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Cardiomyopathy of Aging in the Mammalian Heart is Characterized by Myocardial Hypertrophy, Fibrosis and a Predisposition Towards Cardiomyocyte Apoptosis and Autophagy

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Abstract

Aging is associated with an increased incidence of heart failure, but the existence of an age-related cardiomyopathy remains controversial. Differences in strain, age and technique of measuring cardiac function differ between experiments, confounding the interpretation of these studies. Additionally, the structural and genetic profile at the onset of heart failure has not been extensively studied. We therefore performed serial echocardiography, which allows repeated assessment of left ventricular (LV) function, on a cohort of the same mice every 3 months as they aged and demonstrated that LV systolic dysfunction becomes apparent at 18 months of age. These aging animals had left ventricular hypertrophy and fibrosis, but did not have inducible ventricular tachyarrhythmias. Gene expression profiling of left ventricular tissue demonstrated 40 differentially expressed probesets and 36 differentially expressed gene ontology terms, largely related to inflammation and immunity. At this early stage of cardiac dysfunction, we observed increased cardiomyocyte expression of the pro-apoptotic activated caspase-3, but no actual increase in apoptosis. The aging hearts also have higher levels of anti-apoptotic and autophagic factors, which may have rendered protection from apoptosis. In conclusion, we describe the functional, structural and genetic changes in murine hearts as they first develop cardiomyopathy of aging.

Keywords

Aging; heart failure; cardiomyopathy; cardiomyocyte apoptosis; autophagy

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Introduction

Heart failure affects approximately 5.7 million Americans [1] at an estimated annual cost of \$37 billion [2]. Aging is associated with a dramatic increase in the incidence and prevalence of heart failure: heart failure is four-times more common in those over 85 years, compared to those aged 65–74 years [2]. As our population continues to age [3], the burden of heart failure will increase.

The effect of aging on contractile function of the heart remains controversial. Some have demonstrated overt systolic dysfunction with age [4, 5, 6], but others have not [7, 8, 9, 10]. Still other groups have shown that aging is associated with normal baseline cardiac function, but reduced cardiac response to inotropic stimuli [11, 12]. However, even among those who have demonstrated an age-related cardiomyopathy, the age at which it occurs is still a matter of some debate. Furthermore, the gene transcription profile of the aging heart and the contributions of cardiomyocyte apoptosis and autophagy to the cardiomyopathy of aging have not been fully described. To describe and quantify these pathologies is the first step toward developing disease-specific approaches to the treatment of age-related cardiomyopathy. There may be reversible steps during the development of age-related cardiomyopathy, where interventions could prevent progression to heart failure. Any such treatment would have the potential to improve longevity and quality of life for millions of people each year, and provide significant savings for health-care budgets.

To confirm the presence of age-related contractile cardiomyopathy, and to most accurately describe its age of onset, we followed mice with serial echocardiography as they aged to determine the age at which they first developed systolic dysfunction, and we found this occurred long before they were senescent or dying. The median life expectancy of male C57BL/6 mice is approximately 30 months [13], but we found the development of systolic dysfunction by 18 months. We termed this “aging” rather than “old” or “senescent”, and we focused our studies of cardiac structure, function and gene profile at this age.

Methods

Animals and study groups

Male C57BL/6J were used for all experiments. Young mice were 2 months old and aging mice were 18 months old. Animals were handled according to the guidelines of the Institutional Animal Care and Use Committee at the University of California San Francisco.

Echocardiography

Echocardiography was accomplished under isoflurane anesthesia with the use of a Vevo660 (VisualSonics, Toronto, Canada) equipped with a 30-MHz transducer as previously described [14]. Echocardiograms were obtained at 12, 15, 18 and 21 months in the same animals. A young cohort aged 2 months was used as control.

Electrophysiology Studies

Mice (n=4 young and n=4 aging) were anesthetized, intubated, and the chest cavity exposed and retracted. Peripheral recording electrodes were placed into the skin. Bipolar stimulation electrodes were lowered directly on to the right ventricle by direct visualization. A multiple channel Bloom cardiac stimulator (Fischer Medical Technologies, Inc., Broomfield, CO) was used. Stimulation thresholds were assessed by increasing the stimulation output (milliamperes - mA) to a point of consistent ventricular capture. This level was confirmed by beginning at high output and decreasing until loss of ventricular capture. With a margin for safety, the typical stimulation output used was 2–5 mA. Basic drive cycle lengths were 100–

120 milliseconds for a pacing train of 8–10 beats, and the ventricular effective refractory period (VERP) was assessed for each mouse. Once these basic data were obtained, a ventricular stimulation protocol was used to determine whether the mice exhibited inducible monomorphic ventricular tachycardia. Double and triple extrastimuli were given until the tightest intervals achieved or until ventricular tachycardia was induced. Testing was repeated for multiple trials in order to verify the reproducibility of results. Following ventricular stimulation protocols, each mouse was sacrificed.

