Cardiac Inflammation Contributes to Changes in the Extracellular Matrix in Patients With Heart Failure and Normal Ejection Fraction

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Background—The pathophysiology of heart failure with normal ejection fraction (HFNEF) is still under discussion. Here we report the influence of cardiac inflammation on extracellular matrix (ECM) remodeling in patients with HFNEF.

Methods and Results—We investigated left ventricular systolic and diastolic function in 20 patients with HFNEF and 8 control patients by conductance catheter methods and echocardiography. Endomyocardial biopsy samples were also obtained, and ECM proteins as well as cardiac inflammatory cells were investigated. Primary human cardiac fibroblasts were outgrown from the endomyocardial biopsy samples to investigate the gene expression of ECM proteins after stimulation with transforming growth factor-β. Diastolic dysfunction was present in the HFNEF patients compared with the control patients. In endomyocardial biopsy samples from HFNEF patients, we found an accumulation of cardiac collagen, which was accompanied by a decrease in the major collagenase system (matrix metalloproteinase-1) in the heart. Moreover, a subset of inflammatory cells, which expressed the profibrotic growth factor transforming growth factor-β, could be documented in the HFNEF patients. Stimulation of primary human cardiac fibroblasts from HFNEF patients with transforming growth factor-β resulted in transdifferentiation of fibroblasts to myofibroblasts, which produced more collagen and decreased the amount of matrix metalloproteinase-1, the major collagenase in the human heart. A positive correlation between cardiac collagen, as well as the amount of inflammatory cells, and diastolic dysfunction was evident and suggests a direct influence of inflammation on fibrosis triggering diastolic dysfunction.

Conclusions—Cardiac inflammation contributes to diastolic dysfunction in HFNEF by triggering the accumulation of ECM. (Circ Heart Fail. 2011;4:44-52.)

Key Words: collagen ■ inflammation ■ remodeling ■ diastolic dysfunction

Patients with heart failure with normal ejection fraction (HFNEF) have increased mortality,1–4 and morbidity has also been found to be higher compared with patients with HF and reduced EF.5 One of the underlying mechanisms leading to the clinical symptomatology in patients with HFNEF is diastolic function abnormality with increased diastolic stiffness, but nondiastolic function abnormalities with exercise-induced changes in systolic velocity, chronotropic incompetence, and ventricular-vascular uncoupling have also been demonstrated to contribute to this disease.6–17 Despite the growing prevalence of HFNEF in the past 15 years, a disease affecting about half of the HF population, knowledge about its molecular mechanisms is still limited, especially because the pathways leading to HFNEF are not confined to 1 pathobiology. Intracellular changes with elevated cardiomyocyte resting tension, as well as a shift in titin isoforms, are important in patients with severe HFNEF.19,20 Furthermore, accumulation of cardiac collagen was shown to be present in this disease and to contribute to the aggravation of diastolic function.21–23 Enhanced endothelial migration of inflammatory cells into the myocardium might influence the development of these changes, especially in regard to changes in the extracellular matrix (ECM).24,25

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We hypothesized that inflammation is 1 important trigger of cardiac fibrosis and therefore it plays an important role in HFNEF. We show herein that increased inflammation triggers collagen accumulation in HFNEF patients and that both
inflammation and fibrosis are correlated to diastolic dysfunction. We show that inflammatory cells express transforming growth factor (TGF)-β, which directly induces changes in cardiac fibroblasts as increasing collagen accumulation and decreasing the degradation system (matrix metalloproteinase [MMP]-1 and their tissue inhibitor [TIMP]). This was associated with a transdifferentiation of cardiac fibroblasts to myofibroblasts, known to be a potent contributor of pathological remodelling in diseased tissues. These findings suggest that cardiac inflammation by increased transendothelial migration is a potent trigger of diastolic dysfunction in the HFNEF population.

