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# Mechanical Unloading During Left Ventricular Assist Device Support Increases Left Ventricular Collagen Cross-Linking and Myocardial Stiffness

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**Background**—Left ventricular assist devices (LVADs) induce reverse remodeling of the failing heart except for the extracellular matrix, which exhibits additional pathophysiological changes, although their mechanisms and functional consequences are unknown.

**Methods and Results**—Hearts were obtained at transplant from patients with idiopathic dilated cardiomyopathy (DCM) not requiring LVAD support (n=30), patients requiring LVAD support (n=16; LVAD duration, 145±33 days), and 5 nonfailing hearts. Left (LV) and right ventricular (RV) ex vivo pressure-volume relationships were measured, and chamber and myocardial stiffness constants were determined. Myocardial tissue content of total and cross-linked collagen, collagen types I and III, MMP-1, MMP-9, TIMP-1, and angiotensin (Ang) I and II were measured. LV size, mass, and myocyte diameter decreased after LVAD compared with DCM without LVAD ( $P<0.05$ ). Total and cross-linked collagen and ratio of type I to III collagen increased in DCM compared with nonfailing hearts and increased further after LVAD ( $P<0.05$  versus DCM and nonfailing). Concomitantly, chamber and myocardial stiffness increased with LVAD. The ratio of MMP-1 to TIMP-1 increased in DCM and almost normalized after LVAD, favoring decreased collagen degradation. Tissue Ang I and II also increased during LVAD. There was no significant change in the RV of LVAD-supported heart compared with DCM.

**Conclusions**—LVAD support increases LV collagen cross-linking and the ratio of collagen type I to III, which is associated with increased myocardial stiffness. Decreased tissue MMP-1-to-TIMP-1 ratio (decreased degradation) and increased Ang levels (stimulants of synthesis) are likely mechanisms for these changes. Lack of significant effects on the RV suggest that hemodynamic unloading of the LV (not provided to the RV) might be the primary factor that regulates these extracellular matrix changes. (*Circulation*. 2005;112:364-374.)

**Key Words:** cardiomyopathy ■ collagen ■ heart-assist devices ■ heart failure ■ metalloproteinases

Left ventricular assist devices (LVADs) provide mechanical support for the end-stage failing human heart and have been used as a bridge to cardiac transplantation. We have demonstrated that LVAD support is associated with normalization of diastolic chamber properties as indexed by the passive pressure-volume relation.<sup>1-3</sup> This normalization of diastolic properties results from a regression of myocyte hypertrophy, including reduced LV mass, wall thickness, and myocyte diameter.<sup>4,5</sup> In addition, LVAD support has been associated with a trend toward normalization in cardiomyocyte function,<sup>6</sup> calcium cycling properties,<sup>7</sup> and expression of various genes.<sup>8</sup>

In addition to changes in intrinsic myocardial properties, LVAD use is associated with changes in the characteristics

and metabolism of the extracellular matrix (ECM). However, unlike almost all other aspects of reverse remodeling of the myocardium and ventricular chamber, the ECM changes do not uniformly reflect a return toward normal conditions.<sup>9-11</sup> Changes in myocardial total collagen content, collagen subtypes, and collagen cross-linking are important features of ECM remodeling, and each has specific implications for changes in passive myocardial properties.<sup>12-14</sup> Responsible for fibrillar collagen denaturation and degradation are matrix metalloproteinases (MMPs), a family of functionally related enzymes that cleave matrix components. Therefore, MMPs and the tissue inhibitors of metalloproteinases (TIMPs) play an important role in regulating ECM turnover. Recent data in animal models of hypertension suggest that especially in-

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**Demographic Data and Medication Before Cardiac Transplantation**

	LVAD (n=16)	DCM (n=30)	P
Age, y	46±12	49±12	NS
Male, %	81.3	66.7	NS
LVAD duration, d	145±33	...	...
Medication at transplantation, n (%)			
Inotropic support	2 (13)	25 (83)	<0.01
β-Blocker	4 (25)	9 (30)	NS
ACE inhibitor	6 (38)	23 (77)	<0.05
Digitalis	4 (25)	22 (73)	<0.05
Diuretics	6 (38)	26 (87)	<0.01

creased myocardial collagen cross-linking, rather than total collagen, contributes to enhanced stiffness.<sup>13,15,16</sup> The effect of LVAD-induced hemodynamic unloading on changes in the ECM properties, especially collagen turnover, has not been investigated. Studies in humans, focused on hemodynamic unloading, primarily used patients after aortic valve replacement,<sup>17,18</sup> heterotopic transplantation,<sup>19</sup> or pharmacological intervention in hypertensive heart disease.<sup>20</sup> In these studies, the unique findings were increased fibrosis and chamber stiffness after treatment. However, no studies specifically link changes in the ECM to alterations in passive properties of the human heart after LVAD support.