Tissue preparation and analysis

Animals were humanely sacrificed under general anesthesia. Hearts were arrested in diastole with injection of concentrated KCl and the hearts immediately removed. For RNA and protein analysis, the left ventricle was dissected away from the rest of the heart and rapidly frozen by immersion in liquid nitrogen (n=3 young, n=3 aging). For immunohistochemical analysis, hearts (n=6 young, n=6 aging) were removed, weighed and perfused with 10% neutral buffered formalin via the ascending aorta and then paraffin embedded. For immunohistochemistry, tissue was analyzed by two blinded reviewers and the average of the two analyses is reported. Sections from the mid-ventricular level were examined for fibrosis using picrosirius red staining. Quantification of fibrous tissue was performed on photomicrographs taken using brightfield microscopy, collagen content was measured using polarized light microscopy. Both were quantified by using ImagePro (Media Cybernetics Inc, Bethesda, MD) software. Cardiomyocyte hypertrophy was quantified by measuring the diameter of cardiomyocytes in cross-section at the level of the nucleus. At least 30 cardiomyocytes from throughout the mid left ventricle were measured and averaged per animal.

Histology and Immunohistochemistry

For the Hematoxylin and Eosin stain, the slides were deparaffinized, rehydrated and rinsed in distilled water. The sections were immersed in Harris Hematoxylin (Fisher Scientific) for two minutes. They were then rinsed with subsequent dips in cold tap water, twice for 10 seconds each, Scott's Tap Water (Fisher Scientific) for one minute, and cold tap water for 10 seconds. The slides were then placed in Eosin (Fisher Scientific Protocol) for 30 seconds, rapidly dehydrated through graded ethanol solutions and mounted.

For the Toluidine Blue stain, a stock solution was prepared by dissolving 1 gram of Toluidine Blue (National Aniline & Chemical Co) in 100 ml of 70% ethanol. A fresh 1% sodium chloride solution was made with the pH adjusted to 2.0 using glacial acetic acid, in which the Toluidine Blue stock solution was diluted 1:10 to make a Working Solution. Deparaffinized and rehydrated sections were immersed in the Toluidine Blue Working Solution for 3 minutes and washed in 3 changes of distilled water. The sections were then dehydrated by quick dips in 100% ethanol, cleared and mounted.

For CD68 immunohistochemistry, deparaffinized and rehydrated slides were steamed in a water bath at 100oF for 1 hour in citric acid-based Antigen Unmasking Solution (Vector Labs) and incubated in a 1:200 dilution of rat monoclonal (FA-11) antibody to CD68 (Abcam) for 1 hour at room temperature. The sections were then incubated for 1 hour at room temperature in a 1:200 dilution of Alexa Fluor 488 goat anti-rat secondary antibody (Invitrogen) and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Apoptosis of cardiomyocytes was detected by the colocalization of antibody staining for cardiac troponin-I (Abcam, Cambridge, MA) and TUNEL staining (ApopTag; Chemicon, Temecula, CA) within the same cell. Apoptotic CMs were quantified by blinded reviewers counting the number of positive cells within the entire section. Activation of caspase-3 in cardiomyocytes was detected by the colocalization of antibody staining for activated

caspase-3 (BD Pharmingen, San Jose, CA) and for cardiac troponin-I within the same cell. The percent of the troponin-I positive area that was also positive for caspase-3 was quantified using ImagePro software. Caspase independent cardiomyocyte apoptosis was quantified by co-staining troponin-I with AIF: 1:50 (Cell Signaling), EndoG: 1:500 (ProSci Inc.) and BNIP-3: 1:50 (Abcam). Cardiomyocyte autophagic flux was studied by co-staining troponin-I with Cathepsin D: 1:25–1:40 (R&D Systems). For Hairpin staining, the slides were treated with Proteinase K (Millipore) at 50 ug/ml for 15 minutes. The slides were washed 3 times with PBS before staining for apoptosis with the ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (Millipore) for 16 hours at 22°C. The samples were then washed with PBS for 3 times before counterstaining with ProLong® Gold antifade reagent with DAPI (Invitrogen) and coverslipped.

Quantification of Cardiomyocyte Number

The total number of cardiomyocytes per left ventricle were quantified using the stereological method of Levkai [15]. The mean cardiomyocyte diameter was measured as above. Because of difficulty seeing the ends of cardiomyocytes stained with picrosirius red, we stained with wheat germ agglutinin (WGA) and found this a more reliable way to detect the longitudinal ends of cardiomyocytes. To calculate the absolute number of cardiomyocytes per heart, the volume fraction of cardiomyocytes (Vv Myo) in the heart was determined by the principle of Delesse (area density=volume density). Using image analysis software on Masson's trichrome-stained slides, Vv Myo was calculated as the percentage of cross-sectional area that is occupied by cardiomyocytes (i.e. excluding blood vessels and connective tissue). The mean cardiomyocyte volume (V Myo) was calculated as follows:

$$V \text{ Myo} = (\text{mean diameter}_{\text{cardiomyocyte}} / 2)^2 \times \text{mean length}_{\text{cardiomyocyte}}$$

The absolute number of cardiomyocytes per left ventricle (N Myo) was calculated from the following:

$$N \text{ Myo} = (Vv \text{ Myo} \times \text{LV volume}) / V \text{ Myo},$$

where the total tissue volume of the left ventricle (LV volume) was obtained by dividing its mass on echocardiography by specific gravity (1.0048).

