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Original article

Immune-inflammatory dysregulation modulates the incidence of progressive fibrosis and diastolic stiffness in the aging heart

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ABSTRACT

Diastolic dysfunction in the aging heart is a grave condition that challenges the life and lifestyle of a growing segment of our population. This report seeks to examine the role and interrelationship of inflammatory dysregulation in interstitial myocardial fibrosis and progressive diastolic dysfunction in aging mice. We studied a population of C57BL/6 mice that developed progressive diastolic dysfunction over 30 months of life. This progressive dysfunction was associated with increasing infiltration of $CD45^+$ fibroblasts of myeloid origin. In addition, increased rates of collagen expression as measured by cellular procollagen were apparent in the heart as a function of age. These cellular and functional changes were associated with progressive increases in mRNA for MCP-1 and IL-13, which correlated both temporally and quantitatively with changes in fibrosis and cellular procollagen levels. MCP-1 protein was also increased and found to be primarily in the venular endothelium. Protein assays also demonstrated elevation of IL-4 and IL-13 suggesting a shift to a Th2 phenotype in the aging heart. *In vitro* studies demonstrated that IL-13 markedly enhanced monocyte–fibroblast transformation. Our results indicate that immunoinflammatory dysregulation in the aging heart induces progressive MCP-1 production and an increased shift to a Th2 phenotype paralleled by an associated increase in myocardial interstitial fibrosis, cellular collagen synthesis, and increased numbers of $CD45^+$ myeloid-derived fibroblasts that contain procollagen. The temporal association and functional correlations suggest a causative relationship between age-dependent immunoinflammatory dysfunction, fibrosis and diastolic dysfunction.

1. Introduction

Age-related diastolic dysfunction has a significant impact on the healthy elderly. Left ventricular filling is impaired with normal aging, limiting the maximum exercise that healthy old persons can perform, and reducing their quality of life. The decrease in diastolic filling with age explains up to 60% of their decrease in exercise performance [1]. Perhaps more importantly, age-related diastolic dysfunction strongly predisposes older people to congestive heart failure (CHF), the most common reason for their hospitalization [2]. More than 50% of older men with CHF have preserved systolic function (LVEF>50%) and presumably impaired diastolic function [3]. The proportion of CHF with preserved systolic function is even higher in older women [4]. The prognosis of CHF and preserved LV systolic function is dismal [3]; there are no effective drugs for treatment of diastolic heart failure [5]. Therefore, preventing age-related diastolic dysfunction is likely to have a major additional benefit by decreasing the frequency of CHF with preserved systolic function [6].

Multiple aspects of diastolic function are significantly impaired with age. While active relaxation is impaired due to age-associated decreases in sarcoplasmic reticulum calcium uptake [7–10], passive stiffness also contributes significantly to the impaired diastolic function [11,12]. Stiffness is clearly elevated in the old mouse, old rat, old dog and old human heart and reductions in stiffness in the mouse improve diastolic function [11–14]. For example, in the aged TGF-$\beta1^{+/-}$ mouse, decreased age-related fibrosis and preserved diastolic function are seen [15]. Specifically, in the diastolic pressure/volume relationship, the 24 month wild type heart was almost twice as stiff as the hearts from the six month wild type or 24 month TGF-$\beta1^{+/-}$ mice and this paralleled the lack of age-associated increase in collagen in the aged TGF-$\beta1^{+/-}$ mouse [15]. Therefore aging is associated with increased interstitial fibrosis that increases left ventricular stiffness and impairs (passive) diastolic function.

Importantly, all of the major components of the renin–angiotensin system (RAS) appear to exhibit profibrotic activity, although Ang II may be the hormone most responsible for cardiac fibrosis [16]. Furthermore, there is substantial evidence that the aging heart has an augmented endogenous RAS, including ACE1 and AT1A in old rats

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[17,18] and cardiac Ang II levels in old mice [19] and rats [20]. Hydroxyproline levels, a marker for collagen content, have been known for many years to be elevated in the old rat heart [21]. Basso recently showed that six months of ACE inhibition or AT blockade inhibited the tripling of fibrosis reached by 18 months in the Wistar rat [22]. Only four weeks of candesartan was needed to reduce age-related fibrosis [23]. Similar data in the mouse (increased fibrosis with age that is prevented with ACE1 or AT blockers) were provided by Inserra [24]. The aging heart appears to be sensitive to pathologic effects of the RAS ligands, primarily Ang II, produced locally by ACE1.

In this report, we examined changes in diastolic function in C57BL/6 mice as they aged from 3 months to almost 3 years utilizing Doppler and two-dimensional echocardiography. Increases in left atrial size and altered left ventricular filling correlated with increased interstitial fibrosis; both progressed throughout the animal's life. There was also a progressive increase in induction of MCP-1, IL-4, and IL-13 that correlated both temporally and quantitatively with the fibrosis, cellular procollagen and diastolic dysfunction. *In vitro* studies demonstrated that IL-13 was effectively obligate for monocyte–fibroblast transformation. Thus, we quantitated the presence of $CD45^+$ fibroblasts in the aging myocardium using flow cytometry. The presence of $CD45^+$ fibroblasts in the heart correlated temporally and quantitatively with myocardial fibrosis and chemokine/lymphokine induction over the 13–30 month period. Importantly, the majority of the myeloid-derived fibroblasts contained procollagen and so were actively synthesizing collagen type I. These findings suggest that age-associated interstitial fibrosis and the associated diastolic dysfunction are consequences of immunoinflammatory dysregulation.

