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TGF-β_1 Overexpression: A Mechanism of Diastolic Filling Dysfunction in the Aged Population

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Abstract: The prevalence of cardiovascular disease in the United States dramatically increases with age. A hallmark feature of the aged myocardium is increased fibrosis resulting in diastolic dysfunction. Moreover, the survival of patients subsequent to a myocardial infarction is inversely related to age because of a certain extent to maladaptive remodeling mediated by cardiac fibroblasts. Our hypothesis is that cardiac fibroblast (CF) dysfunction results in overexpressed TGF-β_1 leading to increased cardiac collagen content in the aged population. TGF-β_1 stimulates the synthesis of the extracellular matrix proteins, including collagen in the cardiac tissues. The RT–PCR analysis of mRNA expression of TGF-β_1 of the CF was increased by 43% in the aged mice as compared to the younger. The stiffness of the left ventricle is expressed with the slope of the end-diastolic pressure–volume relationship parameter, β (mmHg/μL). In a mouse model, we demonstrated that β was 0.30 ± 0.05 in the young as compared to 0.52 ± 0.10 in the aged ($p < .05$). The ventricular stiffness was associated with the myocardial collagen content; namely, young versus the aged was 9.5 ± 4.0 as compared to 16.4 ± 2.3% of total protein, respectively ($p < .05$). In conclusion, the gene structure–function relationships support our hypothesis that cardiac fibroblast disregulation contributes to diastolic filling dysfunction in elderly persons. These data provide a potential contributory mechanism for diastolic dysfunction that may be vital in caring for the aged open-heart surgical patient. **Keywords:** TGF-β_1, aging, diastolic function, conductance catheter, fibroblast. *JECT. 2004;36:69–74*

A decrease in diastolic function may contribute to the morbidity and mortality of the cardiac patient (1–3). In rodent and human models, the diastolic dysfunction is related to the collagen content, ratio of types I and III collagen, and the expression of the calcium cycling protein, SERCA2a (4–7). The diastolic dysfunction can be separated into two phases: the isovolumic relaxation phase, and the filling phase. This paper focuses on the age-related changes of the diastolic filling phase characterized by ventricular stiffness.

It is well established that the cardiac fibroblast regulates the extracellular matrix (ECM) composition of the heart (6). Cardiac fibroblasts (CF) constitute the majority of the nonmyocyte cell population in the ventricular myocardium. These cells are located in the interstitium, in areas between and surrounding cardiac myocytes. CF are responsible for the synthesis of such extracellular matrix proteins as fibrillar collagen types I and III, basement membrane type IV collagen, fibronectin, and laminin. In addition to its role in muscle development and myoblast differentiations, extracellular matrix consisting primarily of fibrillar collagen is an intricate and highly organized structure that serves to support cardiac myocytes, to maintain functional integrity of the myocardium and to transduce developed tension.

The balance of synthesis and degradation of the ECM is the key to normal development of cardiac muscle and optimal myocardial contractile and diastolic filling function. Collagen remodeling and accumulation has been demonstrated in several experimental models of cardiac hypertrophy (6). To gain insights into molecular and cellular mechanisms that affect CF function, CF from mice

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were analyzed for the gene expression of transforming growth factor-beta (TGF-β_1). TGF-β_1 is a member of the transforming growth factor family (TGF-β_1, $-\beta_2$, and $-\beta_3$) and is understood to stimulate collagen synthesis by fibroblasts.

This study determined if the diastolic function, ventricular collagen content, and CF TGF-β_1 were related to age. By means of analysis of gene structure–function relationships, our hypothesis was that TGF-β_1-induced CF disregulation contributes to diastolic filling dysfunction in the aged population. We were able to demonstrate that the diastolic filling dysfunction related to overexpression of TGF-β_1 and ventricular collagen content.

MATERIALS AND METHODS

Animals

Four-, sixteen-, and twenty-four-month old female C57BL/6 mice purchased from Jackson Laboratory, Bar Harbor, Maine were cared for in compliance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985) and "Principles of Laboratory Animal Care" (published by the National Society for Medical Research) and with the prior approval by The University of Arizona Animal Review Committee. The mice were conditioned for 2 weeks in the animal facility before the study.