Immunoblot analysis

Hearts were excised from young (n=3) and aging (n=3) mice, and perfused with cold PBS. The left ventricles (LV) were flash frozen in liquid nitrogen and stored in -80°C. For each sample, the LV was homogenized in RIPA buffer with PMSF, complete protease inhibitor cocktail and phosphatase inhibitor cocktail added. Both the RIPA buffer and PMSF were from Santa Cruz Biotechnology and protease and phosphatase inhibitor cocktail tablets from Roche. The lysates were quantified by Pierce BCA protein assay. 15 µg of protein for each sample was separated by 14% Tris-Glycine gels (Invitrogen) and transferred to PDVF membranes (Biorad) overnight at 4°C at 90 mA. The membrane was blocked with 10% (w/v) non-fat dry milk in Tris-Buffered Saline Tween (50mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with primary antibodies then washed 3 times with TBST and incubated with anti-rabbit HRP conjugated antibody for 1 hour at room temperature. The immunoblots were visualized using ECL plus detection system on Amersham Hyperfilm ECL chemiluminescence film (GE Healthcare). Antibodies used were pAKT: 1:500 from Cell

Signaling, Bcl2: 1:100 Santa Cruz Biotechnology, Beclin-1: 1:500 and β -actin (control): 1:5000 from Abcam.

RNA analysis

Total RNA was isolated from left ventricles of young (n=3) and aging (n=3) mice by TRIzol reagent (Invitrogen). Trace genomic DNA in total RNA was removed by DNase I and RNeasy Mini Kit (Qiagen). cDNA was generated from 0.1 mg of total RNA by using SuperScript III First-Strand Synthesis kit (Invitrogen). Microarrays were performed using Affymetrix 1.0 Mouse gene chip. Data were normalized using robust multi-array average (RMA) method. Control and low performing probesets (those with intensity values below a threshold across all samples, the threshold was taken to be the global lowest 25th percentile of intensity values) were excluded from analysis. Also, based on Affymetrix's annotation information, only those probesets which were part of the main design of the array and perfectly matching only one sequence were considered for analysis of differential expression. 20648 out of 35557 probesets remained after filtering

Statistical analysis

Continuous data were compared between young and aging using student's t-test and categorical variables using Chi-square test. Data are presented as mean $\pm$ SD unless otherwise stated. For microarray analysis, moderated t-statistics (as implemented in the limma package in R/Bioconductor) were used to test for differentially expressed probesets. The theoretical (raw) p-values were then adjusted for multiple testing by controlling the false discovery rate (FDR). A cut-off of 0.05 was used on the FDR adjusted p-values to declare a probeset to be significant. Unbiased analyses were performed for single genes as above and using Gene Ontology (GO) terms, which is described in detail on their website <http://www.geneontology.org/>. In addition, we performed Gene Set Enrichment Analysis (GSEA) to look for pre-specified genesets of interest. In brief, this is a biased analysis that calculates an enrichment score (ES) that reflects the degree to which the *a priori* defined geneset of interest is over-represented at the top or bottom of the imputed ranked list of genes [16].

Results

Systolic Contractile Dysfunction Occurs at 18 months of Age

Serial echocardiography was performed on a cohort of aging mice. By 18-months of age, mice exhibit impairment of left ventricular systolic function. Echocardiographic and weight data are presented in Figure 1 and Table 1.

Increased Interstitial Fibrosis and Hypertrophy in Aging Hearts

Aged hearts displayed a significant increase in fibrosis; in particular, collagen levels were increased compared to young animals (Figure 2). In addition to the increased myocardial fibrosis, aged hearts display cardiomyocyte hypertrophy. This phenotype resembles hypertensive heart disease in patients; however, there is no difference in blood pressure (see figure 2). Furthermore, this fibrotic phenotype is not associated with an increased susceptibility to arrhythmia. During electrophysiological testing, no young or aging animals had inducible ventricular tachycardia or ventricular fibrillation and there was a similar ventricular effective refractory period in the left ventricles of the young and aging mice (data not shown).