2. Methods

2.1. Animals

C57BL/6 male wild type (WT) mice of varying age were obtained from NIA (13–30 months of age) or from the barrier facility of Baylor College of Medicine Center for Comparative Medicine (3 months of age). All mice were fed standard mouse chow and water ad libitum. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All animals were treated in accordance with the guidelines of the Baylor College of Medicine Animal Care and Research Advisory Committee. Mice used for the various studies were grouped into different age ranges. For example, the aged groups included animals 13–16 months of age and animals 20–24 months of age. The specific age of the animals used for a particular study was always indicated in the text.

2.2. Protein microarray

Protein was isolated from snap-frozen whole hearts using cell disruption buffer from the Paris Kit (Ambion, Austin, TX) with halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein (250 μg) from each of three young (3 month) and three aged (30 month) hearts was loaded onto mouse cytokine antibody array 1 membranes (RayBiotech, Inc., Norcross, GA). Membranes were processed according to manufacturer's instructions, images on film were scanned, and densitometry was assessed by ImageJ software. Data are expressed as the mean ± SE of the signal compared with background chemiluminescence. A representative membrane is shown in Fig. S1.

2.3. Immunohistochemistry

Hearts were perfused with ZnCl/acetate-tris fixative [25] for 15 min and left in fixative for a total of 4 h before dehydration and embedding in paraffin. Sections (5 μm) were deparaffinized and processed using Vectastain Elite ABC kits with DAB substrate and nickel (Vector Laboratories, Burlingame, CA). The primary antibody was an affinity-purified rabbit anti-collagen type I (Rockland Immunochemicals, Gilbertsville, PA). The negative control was a rabbit monoclonal antibody (DA1E) against an irrelevant antigen (Cell Signaling Technology, Beverly, MA). All sections were processed together and for the same length of time in substrate.

2.4. Quantitative PCR (q-PCR)

Total RNA was isolated from whole hearts with TRizol reagent (Invitrogen, Carlsbad, CA), purified by RNeasy kit (Qiagen, Valencia, CA) and transcribed to cDNA by iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Q-PCR was performed on an iQ5 Multicolor Real Time PCR Detection System (Bio-Rad) using SYBR Green Super mix (Bio-Rad) and specific primers. Gene expression was measured by the comparative CT method to calculate the amount of target mRNA normalized to an endogenous reference (18S). The data were expressed as the fold of mRNA level relative to mRNA expression detected in 3 month old hearts. Each sample was tested in triplicate to assure reproducibility.

Primer sequences were designed using Beacon Designer software version 7 (Premier Biosoft, Palo Alto, CA):

ACE1: sense 5′-GCACTCTCCGTCTCTACC-3′ and antisense 5′-AGCAGGTAATTGATGTCACTC;

AT1A: sense: 5′-GAGAACACCAATATCACT-3′ and antisense 5′-GATTAGGAAAGGGAACAG-3′;

AT2: sense 5′-GTGTATGGCTTGTCTATCCTC -3′, and antisense 5′-ATTCACACCTAAGTATTCAATG-3′;

IL-13: sense 5′-GTAGCCCACTTTATAACA-3′ and antisense 5′-GATGGTCTCTCCTCATTA-3′;

MCP-1: sense 5′-TCCACAACCACCTCAAGCACTTC-3′and antisense 5′-GGCATCACAGTCCGAGTCACAC-3′,

18S RNA: sense 5′-ACCGCAGCTAGGAATAATGGA-3′ and antisense 5′-GCCTCAGTTCCGAAAACCA-3′,

2.5. Cell isolation

Mice were anesthetized via isoflurane inhalation and sacrificed. Hearts were immediately excised, washed to remove excess blood, and finely minced with a scalpel. The minced tissue was then digested with buffered Liberase IV (Roche Applied Science, Indianapolis, IN) with regular trituration by pipet to obtain a single cell suspension. Cell suspensions were filtered to remove large debris, washed with cold HBSS supplemented with taurine and HEPES and stained for immunofluorescence [26].

2.6. Immunofluorescence and flow cytometry

Cells were incubated with antibodies to CD45-PE (clone 30-F11, BD Pharmingen, San Jose, CA) or the appropriate isotype controls followed by streptavidin-PE-Cy5 (Beckman Coulter, Miami, FL). 50 nM calceinAM (which is taken up and forms a fluorescent green product only in metabolically active cells) was added to some samples to delineate living cells before they were analyzed by cytometry. Samples to be analyzed for internal antigens were fixed in 2% paraformaldehyde with 0.1% saponin then incubated with either an antibody to collagen type I (Rockland Immunochemicals, Gilbertsville, PA) or rabbit IgG followed by an anti-rabbit secondary conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR). Cells were analyzed on a Cell Lab Quanta SC flow cytometer (Beckman Coulter) using the Quanta Analysis software.

2.7. Transendothelial migration

Human cardiac microvascular endothelial cells were obtained from Lonza (Walkersville, MD), grown as directed by the supplier and