To test links between LVAD-induced reverse remodeling and changes in the ECM, passive pressure-volume relationships and changes in myocardial and chamber stiffness were correlated to changes in protein expression and activity of MMP-1, MMP-9, TIMP-1; total collagen; insoluble collagen (cross-linked collagen); and the ratio of collagen subtypes I and III in left (LV) and right ventricular (RV) myocardial samples of patients with dilated cardiomyopathy (DCM) with and without LVAD support. Furthermore, myocardial tissue levels of the profibrotic factors angiotensin (Ang) I and II were measured to assess correlations with changes in cardiac collagen deposition. Examination of these same factors in the RV (a chamber that is not unloaded and does not reverse remodel during LVAD support) was performed to test whether hemodynamic unloading might be the primary mechanism responsible for ECM changes during LVAD support. In addition, we suggest that these changes may contribute to the low incidence of global ventricular recovery and lack of permanence of recovery when it is observed.

**Methods**

This study was performed according to the guidelines of the Declaration of Helsinki. All procedures involving human tissue use were approved by the institutional review board of the New York Presbyterian Medical Center. Hearts were obtained at the time of cardiac transplantation from 30 patients with idiopathic DCM without LVAD support and from 16 transplant patients with DCM after LVAD support for a minimum of 30 days (average duration of LVAD support, 144.8±33.1 days with a HeartMate VE LVAD, Thoratec Corp). Five nonfailing (NF) hearts unsuitable for transplantation were also available. In addition, in 11 of the 16 LVAD patients, we were able to obtain LV myocardial tissue samples just before LVAD implantation (LVAD core). The demographic data for all groups are presented in the Table.

**Passive Pressure-Volume Relationship (End-Diastolic Pressure-Volume Relationship) and Estimation of Chamber and Myocardial Stiffness**

All hearts were perfused with cold, hypocalcemic, hyperkalemic cardioplegic solution at explantation. The passive LV and RV pressure-volume relationships of each arrested heart was measured as described previously.<sup>21</sup> In brief, the aortic root, pulmonary artery, and in the case of LVAD-supported hearts, the LVAD inflow cannula were clamp occluded. A metal adapter was attached to the mitral/tricuspid annulus, and a compliant water-filled latex balloon was placed within the LV and RV chambers. Pressure within each balloon was measured with a high-fidelity micromanometer as volume was progressively increased. Pressure was then plotted as a function of volume at each step, resulting in a passive pressure-volume relationship, equivalent to the end-diastolic pressure-volume relationship (EDPVR) of the beating heart. While this relationship was measured in a given chamber (ie, LV or RV), the contralateral chamber was emptied. The size of each chamber was indexed by the volume at which pressure within the ventricle reached 30 mm Hg (LVV<sub>30</sub> and RVV<sub>30</sub>).

LV and RV ventricular chamber stiffness constant ( $\alpha$ ) was calculated from the ex vivo EDPVRs according to the following equation as described by Mirsky and Pasipoularides<sup>22</sup>:  $P = \beta e^{\alpha(V/V_w)}$ , where P is pressure, V is volume, V<sub>w</sub> is myocardial wall volume (calculated from measured LV mass and assumed density of 1.05 g/mL),  $\alpha$  is the chamber stiffness, and  $\beta$  is a scaling constant.

LV myocardial stiffness ( $\kappa$ ) is the slope of the ln(stress) versus ln(strain) relationship for which myocardial stress and strain are estimated assuming a spherical geometry of the heart with an internal radius (a) and an external radius (b).<sup>22</sup> Midwall stress ( $\sigma_m$ ) and midwall strain ( $\epsilon_m$ ) were calculated according to the following equations:  $\sigma_m = 2.03PV/V_w \{2(b/a)/[1+(b/a)]\}^3$ ,  $\epsilon_m = (1/2)[(3/4\pi V)]^{1/3} (1+b/a)$ , and  $(b/a) = (1+V_w/V)^{1/3}$ . Because there is no accepted, simple analytical approach for estimating RV stresses and strains, no attempt was made to estimate RV myocardial stiffness.