Gene Expression Profile of Aging Hearts

Microarray analysis was performed on left ventricular tissue from young and aging hearts. There were 40 significant differentially expressed probesets of which 14 had at least 2-fold change. For the 40 probesets, there were 36 significant Gene Ontology (GO) terms when the genes from the chip were used as reference (Table 2). The differentially expressed genesets were predominantly involved with inflammatory and immune processes. Gene Set Enrichment Analysis (GSEA) for traditional known human pathways involved in fibrosis, cardiomyocyte hypertrophy and heart failure showed no differential expression between young and aged hearts. Transforming growth factor beta (TGFb) has been associated with age-related cardiac fibrosis. We found no differential expression between young and aging hearts in the expression of TGFb subtypes 1, 2 or 3, nor in their receptors.

Pro-apoptotic Profile of Aging Hearts

There are higher levels of activated caspase-3 in cardiomyocytes of aged animals than young animals (See figure 3), but no significant difference in cardiomyocyte apoptosis (7.0 ± 2.0 vs. 5.4 ± 1.9 TUNEL positive CM per section, $p=ns$), suggesting a predisposition to apoptosis but no actual increase in the number of apoptotic CM. We confirmed this using Hairpin-1 staining, which some consider even more specific than TUNEL for apoptotic cell death [17]. There was no difference in the number of Hairpin-1 positive cardiomyocytes between young and aging mice (0.85 ± 1.07 vs 0.50 ± 0.54 respectively; $p=ns$; see Figure 3). Furthermore, there was no difference in cardiomyocyte necrosis, as assessed the number of Hairpin-2 positive cardiomyocytes (0.42 ± 0.53 vs 0.16 ± 0.41 positive CM per section in young and aging hearts respectively; $p=0.35$). Next, we assessed the total number of cardiomyocytes in the left ventricle. There was no difference in the number of cardiomyocytes in the LV of young and aging mice ($21.4 \pm 3.4 \times 10^8$ vs $19.7 \pm 5.0 \times 10^8$ respectively; $p=0.35$). This is consistent with our finding of no difference in cardiomyocyte apoptosis in the aging mice. Gene Set Enrichment Analysis of microarray data identified a significant increase in pro-apoptotic genes using GenMAPP apoptosis pathway (ES 0.42, nominal $p < 0.001$, FDR 0.1, FWER p -value < 0.001). See Figure 4. Thus it appears that aging hearts have predilection for apoptosis, suggested by the pro-apoptotic gene expression and cardiomyocyte expression of activated caspase-3; however, no difference in actual CM apoptosis can be seen compared to young hearts, assessed by two complimentary methods.

To determine whether the discrepancy between the levels of caspase activation and number of apoptotic cardiomyocytes was due to upregulation of anti-apoptotic factors, we studied the anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and phosphorylated Akt (p-Akt). Aging hearts exhibited increased levels of p-Akt and Bcl-2 compared to young animals (Figure 3).

To ensure that the discrepancy between the levels of caspase activation and number of apoptotic cardiomyocytes was not due to a reduction in the amount of caspase-independent apoptosis in aging hearts, we analyzed the number of cardiomyocytes expressing the caspase-independent pro-apoptotic factors: apoptosis inducing factor (AIF), Endonuclease-G (EndoG) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3). There was no difference in the expression of the caspase-independent pro-apoptotic factors between young and aging (AIF 0.30 ± 0.41 vs 0.53 ± 0.50 positive CM/HPF, $p=0.89$; EndoG 7.7 ± 2.6 vs 6.7 ± 1.0 positive CM/HPF, $p=0.88$; BNIP3 11.0 ± 3.3 vs 11.7 ± 2.6 positive CM/HPF, $p=0.82$). See Figure 3. In addition, AIF protein levels did not differ between aging and young hearts.

Autophagy in Aging Hearts

At times of stress, such as nutrient deprivation, autophagy is activated. Aging cardiomyocytes display numerous autophagic vacuoles, detected by electron microscopy (Figure 5), which were not seen in the young heart. Aging hearts had higher beclin-1 protein expression and higher LC3II:I ratio, consistent with induction of autophagic pathways (Figure 5). There was no difference in the expression of any known autophagy genes between young and aging hearts, demonstrating that the changes in protein expression were occurring at a post-transcriptional level. Furthermore, this increase in autophagosomes in the aging hearts was not due to a reduction in autophagic flux, as there was no change in downstream lysosome formation (0.14 ± 0.1 vs 0.15 ± 0.1 cathepsin-D positive CM per HPF; $p=0.94$).

Inflammatory Cells in the Aging heart

There is an established link between inflammation and fibrosis. After finding differentially expressed genesets involving immunity and/or inflammation, we examined the inflammatory cell numbers in the hearts of young and aging mice. In aging mice, there appeared to be clusters of lymphocytes around blood vessels (Figure 6) that were not seen in young hearts. We also examined the number of macrophages and mast cells. Macrophages were rare, as were mast cells and there were no differences between groups (Figure 6).

