restricted only to models of cardiac diseases. Arthritis [48], pulmonary hypertension [49], idiopathic pulmonary fibrosis [50], kidney fibrosis [51] and cancer [52] have been associated with fibroblasts expressing elevated levels of several cytokines, suggesting that the pro-inflammatory phenotype in

fibroblasts may be an important pathophysiologic factor in other connective tissue conditions.

2.3. Myeloid fibroblasts

In our studies of the role of inflammation in interstitial fibrosis, we have previously demonstrated fibrotic mechanisms dependent upon the development of myeloid fibroblasts arising from monocytes in response to dysregulated chemokine signaling [53, 54]. Cardiac fibrosis could be induced in young animals by daily administration of angiotensin II or by daily coronary occlusion for short non-infarctive periods (ischemia/reperfusion cardiomyopathy model, I/RC). These two interventions resulted in the induction of MCP-1, which remained elevated for several weeks before being suppressed by TGF- β . Over that period, monocytes infiltrating the myocardium were initially found to be M1 (pro-inflammatory) but, after a few days, had the phenotype of M2 macrophages (anti-inflammatory, pro-fibrotic) [55]. These M2 macrophages further assume a spindle-shaped appearance, express Col1 and effectively become fibroblasts of myeloid origin (CD45⁺Col1⁺). Genetic deletion of MCP-1 or its receptor (CCR2) demonstrated marked reduction of monocyte uptake and abrogation of interstitial fibrosis [54, 56] stressing the importance of this chemokine in the development of fibrosis.

By employing *in vitro* studies using a transendothelial migration (TEM) assay, which models leukocyte migration through an endothelial barrier and monocyte polarization into various macrophage subtypes, we have learned that macrophages of the M1 phenotype migrate early and then disappear [57]. Another macrophage subtype, M2, migrates later and further polarizes into Col1 expressing M2a macrophages (that are effectively myeloid fibroblasts) (Fig.2). Similar kinetics *in vivo* were observed in an angiotensin infusion study using young animals [55]. However, in the aging heart a continuous presence of M1 and M2a macrophages (Fig. 3) was detected. An increased number of M1 polarized macrophages may be explained by the elevated expression of MCP-1 and continuous leukocyte infiltration seen in the *aging* heart [2]. An increased quantity of M2 on the other hand may be attributed to augmented IL-6 secretion by the mesenchymal fibroblasts. Findings from our laboratory and others revealed that IL-6 facilitates the process of monocyte to myeloid fibroblast transition [26, 58]. The results from other laboratories also suggest that not only monocyte-derived macrophages but also resident macrophages (that are thought to be of embryonic origin [59]) may play a role in fibrosis. As shown by Pinto *et al.* in the resting aging heart there is a population of resident M2 macrophages that have a self-renewing capacity [60], and which we hypothesize may polarize into myeloid fibroblasts via an IL-6 dependent mechanism (Fig. 2).

It is important to note that the cells identified in this review as myeloid fibroblasts are often referred to in the literature as bone-marrow derived fibroblast precursors or fibrocytes. In our studies of the role of inflammation in interstitial fibrosis, we have previously demonstrated fibrotic mechanisms dependent upon the development of fibroblasts from tissue-infiltrating monocytes in response to dysregulated chemokine signaling. We called these cells myeloid fibroblasts, indicating their bone marrow monocyte origin and final maturation status, although they are likely identical to cells called fibrocytes that are of

monocytic origin and have been observed in many organs associated with a fibrotic response [61].

2.4. Role of mesenchymal and myeloid fibroblasts in fibrosis

Interstitial fibrosis in aging is associated with upregulation of two fibroblast populations emanating from different developmental sources, resident mesenchymal fibroblasts and myeloid fibroblasts. Our original study in young mice demonstrated that dysregulation of chemokine expression induced progressive but short-lived interstitial fibrosis requiring the presence of MCP-1 and being limited by suppression of MCP-1 in response to TGF- β [62]. The MSC in aging, however, become resistant to TGF- β stimulation, which accelerates their differentiation (reduces their stemness) and promotes the generation of inflammatory mesenchymal fibroblasts. These inflammatory fibroblasts (CD45^{neg}Col1⁺) demonstrate increased collagen expression, which partially explains the resultant fibrosis. In addition, the secretion of MCP-1 and IL-6, as discussed above, promotes the uptake of monocytes and their differentiation into myeloid fibroblasts (CD45⁺Col1⁺). In models of cardiac injury in young animals, studies using lineage tracing and other approaches have demonstrated that resident fibroblasts and their precursors are the major contributors to fibrosis [63–65]. With respect to the aging heart we have quantified CD44⁺CD45^{neg} resident fibroblasts that are positive for cytoplasmic type I collagen and reported that more of these cells expressed collagen than in the young controls (93% versus 26%) [25]. It is thus likely that, during aging, the same populations of resident fibroblasts and their precursors mediate fibrosis as are seen in post-cardiac injury fibrosis in young animals.