All calculations were performed with custom-programmed commercially available software (Igor Pro 4.01, WaveMetrics Inc).

**Cardiac Tissue Sample Collection**

After measuring the ex vivo passive pressure-volume relationship, we collected myocardial samples from the LV free wall, LV apex, and RV free wall of each heart. The samples were snap-frozen in liquid nitrogen and kept at -80°C for further analysis. Heart samples were also saved for immunohistochemical studies described below. In addition, LVAD core samples from the LV apex were obtained just before LVAD implantation in 11 of the 16 LAVD patients.

**MMP-1, MMP-9, and TIMP-1 Protein Content**

Heart samples were homogenized in 1 mL of lysis buffer (0.05 mol/L Tris-Cl, 150 mmol/L NaCl, 1% Triton, pH 8.0). The homogenate was centrifuged at 3000g for 20 minutes at 4°C, and the supernatant was collected. The supernatant (100 mL) was tested in duplicate for total MMP-1 (free and MMP-1/TIMP-1 complexed), MMP-9 (free and TIMP-1 complexed pro-MMP-9), and TIMP-1 with ELISA kits (Amersham Biotrak) following the manufacturer's instructions. Values of MMP-1, MMP-9, and TIMP-1 are standardized per 1 mg of heart tissue protein.

**Gelatin Zymography and MMP-1 Activity**

Gelatin zymography was performed with myocardial extracts isolated from frozen cardiac samples. Equal amounts of homogenized heart protein were loaded per lane and subjected to 10.0% SDS-PAGE (Bio-Rad apparatus) with 1.0% gelatin (Sigma) in nondenaturing, nonreducing conditions. Gels were washed 3 times in 2.5% Triton X-100 for 20 minutes each time and incubated for 24 hours in 100 mm Tris, pH 7.4, and 1.5 mmol/L CaCl<sub>2</sub> at 37°C, stained with 0.1% Coomassie blue G-250 (Bio-Rad) for 30 minutes in 40% methanol, and then destained in 5% acetic acid until clear gelatinolytic bands were visualized. Band densitometry using Kodak Imageaware software was used to quantify the zymographic results. The

zymogram studies were repeated at least 2 times, and the separately obtained results were combined. All densitometric data are expressed as relative units by dividing the signal intensity by pixel area.

For MMP-1 activity, myocardial samples were pulverized, homogenized in 10 volumes of protein extraction buffer (50 mmol/L Tris, pH 7.4, 1.5 mmol/L CaCl<sub>2</sub>, Triton X-100), and centrifuged for 10 minutes at 2000g to remove cellular debris. The supernatant was assayed for total protein content, and 100  $\mu$ L of each sample was assayed for MMP-1 activity with the MMP-1 Biotrak Activity Assay Kit (Amersham Biosciences) according to the manufacturer's instructions. All measurements were run in duplicate. P-aminophenylmercuric acetate activation was not performed. The levels of activity are reported as nanogram per milliliter per milligram of tissue protein.

### Collagen Characterization

Myocardial collagen can be fractionated into pepsin-soluble and pepsin-insoluble collagens, with the latter reflecting primarily cross-linked collagen. The myocardial pepsin-soluble collagens were extracted overnight with 5 mg/mL pepsin in 0.5 mol/L acetic acid.<sup>23</sup> The soluble and insoluble collagens were separated by centrifugation at 3000g for 10 minutes at 4°C. The soluble collagens included both denatured and undenatured collagens, with the latter quantified by the Sircol collagen assay kit with soluble type I collagen as the standard (Accurate Chemicals).

Total myocardial collagen content was assessed by measuring hydroxyproline content using a modified Stegemann method in hydrolyzed total soluble and insoluble collagens.<sup>24</sup> The extent of collagen denaturation was assessed by the content of undenatured collagen and the ratio of undenatured to total soluble collagens. The ratio of insoluble to total soluble collagen was calculated and used as a measure of cross-linking.

Because the measurement of cross-linked collagen by hydroxyproline is indirect, additional levels of pyridinoline cross-linking were measured in the same samples using Metra Serum PYD EIA kits (Quidel). All samples were diluted 1:10 and 1:20 in assay buffer and then measured following the company's protocol expressed as nanomole pyridinoline per 1  $\mu$ g tissue.