DISCUSSION

With serial assessment of left ventricular function in a cohort of mice, we have described the age at which mice develop systolic dysfunction, and focused our tissue analysis on this timepoint. We have demonstrated that aging male C57/Bl6 mice: 1) develop contractile dysfunction at 18 months of age, and this is associated with structural changes of increased fibrosis and cardiomyocyte hypertrophy; 2) have a different gene expression profile in the left ventricular myocardium from young mice with upregulation of immune/inflammatory genes; and 3) have an increase in activated caspase-3 in cardiomyocytes, with a parallel upregulation of anti-apoptotic factors.

The very existence of an age-related cardiomyopathy is controversial. Some authors have found that with age the murine heart develops contractile dysfunction [4, 5, 6], whereas other have not [7, 8, 9, 10]. Still other groups have shown that aging is associated with normal baseline cardiac function, but reduced cardiac response to inotropic stimuli [11, 12]. However, even among those who have demonstrated an age-related cardiomyopathy, the age at which it occurs is still a matter of some debate. In trying to make sense of such varied and contradictory literature, several issues must be taken into account. Firstly, there are differences in strain and age of the mice in each of these studies, and there will likely be differences in the incidence of heart failure across strains and ages. There may even be differences within strains in the age at which age-related cardiomyopathy occurs, due to different living/cage conditions at various institutions, and different feeding parameters. Secondly, the absence of heart failure at one age in an experiment does not mean it will not occur at a later time in that strain of mouse. Most studies do not serially evaluate cardiac structure and function in the same cohort over time, as we have. We feel that our method of serial echocardiography demonstrating deterioration of function in previously normal hearts is a more robust proof of the existence of age-related cardiomyopathy than simply comparing groups of different animals at different ages. Finally, there are many ways to evaluate cardiac function, including echocardiography, LV pressure measurements and *ex vivo* whole heart perfused preparations. There are many choices for anesthetic agents, and all have some effect on cardiorespiratory function. Thus, the different loading conditions during the different techniques used, and the type of anesthetic agent employed, can have

significant effects on the results of the experiments and make comparisons between studies problematic. Thus, we kept our anesthetic agents and doses the same at each study, used the same echocardiographic machine and echocardiographer, in order to keep the variables to a minimum. This approach strengthens our findings, and we conclude that our results confirm the presence of a murine age-related cardiomyopathy, and describe its age of onset in our laboratory.

Our findings confirm previous studies showing LV fibrosis with increasing age. Interestingly, we have extended these findings to show that this is not associated with upregulation of any known pro-fibrotic genes, or any known fibrotic gene pathways. Although increased cardiac fibrosis has been shown in other disease states in the absence of upregulation of fibrosis-related pathways [18, 19], to our knowledge this is the first description of age-associated increase in cardiac fibrosis without upregulation of any known pro-fibrotic pathway. TGF β is thought to be a crucial mediator of age-related cardiac fibrosis [20]. We showed no change in expression between young and aging hearts in TGF β subtypes 1, 2 and 3, nor any change in their receptor expression. We then used gene set enrichment analysis to determine whether any known fibrosis pathways were involved in our age-related cardiac fibrosis. GSEA is a publicly available resource at www.broadinstitute.org/gsea/ that allows users to analyze their microarray data against known pathways (that are also available on their website). This allows for detection of smaller deviations between groups of known clusters of genes associated with a biological function. Gene expression changes that are too small to be detected using standard multiple comparison testing can be detected with GSEA [16]. The lack of any gene changes or pathway differences suggests that the fibrosis is either due to novel fibrosis pathways, to non-transcriptionally mediated pathways, or to reduction in turnover in the extracellular matrix. We did not see down regulation of collagen turnover pathways such as the tissue inhibitors of matrix metalloproteinases (TIMPS) at the RNA level, although increased clearance of these or other collagenases could potentially account for these findings. Willems et al [21] also demonstrated increased collagen content at 18 months of age in the mouse heart. In contrast to our study, they showed no difference in left ventricular function at the same age despite the increased collagen content. Differences in experimental technique may have accounted for these different findings, as they employed ex-vivo Langendorff preparation to assess ventricular function, whereas we used in vivo echocardiography. The established fibrosis in our study right at the onset of the LV dysfunction suggests that the fibrosis had been occurring for some time before the development of LV dysfunction, and thus is more likely to be involved in the pathogenesis of the cardiomyopathy than reactive to it. The findings of Willems et al are consistent with this hypothesis. It is conceivable that they examined LV function after the onset of fibrosis but before the onset of LV dysfunction. In combination with fibrosis, also we found significant cardiomyocyte hypertrophy. Although the phenotype is similar to hypertensive heart disease, this was not due to elevated blood pressure and there was no increase in the known pathways for LV hypertrophy on microarray analysis. The hypertrophy seen with age may therefore occur via different gene pathways from hypertrophy that occurs in known disease states.