Methods

Study Population

Patients presenting with New York Heart Association class II and III HF, an EF >50%, and diastolic dysfunction on echocardiography were evaluated for participation in this study. Persistent atrial fibrillation and pulmonary diseases were excluded. Significant coronary artery disease or heart valve diseases were ruled out by angiography. Clinically relevant parvovirus B19 infection with a genome equivalent >500 was ruled out in all patients included in the study.26 Twenty patients met the inclusion criteria, were enrolled, and are referred to as the HFNEF group, with respect to recent guidelines.27 In addition, 8 patients without signs of congestive HF who underwent coronary angiography for evaluation of chest pain were also enrolled in this study and served as controls. In all patients, endomyocardial biopsy samples were obtained the day after hemodynamic function was analyzed. Cardiac conditions were stable before catheterization in all patients. All patients gave informed, written consent. The research protocol was approved by the local institutional review board.

Pressure-Volume Measurements and Echocardiography

The conductance catheter was used to assess pressure-volume measurements in all patients, as recently described in more detail.9 In brief, left ventricular (LV) diastolic function was characterized by LV end-diastolic pressure and isovolumetric relaxation (τ). Furthermore, we calculated the exponential curve fit to the diastolic LV pressure-volume points during a transient preload reduction to determine the load-independent diastolic stiffness constant (LV stiffness constant β). We analyzed LV end-systolic pressure and EF as a parameter of systolic function. The slope of the end-systolic pressure-volume relation was calculated as a load-independent parameter for cardiac contractility. Mitral and pulmonary venous Doppler flow velocities were recorded in the apical 4-chamber view with a VingMed System FiVe (GE Healthcare, Chalfont St. Giles, UK) as well as the LV filling index by the ratio of transmitral flow velocity to annular velocity (E/E’ lateral), as previously described.7 Right ventricular systolic pressure (in mm Hg) was measured before the endomyocardial biopsy samples were taken.

Endomyocardial Biopsy

Endomyocardial biopsy samples were obtained from the right side of the ventricular septum of each patient with use of a flexible bioprobe (Westmed) via the femoral vein approach. Right ventricular systolic pressure was measured. The tissue pieces were frozen in LN2 and stored at −70°C for subsequent analysis.28 From all patients, I endomyocardial biopsy sample was used for immunohistological staining, and 1 was used for measuring gene expression. (All measurements were performed once, n=20 in HFNEF and n=8 in controls.)

Figure 1. Box-and-whisker plots showing increased levels of collagen types I and III proteins in endomyocardial biopsy samples from patients with HFNEF compared with controls. Increases in the collagen I to III ratio as well as collagen type I C-telopeptide were measured in serum. The mRNA abundance of collagen types I and III was increased in HFNEF compared with control subjects. AF indicates area fractions. *P<0.05 vs controls.

Cell Culture

Primary cardiac fibroblasts were obtained outgrowth from biopsy specimens from 5 HFNEF patients and were incubated in Iscove’s basal medium (Biochrom AG, Berlin, Germany) containing 10% human serum, 10% fetal calf serum (FCS), 100 U/mL penicillin (Biochrom AG), and 100 μg/mL streptomycin (Biochrom). The cardiac fibroblasts were seeded in 24-well plates, and when the culture was ~80% confluent, they were serum-starved in Iscove’s basal medium containing 0.5% FCS (PAA, Colbe, Germany) for 16 hours. They were then stimulated with 5 ng/mL TGF-β1 (PeproTech, Hamburg, Germany) for 6 or 24 hours compared with no stimulation for 6 or 24 hours. All experiments were done with cells between the second and fourth passage with n=6 wells per experiment for all time points and all patients. For each patient, a mean was calculated from the 6 cell culture wells for each experiment (basal conditions and stimulated for all time points), and the means of all patients are presented in the figures.

Native fibroblasts were stained with antibodies against CD31, desmin, vimentin, P4HB, and α-smooth muscle actin and compared with H1L1 (experimental atrial cardiomyocytes) as well as with endothelial cells. The monocyte cell line THP-1 was cultured in RPMI 1640 medium (PAA) containing 10% FCS (PAA), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA).