### Ratio of Collagen Type I to III

Heart samples were homogenized in an SDS buffer and centrifuged to pellet the collagen and elastin. Purified lyophilized type I and III standards (2 mg) were added to separate 1-mL aliquots of 70% formic acid, and the sample mixtures were heated. Cyanogen bromide (Sigma-Aldrich Corp) was added, and the samples were deoxygenated by bubbling nitrogen gas through them. After incubation, 1-mL samples were placed individually into 10 000 molecular-weight cutoff Slide-A-Lyzer dialysis cassettes (Pierce Biotechnology Inc) and dialyzed twice against PBS for 12 hours each time. The supernatant samples were concentrated 10-fold with YM-10 Centricon filters (Millipore). Samples mixed 1:1 with 2 $\times$ SDS loading buffer were loaded onto a 15% acrylamide gel (40  $\mu$ L/well) and run for 2 hours at 130 V. The gel was stained with silver (Bio-Rad). The peptides chosen for quantification were  $\alpha_1$ (III)-CB5 for type III collagen and  $\alpha_1$ (I)-CB8 for type I collagen.<sup>25</sup> Densitometric analysis was conducted with Bio-Rad software, and the data are reported as the ratio of type I to type III collagen.

### Immunohistochemical Staining of Myocardial Type I and III Collagen and Myocyte Diameter

Freshly collected LV and RV myocardial samples were immediately fixed in buffered formalin (10%), embedded in paraffin, and mounted on glass slides. Double immunofluorescent staining of type I and type III collagen (Rockland) was performed by routine protocols in our laboratory. A methodical negative control went through every step of the procedure except for incubation with the primary antibody. Images were viewed on a Nikon Optiphot-2 microscope (Nikon Corp) with an MTI 3CCD digital camera (Dage-MTI Inc). Digitally acquired images were analyzed with Image Pro Plus V3.0 analysis software (Media Cybernetics) using color-cube-based selection criteria for positive staining. On each

section, a density range was set to resemble collagen content. The number of pixels included in this range was divided by the total number of pixels in this field. Ten fields at  $\times$ 20 magnification per sample were analyzed, and results were then averaged.

To quantify myocyte diameter, samples were prepared with Masson's trichrome stain. At  $\times$ 20 magnification, 2 orthogonal diameters were obtained per myocyte and then averaged. On average, we analyzed 6 fields per slide per patient. In each field,  $\approx$ 10 myocyte diameters were determined. Only sections cut in cross section were analyzed.

### Ang I and II Myocardial Tissue Levels

Tissue Ang I and II levels were measured by radioimmunoassay after SepPak extraction and high-performance liquid chromatography separation.<sup>26</sup> A known amount of <sup>125</sup>I-Ang I was added as an internal standard before the extraction procedure, and the recovery of <sup>125</sup>I-Ang I after high-performance liquid chromatography separation was used to correct for losses (maximally 20% to 30%) that occurred during extraction and separation.<sup>27</sup>

### Myocardial Force Generation

Myocardial force data were obtained from LV free wall trabeculae as described previously.<sup>6,21</sup> In brief, immediately after cardiectomy, trabeculae <1 mm in diameter were excised and immersed in oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) ice-cold Krebs-Ringer solution with 30 mmol/L 2,3-butanedione monoxime. For the mechanical measurements, muscle strips were mounted in a bath, with one end connected to a force transducer and the other connected to an adjustable-length micrometer gauge using fine steel hooks. During superfusion with 37°C oxygenated Krebs-Henseleit buffer (rate, 1 mL/L; bath volume, 1 mL), trabeculae were stimulated at 1 Hz and allowed to equilibrate for at least 1 hour at slack length. After the resting period, isometric twitches were evoked with stimulation voltage 20% above threshold (duration, 5 ms). The trabeculae were then progressively stretched to the length of maximal force generation ( $L_{max}$ ) at 1-Hz frequency. Adequate muscle oxygenation was confirmed as detailed previously.<sup>6</sup> Myocardial force generation was calculated after normalization to cross-sectional area.