2.5. The phenotype of bone wall MSC and fibroblasts derived from them in young and aging mice

It is not clear in what circumstances bone marrow derived MSC can contribute to the fibroblast pool in the heart after injury. Reports demonstrating opposite results have been presented [66, 67] and one reason for the discrepancy may be dependent on the type of experimental design (permanent occlusion vs. reperfusion). Since the environment of the *aging* heart in regards to fibroblast activation and leukocyte infiltration resembles (to a lesser extent) the setting of the injured young heart, we decided to analyze bone wall MSC (BW-MSC) derived from young and *aging* mice. Bone wall, rather than bone marrow, has been shown to be a major source of MSC in the adult mouse, so therefore we isolated these cells according to a protocol developed by Zhu and colleagues [68]. Surprisingly, BW-MSC isolated from *aging* animals (24–30 month-old) did not display the same defects as observed in cardiac resident MSC; their expression of Nanog was comparable to the levels expressed by cells isolated from young animals and fibroblasts derived from these BW-MSC did not display differences in T β RI, RasGrf1, MCP-1 and IL-6 levels between these two age groups (unpublished observation, n= 6, 4 for cells derived from 3 and 24–30 month-old mice respectively) as opposed to fibroblasts derived from cardiac MSC [26, 27]. These results imply that the alteration of the environment in the aging heart (or circulating factors) may be responsible for the change of cardiac MSC phenotype, since BW-MSC are unaffected by age.

3. Potential therapeutic strategies targeting inflammatory mediators released by fibroblasts

Both clinical and experimental studies of aging have suggested potential roles for inflammation in the cardiac disease of aging associated with heart failure and cardiac fibrosis. Our studies have demonstrated increases in the renin angiotensin axis and we reported an increase in the endogenous renin angiotensin system in aging animals [2]. Angiotensin is known to generate a pro-inflammatory environment in young animals. Part of the angiotensin-induced inflammation may be due to reactive oxygen species production. However, the effects of reactive oxygen based strategies has been widely studied in aging without any definitive results; to be more specific, strategies aimed at the origins of increased reactive oxygen in the heart may be necessary.

Our previous and current work led us to delineate the mechanistic link between the upregulated Ras-FTase-Erk pathway and fibrosis. Below we list potential therapeutic strategies that may provide benefits for the aging heart based on these findings (see also Fig. 4).

3.1. Insulin

A majority of *aging* mice on standard laboratory chow and the majority of old patients are obese [69, 70]. In aging this is associated with increased circulating insulin levels [25, 35] that appear to participate in the downstream signal dysregulation. Thus far, our studies have shown that pathophysiologic concentrations of insulin result in the reduction of Nanog expression in MSC and stimulation of collagen synthesis and FTase activity in fibroblasts. In obese or *aging* mice, we have also previously demonstrated increased myocardial fibrosis and defective cardiac scar formation [71, 72]. Therefore a pharmacological approach to reduce the insulin resistance and normalize the circulating insulin levels may moderate the progression of interstitial fibrosis. The potential usefulness of this direction is also bolstered by the large amount of literature demonstrating that calorie restriction is associated with improved healthspan in patients [73].

3.2. FTase

Aberrant activation of the FTase-Erk pathway as seen in fibroblasts derived from *aging* hearts mediates augmented expression of Col1, IL-6 and MCP-1 [25, 26]. Treatment with statins, inhibitors of cholesterol biosynthesis that also interfere with synthesis of farnesyl chains (therefore inhibit signaling transduction mediated via farnesylation) have been shown to reduce inflammatory mediators *in vitro*. This may explain some of the pleiotropic effects of statins; however, a direct targeting of FTase may be a more specific therapeutic strategy.

3.3. RasGrf1 and Ras pathway

Our data strongly suggest that RasGrf1 is an attractive target for intervention; RasGrf1 is a signaling molecule located downstream from the insulin receptors and its activity has been shown to be upregulated by insulin [74]. This suggests that insulin may promote the exaggerated activation of the Ras pathway in the aging heart. In other laboratories, genetic deletion of RasGrf1 is associated with reduced circulating insulin levels and also appears to

reduce beta cell proliferation in the pancreas but does not induce diabetes [75]. Other work has suggested that RasGrf1 expression in stem cells leads to their loss of stemness [76] which has potential implications in poor myocardial infarction repair in aging, as we previously reported [72]. Interestingly, as we have correlated an elevated expression of RasGrf1 with cardiac fibrosis, the downregulation of another enzyme that is associated with the Ras pathway, Rasal1, (that catalyzes hydrolysis of RasGTP to RasGDP and inactivates Ras), has been associated with kidney fibrosis [77], suggesting that an aberrant activation of the Ras pathway may be a common pro-fibrotic mechanism.