Our gene expression data revealed an increase in several gene ontology terms associated with immunity and/or inflammation. Mast cells are associated with myocardial fibrosis [22], and the number of mast cells in the heart has been shown to correlate with the amount of myocardial fibrosis [23]. To explore this link between mast cell number and fibrosis in age-related cardiomyopathy, we examined the number of mast cells in the hearts of young and aging animals but found no difference. Macrophage infiltration in the heart is also associated with pathological fibrosis in experimental models [24, 25]. We therefore examined our heart tissues for macrophages and found there were very few macrophages at all, with no

difference in the numbers between young and aging mice hearts. We detected clusters of lymphocyte-like cells in the peri-vascular regions of aging hearts. The association between lymphocyte infiltration and myocardial fibrosis in cardiac transplant rejection is well known. Our gene expression data shows upregulation of complement components [26] and CCR2 [27] that can regulate lymphocyte function. We hypothesize that lymphocyte infiltration may be the link between the inflammatory gene profile and the myocardial fibrosis we have observed, but further experiments are required to confirm that these cells are truly lymphocytes and that this observed link is causal. Our finding are congruent with others in the field [28], who have also shown upregulation of inflammatory/immune pathways in the aging mouse heart, although the actual genes they list are slightly different from ours. This may be because of strain differences between the studies. In a large study, Swindell [29] combined numerous microarray studies of murine cardiac aging, and reported upregulation of numerous GO terms (over 100 biological processes were upregulated). These are also largely related to inflammation and immunity, similar to ours. The difference between our data and that of Swindell may be due to two factors: he combined data from 2 different strain of mouse, and he used 24 month-old mice, whereas we used only c57Bl6 at 18 months of age. In another study, Park and colleagues [30] used microarray technology to assess the aging heart. They compared seven different mouse strains and found 6 GO terms were consistently upregulated across at least 6 of the 7 strains they tested. Of these 6 terms, 5 involved inflammation/immunity, and all these 5 were also upregulated in our study. Although they tested strains that were different from ours, the consistency of the finding of immune/inflammatory gene upregulation across strains strengthens our microarray findings. To our knowledge, however, ours is the first study to describe both inflammatory cell infiltration to the heart and inflammatory gene upregulation in aging mouse hearts. Future studies are required to address the links between inflammatory gene induction, cardiac fibrosis and the cardiomyopathy of aging.

The propensity of aging cardiomyocytes toward apoptosis may contribute to the known susceptibility of the elderly to diseases such as myocardial infarction and heart failure. At this early time-point in the evolution of age-related cardiomyopathy, we cannot detect differences in the number of apoptotic CM, yet we detect a significant increase in active caspase-3. The presence of active caspase 3 is considered to be a surrogate measure for apoptosis, so how can we reconcile this discrepancy? One possible explanation is that the process of apoptosis is brief, and it is relatively uncommon to see long-lived cells like cardiomyocytes undergoing apoptosis [31]. Thus, because the event is so brief, finding true differences between groups is difficult and we are likely to underestimate the true contribution of this brief cellular event to the organ as a whole. However, our finding of similar total numbers of cardiomyocytes between young and aging mice would argue against this, and would suggest that there is truly less apoptosis. Furthermore, we believe our findings are strengthened by the use of two methods to assess apoptosis: Hairpin-1 and TUNEL. Both these methods assess for DNA cleavage associated with apoptosis, but Hairpin-1 is more specific for DNA double-strand breaks with single base 3' overhang, and is thus thought to be more specific for apoptosis [32, 17]. Another reason that increased caspase-3 may not translate into increased apoptosis is that other caspase-independent pathways of apoptosis may become downregulated. To our knowledge, caspase-independent pathways to cardiomyocyte apoptosis have not previously been described in the aging murine heart. We assessed three well-characterized caspase-independent mediators of apoptosis (AIF, EndoG, and BNIP3) and found they were not suppressed compared to young hearts. Ljubicic et al recently demonstrated increased AIF in the hearts of senescent rats [33]. There may be other caspase-independent apoptotic pathways that are downregulated to account for our findings. An alternative explanation is the upregulation of anti-apoptotic pathways that counteract the activation of caspase-3, resulting in no increase in apoptosis. We showed an increase in pAKT and Bcl-2, which may account for this, but numerous other

anti-apoptotic pathways exist. Possibilities for such pathways include IAPs (inhibitor of apoptosis proteins), heat shock proteins, calpains, proteases, phosphoinositides and Bcl-2 family members, which can all antagonize caspase signaling. Other anti-apoptotic pathways downstream of caspase-3 may also be upregulated. It bears mentioning that we analyzed apoptosis and activated caspase-3 in cardiomyocytes, not in the entire tissue. Therefore our results reflect apoptosis in CM, and are not confounded by signaling in other cell types. Our finding of cardiomyocytes “primed” for apoptosis suggests that anti-apoptotic strategies are an important research direction for heart disease in the aging. This time-point, where there is a predilection towards, before there is no actual apoptosis, may be a particularly good time to intervene to prevent apoptosis.