### Statistical Analysis

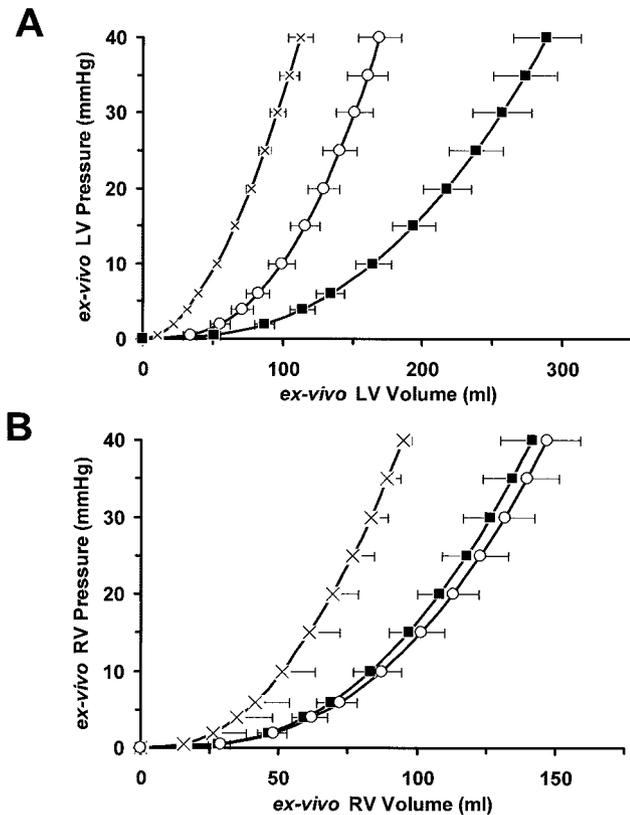
Results are presented as mean $\pm$ SEM. One-way ANOVA was used to select differences between groups, followed by Tukey-Kramer post-hoc testing. A paired *t* test was used to compare paired pre- and post-LVAD samples. The statistical analysis was conducted with a commercially available statistical software package (SPSS 11.5). Values of  $P < 0.05$  were considered statistically significant.

## Results

### LV and RV Ex Vivo Passive Pressure-Volume Relationships and Mass

Ex vivo EDPVRs are shown in Figure 1. A marked difference between NF and DCM hearts is evident in the LV, with the average curve derived from LVAD-supported hearts close to that of NF hearts. Ventricular size, indexed by  $V_{30}$ , is summarized in Figure 2A. The mean  $L_{V_{30}}$  of hearts supported by LVADs for >30 days was significantly smaller than that of DCM hearts without LVAD support ( $P < 0.05$ ) and reached values close to those of the NF hearts. In contrast, there was no significant difference between DCM and LVAD-supported hearts with respect to the RV EDPVR and mean  $R_{V_{30}}$ , indicating that LV unloading is the main trigger (Figures 1 and 2A).

After LVAD support, LV mass was significantly reduced compared with nonsupported DCM hearts ( $P < 0.05$ ; Figure 2B) and reached values similar to NF hearts. RV mass was increased in DCM hearts ( $P < 0.05$  versus NF) but showed no significant reduction after LVAD support.



**Figure 1.** A, LV passive EDPVR for NF (×), DCM (■), and LVAD-supported (○) hearts. After LVAD support, EDPVR is shifted left toward NF hearts. B, RV EDPVR. No change in EDPVR is evident between DCM and LVAD-supported hearts.