Groups

The mice were categorized by age as: 4-month-old (young), 16-month-old (aged), and 24-month-old (senescent). In study I, the diastolic function was analyzed in 4- and 16- month-old mice (n = 15/group). The number of mice used per group was determined by our previously reported work on aging in the murine model (8). After the hemodynamic analysis, six hearts from each age group were randomly selected and assayed for collagen content. In study II, six hearts from 4-, 16-, and 24-month-old mice were randomly selected and processed for isolation of CF. RT-PCR was used to compare the mRNA expression of each age group in the cardiac fibroblasts.

Quantification of Ventricular Mechanics

The left ventricular mechanics of Group I was performed before CF isolation. The methods that were used are described in detail by Yang and Larson (8–11). The ventricular mechanics parameters acquired with the Millar Conductance Catheter System are defined as follows. The dP/dt_{min} is preload-dependent derivatives of the isovolemic relaxation. Tau-Weiss (τ) is the parameter that describes the afterload-independent isovolemic diastolic function mediated by SERCA2a and phospholamban. Beta (β) is the slope of the end-diastolic pressure volume relationship and describes the ventricular stiffness ventricular collagen content and type (8).

Fibroblast Isolation

This protocol was provided by Thomas Borg, Ph.D., University of South Carolina to our laboratory and has provided fibroblasts of 88% purity using the CF-specific DDR2 antibody with flow cytometry. The mice were sacrificed using cervical dislocation, and the hearts were quickly removed. The hearts were minced and rinsed in four washes of warm sterile Krebs–Henslett buffer (KHB). A digestion preparation solution was made by dissolving 0.54 mg Liberase 3 enzyme (Roche Biochemical) in 60 mL warm sterile KHB. Ten mL of digestion solution was added to the heart tissue in a 15-mL conical tube and placed in a rotator in the incubator (37°C) for 15 minutes. The tube was vortexed for 30 seconds, the supernatant (containing fibroblasts) was centrifuged at 1000 rpm for 10 min, and resuspended. The first two digestions were discarded, and the subsequent 10 digestions were retained for adherence of the CF to culture flasks. The isolated fibroblast concentrate was plated in a T75 with 20 mL of DMEM/Hepes with 10% FCS medium. After 2 hours, the adherent cells (CF cells) were retained, and the floating cells (noncardiac fibroblast cells) were discarded. At this time, the adherent primary CF cells were processed for RT-PCR for the ex vivo analysis.

Semiquantification of Age-Related IL-10 mRNA Expression in Cardiac Fibroblasts with RT-PCR

Isolated cardiac fibroblasts were processed for determination of differential expression of TGF-β_1. The mRNA was extracted with TRIZOL reagent (Gibco BRL), and mRNA concentrations were determined using a BIO-RAD Biophotometer. RNA was reverse-transcribed (RT) from 5 mg of total RNA in a final volume of 20 μL RT mixture for 2 hours at 37°C. The RT mixture consisted of 4 μL 5 × RT buffer, 1μL dNTPs, 20 U RNAse inhibitor, 3μg random primers, and Superscript RT enzyme. PCRs were performed in a 50 μL reaction volume containing 1 mL cDNA, 5 μL 10 × PCR buffer, 1 mL 10 mM dNTPs, 2 μL 50 mM $MgSO_4$, 1 μL 10 mM of TGF-β_1 and 1 U Taq DNA polymerase. The murine TGF-β_1 primers were purchased from Integrated DNA Technologies, Coralville, IA. The RT-PCR was quantified with 18s rRNA as an internal standard using primers and competimers purchased from Ambion, Austin, TX 78744-1832.

Linearity of TGF-β_1 was determined before assay using cycles between 24 and 32. A hot start was applied for 4 minutes at 95°C, and the cycle (denaturation step at 95°C for 30 sec, annealing step at 60°C for 30 sec, and an elongation step at 72°C) was repeated for 30 cycles. PCR products were then analyzed by horizontal electrophoresis in a 1% agarose gel. The gels were photographed on a UV light source and analyzed with a Bio-Rad GS-800 Calibrated Densitometer.