Autophagy has been described as a part of normal aging [34, 35, 36, 37, 38]. Whether autophagy is physiological, pathological, or a reaction to other pathologies is not known. Furthermore, whether it contributes to the cardiomyopathy of aging is also not known. We showed increased CM autophagy in the aging heart. Increased early autophagic markers beclin-1 and LC3II:I ratio (markers of the autophagosome) may represent increased autophagosome formation or decreased clearance (fusion with lysosomes i.e. autophagic flux). Here we show no impairment in the lysosome compartment by cathepsin-D staining, so the increase in autophagosomes is not due to reduction in the lysosome compartment. We did not detect differences in autophagy-related genes. Therefore, the autophagic response is likely at the posttranslational level and is more likely to be a secondary response than a constitutively activated major pathogenic cause of the cardiomyopathy. Autophagy has been associated with rescue from apoptosis in cardiomyocytes *in vitro* [39]. It is enticing to speculate that the increases seen in anti-apoptotic and autophagic factors were able to rescue the cardiomyocytes from caspase-dependent apoptosis *in vivo*.

Whereas studying a very old, senescent animal may show cellular and molecular changes that are secondary to heart failure, we believe that studying this time-point early in the evolution of heart failure is more likely to identify gene expression differences that are causally linked to the development of heart failure. Our findings are observational in nature, however we describe in detail the phenotype of age-related cardiomyopathy at a structural, functional, cellular and molecular level at the onset of the disease. These observations set the scene for future studies of therapies aimed at preventing or reversing the early stages of age-related heart failure.

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Decline in Left Ventricular Function with Age

Figure 1. Serial echocardiography demonstrates a decline in left ventricular systolic function at 18 months of age.

Figure 2.

Young hearts (A) demonstrate scant extracellular matrix (red color) on picrosirius red staining. Aging hearts (B) show expansion of extra cellular matrix. Polarized light microscopy (C young, D aging) shows that this is predominantly collagen, exhibiting birefringence. Quantification is shown below. Note that the increase in fibrosis and collagen is accompanied by cardiomyocyte hypertrophy, but there is no difference in blood pressure. Grey bars represent young, black bars represent aging. * $p < 0.01$ vs young.

Figure 3.

Aging hearts have higher cardiomyocyte expression of activated caspase-3 (A). Although activated caspase-3 can be seen in non-cardiomyocytes (troponin-I negative cells) in the young, there is little staining of cardiomyocytes. In the aged hearts, co-localization of troponin-I and activated caspase-3 (white arrows) indicates increased cardiomyocyte expression of active caspase-3. However, this does not translate into higher levels of cardiomyocyte apoptosis assessed by Hairpin-1 staining (B). Apoptotic cardiomyocytes were rarely found (white arrow), and were not different between young and aging hearts. Caspase-independent mediators of apoptosis are not different in young and aging hearts (C). Characteristic cytoplasmic staining of AIF and BNIP-3, and peri-nuclear staining of EndoG

are seen (white arrows). Anti-apoptotic protein expression appears higher in the aging heart (D). This suggests parallel activation of pro-apoptotic and pro-survival signals in aging cardiomyocytes, which results in no difference in the overall number of apoptotic cardiomyocytes. AIF = apoptosis inducing factor; EndoG = Endonuclease-G; BNIP3 = BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; Bcl-2 = B-cell lymphoma 2; p-Akt = phosphorylated Akt.

Figure 4. Pro-apoptotic gene expression profile in aging hearts. Heatmap and gene list of apoptotic genes differentially expressed in young and aging hearts derived from geneset enrichment analysis. Red signifies increased expression, blue signifies reduced expression.

Figure 5.

Activation of autophagy is seen in aging cardiomyocytes. (A,B) Electron microscopy demonstrates frequent autophagosomes in the aging heart (white arrowheads), but these are rare in the young heart. Autophagosomes are generally seen in close proximity to mitochondria. (D) Beclin-1 protein, a component of the autophagosome membrane, is upregulated in aging hearts. Aging heart demonstrate a shift from LC3 I to LC3 II, which is associated with induction of autophagy. Importantly, there is no blockage in autophagic flux to account for this finding, as demonstrated by similar levels of cathepsin D positive cardiomyocytes in young and aging hearts (C).

Figure 6.

Inflammatory cells in the heart. A. Aging animals showed clusters of lymphocyte-like cells around blood vessels (green arrows) but young animals did not. B. Macrophages identified by CD68 staining were rarely seen in either the aging or young hearts. C. Mast cells, identified by toluidine blue staining (white arrow) were also rarely seen in either the aging or young hearts.