3.4. Epigenetic modifications

The expression of RasGrf1 and Rasal1 is regulated by DNA methylation [77, 78]. The incidence of altered DNA methylation and histone post-translational modifications increases with age [79, 80]. The level of DNA methylation and histone acetylation are linked, as methylated DNA binding proteins are capable of recruiting histone deacetylase (HDAC) and repressing transcriptional activity [81]. Recently, HDAC inhibitors have been shown to block cardiac fibroblast activation, maturation of myeloid cells into M2a macrophages [82] and IL-6 production [83], suggesting that chromatin modification may be a common factor contributing to age and inflammatory related diseases.

3.5. Inflammatory cytokines

Our initial data demonstrated that TGF β -resistant fibroblasts were the source of elevated MCP-1 that persisted in the hearts of the aging mice [27]. Since MCP-1 appeared to be necessary for the generation of the monocyte-derived myeloid fibroblast, the data suggested a direct linkage between fibrosis and inflammatory mediators secreted by fibroblasts arising from MSC. We further demonstrated the critical importance of aberrant synthesis and secretion of IL-6 and stimulation of the formation of myeloid fibroblasts downstream of MCP-1 [26]. However, we have reported that these dysregulated mesenchymal fibroblasts make other inflammatory mediators that may regulate adverse remodeling as well [26].

4. Conclusions

In this review we analyzed the consequences of dysregulation of one type of cell (MSC) and its contribution to cardiac inflammation. We have discussed that, in the aging mouse heart, increased circulating insulin levels or acquired epigenetic modifications cause resident MSC to change their phenotype and differentiate into inflammatory fibroblasts. Although fibroblasts in the young heart can also acquire a pro-inflammatory phenotype after ischemia reperfusion injury, this activation is controlled with time and the level of inflammatory mediators soon return to baseline [84]. Therefore an impaired control mechanism as well as an imprinted phenotype in the aging heart may be responsible for the observed defects. Elevated inflammation in aging has been noticed by others as well. Fifteen years ago Claudio Franceschi proposed the term “inflammaging” to explain the association between advanced age and elevated low levels of systemic inflammation [85]. Further, many pathological conditions such as type 2 diabetes, metabolic syndrome, osteoporosis, sarcopenia, atherosclerosis, frailty and dementia, that are associated mostly with advanced age, manifest with increased circulating levels of inflammatory cytokines and acute phase

proteins [86]. This review emphasizes that local inflammation at a relatively low grade may be equally as detrimental as systemic inflammation and may predispose the aging heart to failure.

There are many excellent publications relevant to the subject of this review that due to the limited space the authors regrettably were unable to cite.

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Glossary

MSC	mesenchymal stem cells
TGF-β	transforming growth factor- β
TβRI	TGF- β receptor I
MCP-1	monocyte chemoattractant protein-1
RasGrf1	Ras protein-specific guanine nucleotide releasing factor-1
FTase	farnesyltransferase

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Highlights

- Fibrosis in the aging heart develops due to MSC dysfunction
- Evidence points to elevated circulating insulin levels as a primary source of MSC dysregulation in aging
- MSC in the aging heart differentiate into fibroblasts that acquire an inflammatory phenotype
- Inflammatory mesenchymal fibroblasts derived from old hearts secrete higher levels of MCP-1 and IL-6 due to an aberrant activation of the FTase-Ras-Erk pathway
- MCP-1 induces influx of monocytes from the blood and IL-6 facilitates their polarization into myeloid fibroblasts

Figure 1.

In the aging mouse heart aberrant MSC differentiate into fibroblasts that secrete higher levels of cytokines via the FTase-Ras-Erk pathway. *The red color denotes increased RNA/protein expression in the aging heart.*

Figure 2.

Elevated levels of MCP-1 attract leukocytes through an endothelial barrier into the heart, where they polarize into M2a macrophages/myeloid fibroblasts, a process that is augmented in the presence of IL-6. M ϕ denotes macrophage.

Figure 3. Presence of M1 macrophages (A) and M2 macrophages (B) in the aging mouse heart. Arrows point to M1 (CD86⁺procollagen type I^{neg}) and M2 (CD301⁺procollagen type I⁺) macrophages. Heart sections from 3 animals per age groups (3 and 30 month-old) were analyzed.

Figure 4.
Possible pharmacological interventions to prevent age-related cardiac fibrosis. Targets are marked by red color.