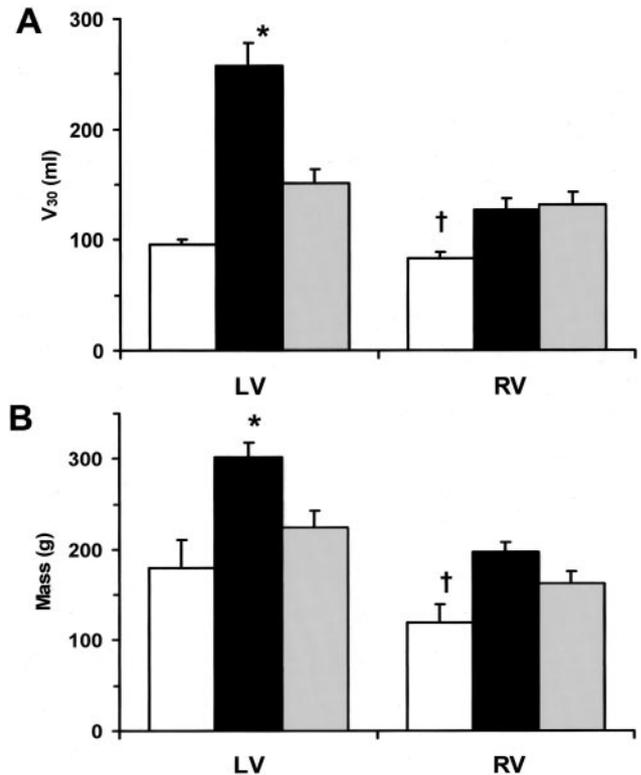
### Chamber and Myocardial Stiffness

The LV dimensionless chamber stiffness constant ( $\alpha$ ) was significantly increased after LVAD support compared with DCM ( $P < 0.05$ ; Figure 3A) and reached an almost normal value. In contrast, RV chamber stiffness constant was decreased significantly in LVAD-supported hearts compared with NF and DCM hearts ( $P < 0.01$ ).

LV myocardial stiffness constant ( $\kappa$ ) increased significantly in DCM compared with NF hearts ( $P < 0.05$ ; Figure 3B). However, after LVAD support, myocardial stiffness increased further ( $P < 0.05$  versus DCM and  $P < 0.01$  versus NF hearts).

### Protein Expression of MMP-1, MMP-9, and TIMP-1

MMP-1 protein expression increased significantly in the DCM group versus NF hearts in both the LV and RV (Figure 4A;  $P < 0.05$ ). After LVAD support, MMP-1 protein expression trended toward lower levels in the LV but did not change in the RV. In contrast, TIMP-1 expression, which was reduced in DCM hearts, increased tremendously during LVAD support, reaching almost normal levels in the RV and levels higher than normal in the LV (Figure 4B;  $P < 0.05$  versus DCM). Hence, the MMP-1-to-TIMP-1 ratio was normalized in the LV ( $P < 0.05$  versus DCM) and trended toward normal in the RV (Figure 4C). MMP-9 protein expression was elevated in end-stage heart failure in both the



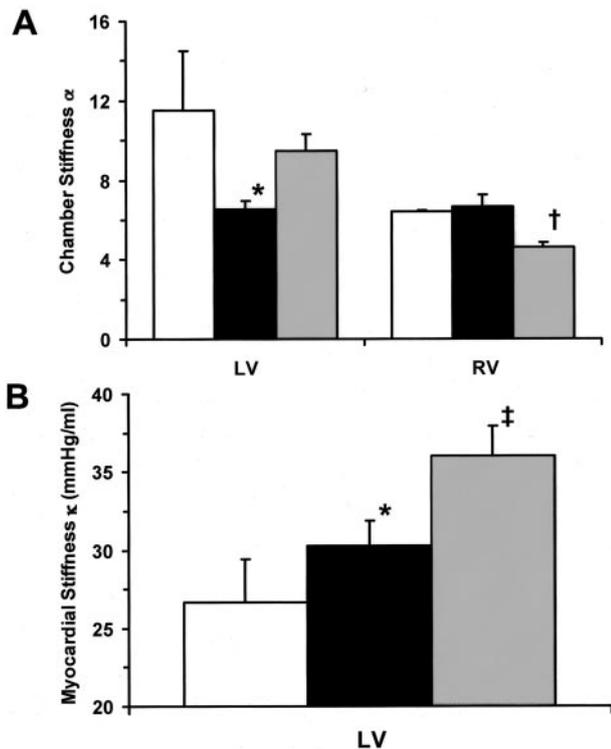
**Figure 2.** A,  $V_{30}$ , ex vivo volume providing pressure of 30 mm Hg, is index of ventricular size.  $LVV_{30}$  was decreased after LVAD support for  $>30$  days and reached values close to NF hearts.  $RVV_{30}$  did not change significantly after LVAD support. B, Significant decrease in mass was evident only in LV. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs LVAD and NF; † $P < 0.05$  vs LVAD and DCM.

LV and RV ( $P < 0.05$  versus NF hearts) and trended to be downregulated in the LV but not RV after LVAD support (Figure 4D).

### Gelatin Zymography and MMP-1 Activity

Gelatin zymography detected 2 major gelatinolytic bands in the myocardial extracts that were generated by MMP-2 and MMP-9 (Figure 5A). MMP-2 gelatinolytic activity was increased in pre-LVAD samples, but did not change after LVAD support in both the LV and RV. In contrast, MMP-9 gelatinolytic activity was significantly decreased in LV samples after LVAD support to almost normal activity ( $P < 0.05$  versus DCM) but did not change in RV samples compared with DCM (Figure 5B). Results of densitometric analysis provided values (in relative, unitless values) for MMP-9 of 34 for NF hearts, 59 for DCM hearts, and 31 for LVAD hearts. For MMP-2, the densitometric relative values are 40, 66, and 35, respectively.