Type I Carboxy-Terminal Telopeptide of Collagen and Propeptide of Procollagen Type I in Serum

Serum was collected 1 hour before invasive measurements, and type I carboxy-terminal telopeptide as a breakdown product of collagen type I, as well as procollagen type I as a marker of type I collagen synthesis, was measured as described previously.29

RNA Isolation

Total RNA was extracted from the myocardial biopsy samples or cell culture wells by the Trizol method (GIBCO BRL, Carlsbad, Calif).
Additional purification was performed with the ChargeSwitch total RNA cell kit (Invitrogen, Karlsruhe, Germany). The yield of purified total RNA was analyzed by checking the UV absorbance at 260 nm on a NanoDrop ND-1000 (Agilent Technologies, Boeblingen, Germany) spectrophotometer.

Real-Time Reverse Transcription–Polymerase Chain Reaction

The ABI-inventoried TaqMan gene expression assays (each included forward and reverse primers as well as a fluorescently marked probe) used for preamplification (from the biopsy samples) and for the real-time reverse transcription–polymerase chain reaction were obtained from Applied Biosystems. Prior real-time reverse transcription–polymerase chain reaction cDNA samples (1 to 250 ng) of the biopsy samples were preamplified with pooled gene expression assays with the TaqMan PreAmp Master Mix (early access) in a final volume of 25 µL. Quantification of housekeeping CDKN1B transcripts as an internal control for the amount and quality of cDNA was performed for all samples. Gene expression for collagen types I and III was analyzed from cardiac biopsy samples (primers from Applied Biosystems). Data for cell culture were also normalized to human CDKN1B mRNA levels as an endogenous control (unaffected by TGF-β treatment) and are expressed relative to untreated controls according to the formula $2^{-\Delta\DeltaCT}$.

Figure 2. Decreases in protein levels of MMP-1 and increases in levels of MMP-2 and TIMP-1 were found in endomyocardial biopsy samples from patients with HFNEF compared with controls. Increased oxidative stress by DHE staining was documented in HFNEF compared with control subjects. AF indicates area fractions. *$P<0.05$ vs controls.

Histology

Immunohistological staining was performed by standard techniques. The antibodies were purchased from Chemicon (anti-MMP1, CD3, CD11a, CD45, vascular cell adhesion molecule [VCAM]-1, and collagen types I and III) and Calbiochem (anti-TIMP-1 and anti–MMP-2). Immunohistochemical staining images were quantified by digital image analysis. For double immunofluorescence, we used Tissue Tec–embedded cryosections. The following primary antibodies were used: mouse anti–α-sarcocrem actin (1:50, Santa Cruz) and mouse–anti–TGF-β (1:50, Serotec). The secondary antibodies were labeled with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate (Sigma, St. Louis, Mo). Nuclei were visualized with 4',6-diamidino-2-phenylindole (Sigma). Slides were embedded in fluorescent mounting medium (DakoCytomation, Glostrup, Denmark), coverslipped, and analyzed by fluorescence microscopy (Zeiss, Jena, Germany). Sections were incubated with 10 µmol/L dihydroethidium to detect O$_2^-$ and visualized by fluorescence microscopy, whereby DHE-positive nuclei were counted with respect to the number of total nuclei.

Statistical Analysis

Data are shown in box-and-whisker plots (Figures 1 through 3) and as mean±SEM (Figures 4 and 5). For comparison of the HFNEF

Figure 3. Increased numbers of CD3$^+$, CD11$^+$, and CD45$^+$ cells were found in endomyocardial biopsy samples from patients with HFNEF compared with controls. *$P<0.05$ vs controls.
group with the control group, the nonparametric Mann–Whitney U test was used for data that were not normally distributed. Fisher’s exact test was used to analyze categorical variables. A probability value $<0.05$ was considered statistically significant. Data were analyzed with Graphpad 5.01 (PRISM, San Diego, Calif) and SPSS (version 15.0; SPSS Inc, Chicago, Ill).