Collagen Assays

The lower third of the left ventricle was removed for collagen content determination. This apical portion did not have valve or vascular tissues that would falsely increase the collagen content analysis. Collagen content provides an indication of CF synthesis and degradation of collagen. Undenatured collagens were measured in tissue homogenates using our established Sircol collagen assay (Biocolor Ltd., Belfast, Ireland) following the Biocolor Ltd. package insert method. Briefly, test samples were mixed gently in 1.0 mL of 0.1% picrosirius red for 30 minutes at room temperature. During this time, the picrosirius red dye binds with collagen, and the collagen-dye forms a precipitate. The samples were then centrifuged for 5 minutes at 10,000 *g*, and the supernatant discarded. The pellet was resuspended in 1 mL of 0.5 M NaOH, and the absorbance of the sample was read at 550 nm. Total protein concentration was determined with a BCA kit (Pierce, Rockford, IL). Test samples were compared to a standard curve of known concentrations of collagen and reported as percentage of total cardiac protein.

Statistical Analysis

Analysis of variance (ANOVA) with multicomparison procedures was used to test the differences among the defined groups with SPSS version 11.5. The differences were calculated with a significance level of (0.05) and power (0.8). Values obtained from treatment groups were compared to control values using the Student's *t*-test. Comparable nonparametric tests (Kruskal–Wallis and the rank sum test) were substituted when tests for normality and equal variance failed. All data are reported as means ± standard error of the mean.

RESULTS

Table 1 compares the diastolic hemodynamic parameters of the young and old mice. The dP/dt_{min} was reduced by 35% ($p < .05$) in the older mice compared to the young. However, dP/dt_{min} can be influenced by preload and afterload (8) and, therefore, tau (τ) and beta (β) were determined to define the regional portion of the diastolic curve of a pressure–volume loop. The isovolumic relaxation phase of the pressure–volume loop is described by τ, and the stiffness of the left ventricle during the left ventricular filling phase is described by β.

Table 1. The left venctricular diastolic function and collagen content related to age.

Parameter	Unit	*n*	Young	Old-WT
Age	months	15	4 to 6	16 to 17
Body Weight	grams	15	32.05 ± 0.94	36.12 ± 0.62*
Heart Rate	bpm	15	567 ± 9	532 ± 16
dP/dt_{min}	$mmHg*sec^{-1}$	15	–7991 ± 977	–5200 ± 479*
Tau-Weiss	msec	15	5.66 ± 0.27	7.08 ± 0.40*
Beta (β)	mmHg/μl	15	0.30 ± 0.05	0.53 ± 0.10
Collagen Content	% total protein	6	9.5 ± 4.0	16.4 ± 2.3*

dP/dt_{min} = Maximum derivative of change in diastolic pressure over time.
Tau (τ) weiss = Time constant of isovolumic relaxation.
Beta (β) = Slope of end-diastolic pressure-volume relationship.
*$p < .05$.

The parameter τ, a measurement of ventricular relaxation during the phase, when no volume is being transferred from the left atrium into the left ventricle, is normally independent of preload and afterload. There was a 25% ($p < .05$) increase in time (msec) of τ that suggests a decrease in the rate of calcium reuptake by the SERCA2a calcium cycling protein in the older mice as compared to the younger. More striking was a 76% ($p < .05$) increase in ventricular stiffness as described by β in the older mice as compared to the younger. The increase in β corresponded to an increase in left ventricular collagen content of 73% ($p < .05$) when comparing the older mice with the younger. The increased cardiac collagen content in the aged is consistent with increased left ventricular stiffness and accounts for the most significant contributing factor to diastolic filling dysfunction in the aged group.

Figure 1 shows that there was an age-related increase in TGF-β_1 expression by the CF. These data suggest that a localized increase in TGF-β_1 mRNA expression by the CF may be associated with the increased collagen content and increased ventricular stiffness in the aged.