To measure the levels of MMP-1 activity in addition to the MMP-1 protein expression, we used a commercially available kit to quantify MMP-1 activity in paired pre- and post-LVAD samples. In pre-LVAD core samples, MMP-1 activity was elevated, similar to the elevation seen in total MMP-1 content (Figures 4A and 5C). After LVAD support, MMP-1 activity trended to be downregulated in the LV ( $P = 0.08$ ).



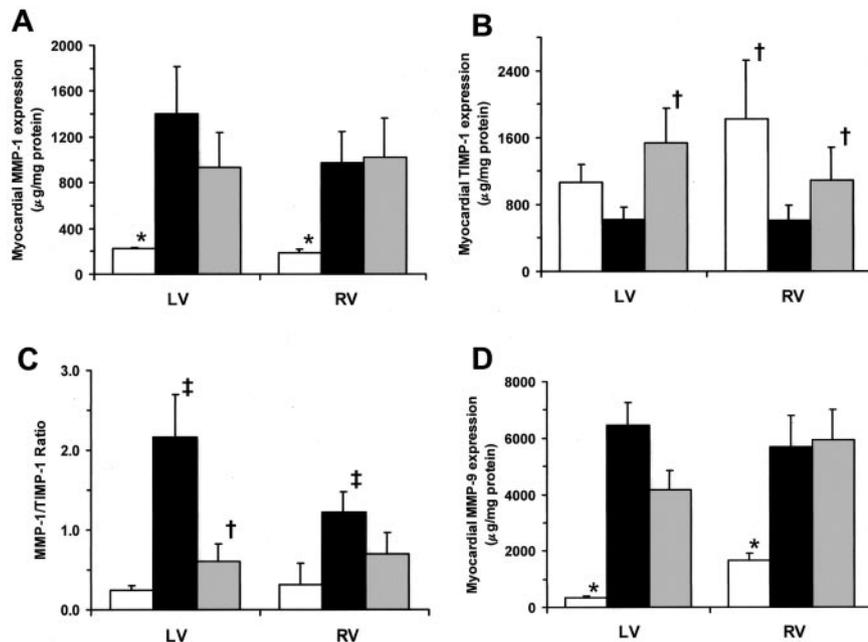
**Figure 3.** A, Dimensionless chamber stiffness constant ( $\alpha$ ) was almost normalized after LVAD support in LV and reduced in RV. B, LV myocardial stiffness constant ( $\kappa$ ) was increased in DCM and increased further after LVAD support in LV. RV myocardial stiffness was not calculated because there is no accepted, simple analytic approach for estimating RV stresses and strains. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs NF and LVAD; † $P < 0.01$  vs NF and DCM; ‡ $P < 0.05$  vs DCM;  $P < 0.01$  vs NF.

**Collagen Content and Cross-Linking**

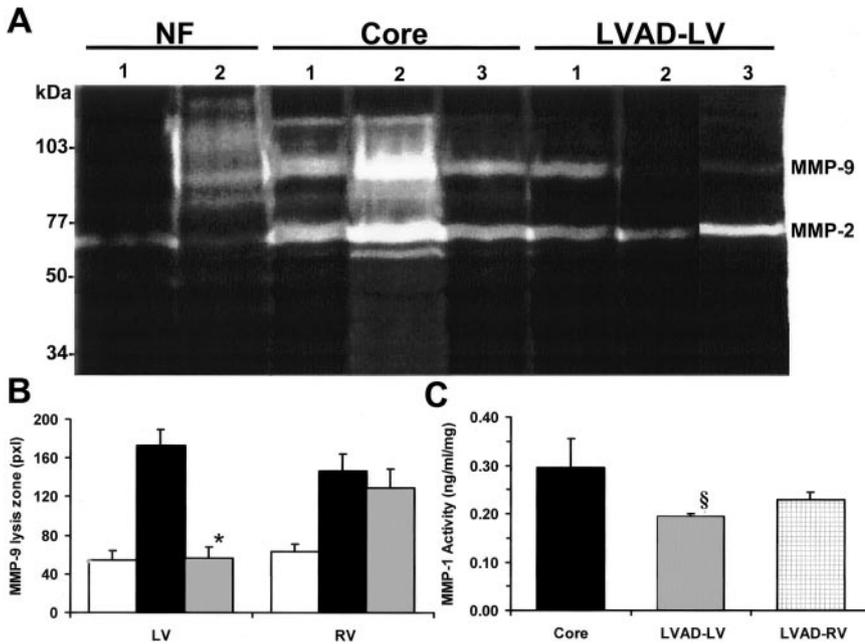
The goal of this study is to compare ECM components and stiffness in the LV and RV after LVAD support. State-of-the-art collagen measurements should be performed in paired