Results

Patient Demographics

Patient characteristics are summarized in Table 1. There were no significant differences between HFNEF patients and controls with respect to age, sex, or body surface area. The prevalence of hypertension and diabetes mellitus was not statistically different between the groups (Table 1).

Hemodynamic Data

Systolic function was not changed in HFNEF compared with controls. In contrast, diastolic dysfunction was evident in the HFNEF group, with an increased LV stiffness constant $\beta$, increased LV end-diastolic pressure, and prolonged isovolumetric relaxation. Echocardiography revealed that the cardiac dimensions of both groups were not significantly different. Diastolic dysfunction with increased $E/E'$ lateral was documented in the HFNEF group. Moreover, significant cardiac hypertrophy was documented in HFNEF compared with control subjects (Table 2). Right ventricular systolic pressure, as 1 parameter of pulmonary hypertension, was not significantly different in HFNEF and controls (29.7; range, 24 to 33 mm Hg, vs 25; range, 21 to 28.5 mm Hg; $P=0.4$).

Figure 4. A, Representative histologic image with double staining of CD11$^+$ cells with the profibrotic growth factor TGF-$\beta$. B, In vitro experiments with phorbol 12-myristate 13-acetate–activated monocytes (THP-1 cells) showed increased production of TGF-$\beta$ mRNA and protein levels of TGF-$\beta$ in a time-dependent manner. *$P<0.05$ vs individual controls.

Figure 5. In vitro cell culture experiments of primary cardiac fibroblasts stimulated with TGF-$\beta$ for 6 or 24 hours. Increased gene expression of $\alpha$-smooth muscle actin (a-SMA), connective tissue growth factor (CTGF), and collagen type I (Col I) were found in stimulated cells compared with unstimulated cells. MMP-1 was decreased after stimulation with TGF-$\beta$. MMP-2 was increased after stimulation. *$P<0.05$ compared to the relative controls.
Type I carboxy-terminal telopeptide, as 1 serum marker for collagen degradation, was increased in the sera of HFNEF patients compared with controls (mean±SEM, 5.9±2.1 vs 2.3±1.4 μg/L; P=0.025). Procollagen type I, a serum marker for collagen production, was also increased in the HFNEF group compared with controls (mean±SEM, 128.9±26 vs 65.1±21 μg/L; P=0.018).

### Fibroblast Cell Culture

After stimulation with TGF-β for 6 or 24 hours, connective tissue growth factor and collagen type 1A1 mRNAs were upregulated compared with controls. The transdifferentiation to myofibroblasts, as suggested by α-smooth muscle actin mRNA expression, was observed after 24 hours of stimulation with TGF-β. MMP-1 was not increased after 6 or 24 hours, but MMP-2 was upregulated after 24 hours. With an increased TIMP-1 expression, the MMP-1 to TIMP-1 ratio as 1 indicator of MMP-1 activity was downregulated after stimulation with TGF-β for 6 or 24 hours (Figure 5).

### THP-1 Cell Culture

Activated THP-1 cells produced TGF-β mRNA compared with their nonactivated controls. This was followed by a time-dependent production of TGF-β protein, which showed significantly increased protein levels 24 hours after stimulation of the THP-1 monocytes (Figure 4B).

### Discussion

The salient finding of the current study is that cardiac inflammatory cells documented in endomyocardial biopsy samples from patients with HFNEF induce extracellular remodelling with increased accumulation of collagen. TGF-β, excreted by inflammatory cells, is 1 potent stimulus for the regulatory changes of the ECM, as shown in cell culture experiments with primary cardiac fibroblasts derived from endomyocardial biopsy samples of patients with HFNEF. These changes contribute to diastolic dysfunction, which is 1 of the underlying pathologies of HFNEF, which was documented by a correlation between collagen (as well as inflammatory cells) and diastolic dysfunction.
The molecular changes leading to diastolic dysfunction are still debated, and different pathways can affect the pathology of HFNEF. Altered isoform expression of the giant muscle protein titin can determine the elastic properties of the heart. Moreover, increased myocyte tension, which can be prevented by experimental phosphorylation of titin, contributes to diastolic dysfunction in patients with severe HFNEF. Additional modulatory effects on titin stiffness may arise from disulfide bonding under oxidant stress also affecting LV compliance. Nevertheless, the steep part of the diastolic pressure-volume relation is mainly modified by the ECM, as can be observed when comparing stretch lengths between whole-muscle strips and single myocytes, suggesting that excess collagen might further aggravate diastolic dysfunction.