DISCUSSION

These gene-structure-function relationships support our hypothesis that CF dysregulation accounts for diastolic filling dysfunction in the aged. The overexpression of the TGF-β_1 gene was demonstrated in the aged mouse. The ventricular collagen content increased with age that seemed to be related to the decrease in diastolic function. These relationships suggest that TGF-β_1 stimulation of CF collagen formation leads to a reduction in cardiac diastolic function.

TGF-β_1 has regulatory effects on a broad spectrum of cell types. It is a multifunctional cytokine, acting by both autocrine and paracrine mechanisms. This molecule is both a stimulator and inhibitor of cellular replication, and it possesses the capacity to control the production of many components of the ECM (12). The cytokine TGF-β_1 has been shown to correlate with the increase in collagen content of the heart (13). The basis for understanding TGF-β_1 in the regulation of collagen formation in the heart resides in wound healing (14,15). TGF-β_1 has been implicated in the development of interstitial fibrosis in cardiac hypertrophy (13,16,17). The regulatory roles of TGF-β include stimulation of the ECM, decreasing the matrix metalloproteinase (MMP) expression, and increasing the expression of inhibitors of MMP (TIMP) (18). A consequence of

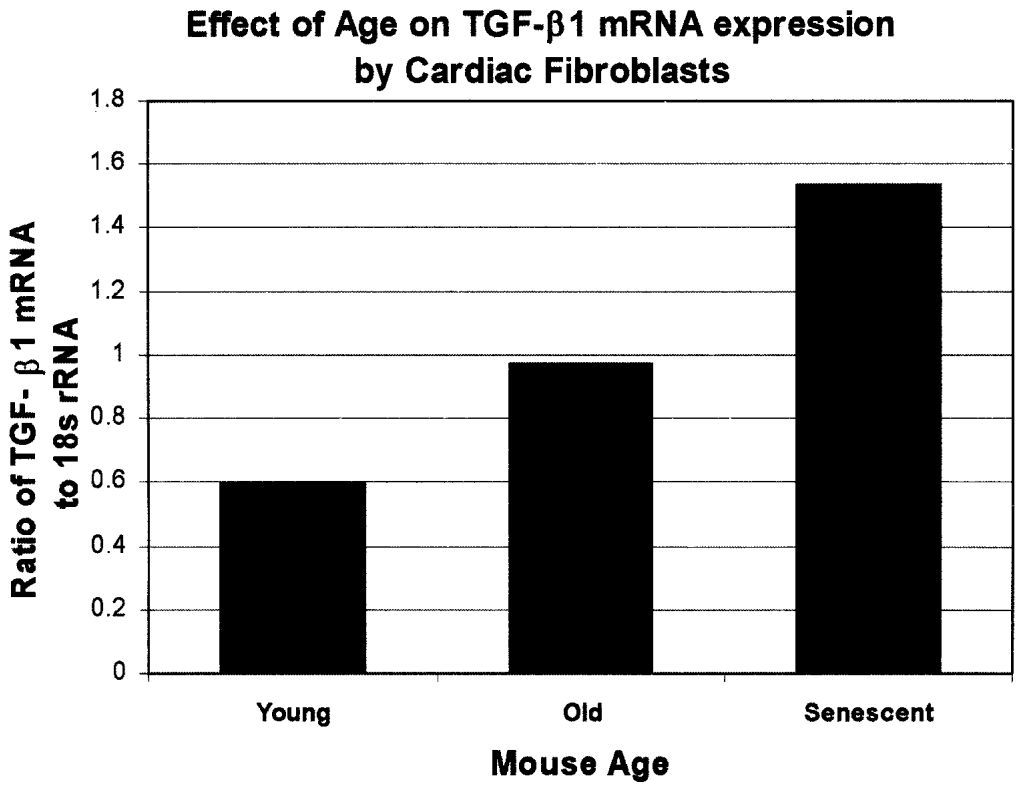

Figure 1. RT-PCR of cardiac fibroblast mRNA for TGF-β_1. This graph represents the RT-PCR performed three times where there was a markedly increased expression of TGF-β_1 by primary cardiac fibroblasts related to age. This gene expression is consistent with the increase in ventricular structural collagen and the functional parameter β.