Table 1

Echocardiographic and clinical data of young and aging mice

	Young	Aging	p-value
Age (months)	2	18	-
LVEDV (μL)	70.0 $\pm$ 9.3	86.5 $\pm$ 5.2	<0.05
LVESV (μL)	33.8 $\pm$ 7.4	46.4 $\pm$ 2.9	<0.05
LVEF (%)	54.9 $\pm$ 7.8	46.3 $\pm$ 2.2	<0.05
LVID-d (mm)	4.3 $\pm$ 0.3	4.4 $\pm$ 0.1	NS
LVID-s (mm)	3.0 $\pm$ 0.3	3.3 $\pm$ 0.2	<0.05
FS (%)	29.7 $\pm$ 2.8	24.1 $\pm$ 4.0	<0.05
E:A ratio	1.41 $\pm$ 0.16	1.18 $\pm$ 0.04	<0.05
Systolic BP (mmHg)	65 $\pm$ 13	74 $\pm$ 15	NS
Diastolic BP (mmHg)	54 $\pm$ 11	59 $\pm$ 9	NS
HW (g)	0.10 $\pm$ 0.01	0.12 $\pm$ 0.01	<0.05
BW (g)	27.1 $\pm$ 4.2	38.7 $\pm$ 6.8	<0.05
HW:BW ratio	0.36 $\pm$ 0.03	0.31 $\pm$ 0.06	NS

LV = left ventricular; EDV = end-diastolic volume; ESV = end-systolic volume; ID-d = internal diameter in diastole; ID-s = internal diameter in systole; FS = fractional shortening; E:A ratio = early filling:atrial contraction ratio of trans-mitral Doppler flow; BP = blood pressure; HW = heart weight; BW = body weight.

Table 2

Gene Ontology Terms Differentially Expressed in Young and Aging Left Ventricles.

Best GOs	Term	Genes	Gene Count	P-Value
GO:0006959	humoral immune response	ccr2 c3 c1s c4b serping1	5	2.17E-05
GO:0006958	complement activation, classical pathway	c3 c1s c4b serping1	4	3.35E-05
GO:0002455	humoral immune response mediated by circulating immunoglobulin	c3 c1s c4b serping1	4	4.20E-05
GO:0002541	activation of plasma proteins involved in acute inflammatory response	c3 c1s c4b serping1	4	5.99E-05
GO:0006956	complement activation	c3 c1s c4b serping1	4	5.99E-05
GO:0002526	acute inflammatory response	c3 c1s c4b serping1	4	0.000448
GO:0016064	immunoglobulin mediated immune response	c3 c1s c4b serping1	4	0.000571
GO:0019724	B cell mediated immunity	c3 c1s c4b serping1	4	0.000571
GO:0002253	activation of immune response	c3 c1s c4b serping1	4	0.000571
GO:0045087	innate immune response	c3 c1s c4b serping1	4	0.000911
GO:0002449	lymphocyte mediated immunity	c3 c1s c4b serping1	4	0.000911
GO:0050778	positive regulation of immune response	c3 c1s c4b serping1	4	0.000911
GO:0002250	adaptive immune response	c3 c1s c4b serping1	4	0.000911
GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	c3 c1s c4b serping1	4	0.000911
GO:0002684	positive regulation of immune system process	c3 c1s c4b serping1	4	0.000911
GO:0002443	leukocyte mediated immunity	c3 c1s c4b serping1	4	0.00101
GO:0006954	inflammatory response	ccr2 c3 c1s c4b serping1	5	0.00111
GO:0050776	regulation of immune response	c3 c1s c4b serping1	4	0.00143
GO:0002682	regulation of immune system process	c3 c1s c4b serping1	4	0.00149
GO:0051240	positive regulation of multicellular organismal process	c3 c1s c4b serping1	4	0.0017
GO:0002252	immune effector process	c3 c1s c4b serping1	4	0.00313
GO:0009611	response to wounding	ccr2 c3 c1s c4b serping1	5	0.00504
GO:0002376	immune system process	ccr2 c3 c1s prkcq c4b	7	0.00504
GO:0051239	regulation of multicellular organismal process	serping1 skap2	5	0.00558
GO:0006952	defense response	c3 c1s prkcq c4b serping1	5	0.011
GO:0006955	immune response	ccr2 c3 c1s c4b serping1	5	0.0159
GO:0004866	endopeptidase inhibitor activity	c3 c4b serping1	3	0.0226
GO:0030414	peptidase inhibitor activity	c3 c4b serping1	3	0.0233
GO:0009605	response to external stimulus	ccr2 c3 c1s c4b serping1	5	0.0276
GO:0016024	CDP-diacylglycerol biosynthetic process	cds1	1	0.0345
GO:0005436	Sodium phosphate symporter activity	slc17a7	1	0.0345
GO:0004925	prolactin receptor activity	prlr	1	0.0345
GO:0042137	sequestering of neurotransmitter	slc17a7	1	0.0345
GO:0016942	insulin-like growth factor binding protein complex	igfbp5	1	0.0345
GO:0015319	Sodium inorganic phosphate symporter activity	slc17a7	1	0.0345
GO:0046341	CDP-diacylglycerol metabolic process	cds1	1	0.0345