Cardiac collagen is a stable protein with a low turnover (80 to 120 days), but its balance can be disrupted in pathological conditions. Next to ischemia, which may lead to replacing fibrosis, for example, increased wall stress, angiotensin II, and TGF-β may induce profibrotic processes leading to pathological tissue fibrosis. The tensile strength of collagen type 1 approximates that of steel. Therefore, LV chamber stiffness in vivo will be affected by the amount of cardiac ECM. In this study, we showed that total cardiac collagen content was increased in patients with HFNEF, in agreement with other work that showed increased fibrosis in this disease. Furthermore, not only was collagen type I found to be increased in this study population, but also there was a change in the collagen type I to III ratio in favor of the stiffer collagen type I, similar to that in patients with systolic HF. In addition to increased protein levels of collagen types I and III, their mRNA abundance was also increased in endomyocardial biopsy samples from patients with HFNEF compared with controls. Another marker of excessive collagen production is type I carboxy-terminal telopeptide, a degradation product of collagen with increased collagen turnover together with propeptide of procollagen type I, a serum marker of collagen production, both of which were increased in the HFNEF group. Oxidative stress was increased in HFNEF patients, as DHE staining revealed, which might be the result of increased LV stiffness.

Multiple studies have shown that 1 of the best-known inducers of collagen production is the profibrotic growth factor TGF-β. It also has profound effects on ECM homeostasis, in part through its ability to alter the balance between MMPs and their TIMP inhibitors. The endogenous collagen degradation system is regulated by increased activity of MMPs overcoming their tissue inhibitors. Nevertheless, there are different MMPs, and the substrate affinity of those proteases is different. MMP-1 (interstitial collagenase) is known to degrade collagen fibers, and therefore it likely favors collagen degradation. Through the activity of the activator protein-1 transcription factor, TGF-β can, on one hand, repress MMP-1 gene expression and, on the other hand, increase TIMP-1 expression. Congruent with these ex vivo data, we have show an upregulation of TIMP-1 protein and a downregulation of MMP-1 protein levels in the biopsy samples from HFNEF patients, which leads to a significant decrease in the MMP-1–TIMP-1 ratio. This inhibition of the collagen degradation system could be 1 mechanism contributing to the accumulation of ECM in HFNEF patients and the initiation of diastolic dysfunction over a longer time period. Interestingly, Lopez and colleagues showed that this ratio was increased in patients with systolic HF, whereas it was unchanged in their hypertensive HF group. Whether this process is dynamic or changes with time, or whether this mechanism represents a distinct difference between both HF subtypes remains to be clarified.

Furthermore, and in contrast to the activator protein-1–mediated downregulation of MMP-1, we found increased levels of MMP-2 (controlled by activator protein-2), which is a known gelatinase and has substrate affinity for denatured fibrillar collagen as well as for the basement membrane. Some studies have described MMP-2 serum levels in patients with hypertensive and diastolic HF, but the results are inconsistent, with some studies showing an increase, and others showing no difference or even a decrease in MMP-2. Recently, increased levels of MMP-2 were shown to predict HF in patients with diastolic dysfunction and hypertension. In that study, MMP-2 was a better prognostic marker than the well-known HF biomarker brain natriuretic peptide. Several experimental studies in MMP-2–knockout animals have helped to advance understanding of the molecular function of MMP-2. Matsumura and colleagues showed in MMP-2–knockout mice a decrease in invading inflammatory cells and a decrease in LV rupture after myocardial infarction. Those authors demonstrated that destruction of basement membrane proteins facilitates the transendothelial migration of immunocompetent cells, thereby triggering cardiac inflammation.