the increased TGF-β activity could be an increased collagen formation in the heart. Moreover, TGF-β induces a phenotypic change in the fibroblast to myofibroblasts (MyoFb), and these α-smooth actin-expressing cells induce collagen matrix contraction (19). An important issue arises: in the environment of higher concentrations of TGF-β_1 in the heart, a conversion of CF to MyoFb, would the increased numbers of MyoFb cause collagen contraction and a second mechanism for a less compliant diastolic function of the heart? This MyoFb concept is supported by the report that TGF-$\beta_{1,3}$ markedly induce fibroblast 3D collagen lattices (20). The TFG-β_1 activation of MyoFb has been shown to be associated with maladaptive remodeling subsequent to a myocardial infarction (14). Thus, our demonstration that the secretory dysfunction of the CF may induce an autocrine and paracrine overexpression of TGF-$\beta_1$that accounts for the increased collagen content in the left ventricle of the aged. Clearly, further studies must be performed to characterize the MMP and TIMP activities in addition to the ratio of Types I and III collagen.

CFs are the cellular source of collagen for the ECM, therefore, the CF function is the main determinant of ventricular stiffness (17). The major types of collagen in the heart are Types I and III, and Type I is the thicker form that provides the tensile strength where as Type III fibers are thin and provide elastic properties of the heart. In the heart, 80% of the collagen is Type I and about 11% is Type III (4). Therefore, the relationship among CF gene expression, collagen content, and ventricular diastolic stiffness is demonstrated in this study.

There is substantial evidence that there is a modified fibroblast function related to aging. Direct evidence of changes in fibroblast function with age was described using microarray analysis (21). Shelton et al. showed that replicative senescent fibroblasts demonstrated higher levels of matrix-regulating proteins and inflammatory mediators when compared with early-passage fibroblasts (21). Mogford et al. demonstrated that aged human fibroblasts have a markedly reduced migratory capacity in response to hypoxia as compared to younger dermal fibroblast (22). A study of tissue injury demonstrated that MyoFb harvested from aged rats caused an increased gel contraction as compared to younger rats (23). Evidence for age-related effects on wound healing and fibroblast function have been achieved from observations without adjustment for other factors than age (24). These reports of rodent and human fibroblast function in the aged provide only indirect evidence that chronological age senescence may apply to our understanding of the cardiac fibroblast.

Diastolic filling dysfunction in the aging is associated with echocardiographic parameters, such as decreased peak velocity of early diastolic mitral inflow, increased isovolumic relaxation time, and early diastolic deceleration time (25,26). The abnormal left ventricular diastolic function can lead to appreciably elevated left atrial pressures (27). Impaired diastolic function is also related to

diabetes (28). Conversely, without apparent cardiac disease, such as coronary disease, hypertension, and systolic dysfunction, the isovolumic relaxation remains normal in adults up to the eighth decade (29). The isovolumic relaxation corresponds to our measured τ parameter that is independent of the filling phase and in ECM composition. These clinical reports suggest that diastolic filling dysfunction occurs related to aging but is not associated with the isovolumic relaxation phase of diastole. Moreover, overexpression of TGF-β_1 has been associated with hypertension and end organ damage especially in patients who have specific genotypes of the promoter region of the TGF-β_1 gene (30,31). Overexpression of TGF-β_1 has also been associated with allograft diastolic dysfunction in heart transplantation patients, affecting survival of the graft (32). These clinical reports support our findings that there is stiffening of the left ventricle related to aging that may be attributable to CF dysfunction.

CONCLUSION

Diastolic function is related to SERCA2a myocyte content and the concentration and types of collagen in the extracellular matrix. We have shown that the diastolic filling dysfunction associated with chronic myocardial stiffness may be caused by CF dysfunction leading to increased collagen content in the extracellular matrix. As more cardiac surgical procedures are being performed on elderly patients, an understanding of the accompanying cardiac function is fundamental for successful outcomes.

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