In light of such findings, we investigated the number of inflammatory cells in our patients and showed that HFNEF is associated with increased cardiac inflammation, with high numbers of CD3+ , CD11a+ , and CD45+ cells. Moreover, the adhesion molecule VCAM-1, which attracts immunocompetent cells to the endothelium and initiates transendothelial migration, was also increased in the HFNEF group. This result is especially interesting, because VCAM-1 is upregulated by angiotensin II, which may be increased in regard to known risk factors like hypertension and diabetes mellitus in HFNEF. Recently, it was shown that immunocompetent cells like T-cells (CD3+) can indeed alter tissue remodelling in vitro, and we have shown that cardiac inflammation is associated with excessive collagen accumulation in experimental diabetic cardiomyopathy in 1 animal model of HFNEF. There is experimental and clinical evidence that inflammatory cells might modulate cardiac function in HF with reduced EF and normal EF. The direct effects of these cells are still under debate, but it has been suggested that increased inflammation is associated with the development of systolic HF by distinct changes in the ratio of MMP to TIMP. In line with these data, we have demonstrated herein that these inflammatory cells express TGF-β in cardiac tissue, as shown by immunohistochemical double staining. Moreover, THP-1 cells, when they become acti-
vated, which is 1 hallmark of transendothelial migration, produce TGF-β on the mRNA and protein level, as evidenced by in vitro studies of phorbol 12-myristate 13-acetate–activated THP-1 cells. In addition, TGF-β gene expression was increased in the HFNEF biopsy samples. To test the direct effect of TGF-β expressed by inflammatory cells in this study, we performed experiments in a cell culture system with primary human cardiac fibroblasts derived from endomyocardial biopsy samples of HFNEF patients. After stimulation with TGF-β, fibroblasts expressed increased amounts of α-smooth muscle actin, 1 marker for their transdifferentiation into pathologically activated myofibroblasts. Connective tissue growth factor was concomitantly increased, leading to higher collagen gene expression after 6 and 24 hours, which explains the collagen accumulation found in the endomyocardial biopsy samples. On the other hand, the degradation system of collagen accumulation found in the endomyocardial biopsy gene expression after 6 and 24 hours, which explains the basal membrane, this might induce a vicious circle of inflammation triggering fibrosis and ultimately, to progression of the disease.

Conclusions
In this study, HFNEF was characterized by an increase in cardiac inflammation. This inflammatory process triggers cardiac collagen accumulation by inducing collagen gene expression and inhibiting the cardiac degradation system. Inhibiting the transendothelial migration of inflammatory cells into cardiac tissue might be a future therapeutic concept in HFNEF.

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Disclosures
None.

References


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CLINICAL PERSPECTIVE

Heart failure with normal ejection fraction (HFNEF) can be diagnosed in ≈50% of patients with the clinical syndrome of HF. Nevertheless, the HFNEF syndrome seems to be heterogeneous and therefore will present with different pathologies leading to the disease. It is assumed that an accumulation of cardiac collagen is 1 important mediator in the development of HFNEF. We show here that increased transendothelial migration of inflammatory cells triggers a profibrotic phenotype by activating fibroblasts into myofibroblasts in cardiac tissue. Activated myofibroblasts, characterized by α-smooth muscle actin, are well known for excessive collagen production in other diseased tissues and express significantly more collagen compared with normal cardiac fibroblasts. Moreover, inflammatory cells reduced the expression of the collagen degradation system, the matrix metalloproteinases, again promoting cardiac fibrosis as 1 trigger for a stiff and noncompliant ventricle. This transdifferentiation was induced by transforming growth factor-β, which was expressed by inflammatory monocytes in the heart. Interestingly, we document here that these inflammatory cells can be found more frequently in cardiac tissue of the HFNEF group than in the control group. Therefore, continuous low-grade inflammation might be 1 key player in the development and/or progression of HFNEF. This would make anti-inflammatory strategies inhibiting transendothelial migration of cells into the cardiac tissue an interesting approach in treating HFNEF, a disease plagued by limited data.
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