samples from the same patient before and after LVAD implantation. However, because EDPVR measurements and stiffness estimation are possible only from whole hearts and because RV myocardial tissue is not routinely available from pre-LVAD patients, we performed a subanalysis to demonstrate that collagen content in pre-LVAD samples from the LV apex is similar to the DCM apex and free wall samples. In 11 of the 16 patients from the LVAD group, paired samples (pre- and post-LVAD implantation) were available. In 9 of these 11 patients, total and cross-linked collagen increased after LVAD support. There were no significant differences between pre-LVAD samples from the LVAD group and samples from the DCM group (total collagen,  $9.72 \pm 0.40$  versus  $8.73 \pm 0.72$   $\mu\text{g}/\text{mg}$ ; cross-linked collagen,  $2.69 \pm 0.42$  versus  $2.68 \pm 0.25$   $\mu\text{g}/\text{mg}$ , respectively; both  $P = \text{NS}$ ). In addition, total and cross-linked collagen were the same in tissue samples obtained from LV apex and LV free wall in DCM patients (total collagen,  $9.15 \pm 0.59$  versus  $8.47 \pm 0.19$   $\mu\text{g}/\text{mg}$ ; cross-linked collagen,  $2.32 \pm 0.54$  versus  $2.85 \pm 0.40$   $\mu\text{g}/\text{mg}$ , respectively; both  $P = \text{NS}$ ).

Total LV myocardial collagen content (assessed by hydroxyproline) was increased in patients with DCM compared with those with NF hearts ( $P < 0.05$ ; Figure 6A). However, total collagen content increased further after LVAD support ( $P < 0.05$  versus DCM,  $P < 0.01$  versus NF hearts). In particular, cross-linked collagen showed a tremendous increase after LVAD support compared with DCM and NF hearts ( $P < 0.01$  versus DCM and NF hearts; Figure 6B). There was no change in soluble collagen between DCM and LVAD-supported hearts (Figure 6C). To avoid errors resulting from the indirect measurement of cross-linked collagen by the hydroxyproline assay, we also determined collagen cross-links by direct measurement of pyridinoline. Similar to the hydroxyproline measurement, LV pyridinoline cross-linking was increased in DCM compared with NF hearts ( $P < 0.05$ ) and increased significantly after LVAD support ( $P < 0.05$  versus DCM,  $P < 0.01$  versus NF hearts; Figure 6D).



**Figure 4.** MMP-1, MMP-9, and TIMP-1 protein expression in NF, DCM, and LVAD-supported hearts. A, MMP-1 expression is significantly increased in DCM hearts, and is slightly decreased after LVAD support in LV, but does not change in RV. B, Increase in TIMP-1 protein expression in LVAD-supported hearts in LV and RV. C, Normalization of ratio of MMP-1 to TIMP-1 after LVAD support in LV. D, MMP-9 protein expression tended to be downregulated after LVAD support in LV but not RV samples. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs DCM and LVAD; † $P < 0.05$  vs DCM; ‡ $P < 0.05$  vs nonfailing.



**Figure 5.** Reduced MMP activity in LVAD-supported failing hearts. A, Representative gelatin zymography for MMP gelatinolytic activity in NF, before (core), and after LVAD support (LVAD). MMP-2 did not change, but MMP-9 decreased after LVAD support. B, MMP-9 activity was significantly reduced in LV samples after support but not RV samples. C, MMP-1 activity in paired samples before and after LVAD support for LV (n=15 per group) and RV (n=10). After LVAD support, MMP-1 activity trended to be reduced in LV. \* $P < 0.05$  vs DCM; § $P = 0.08$  vs core.

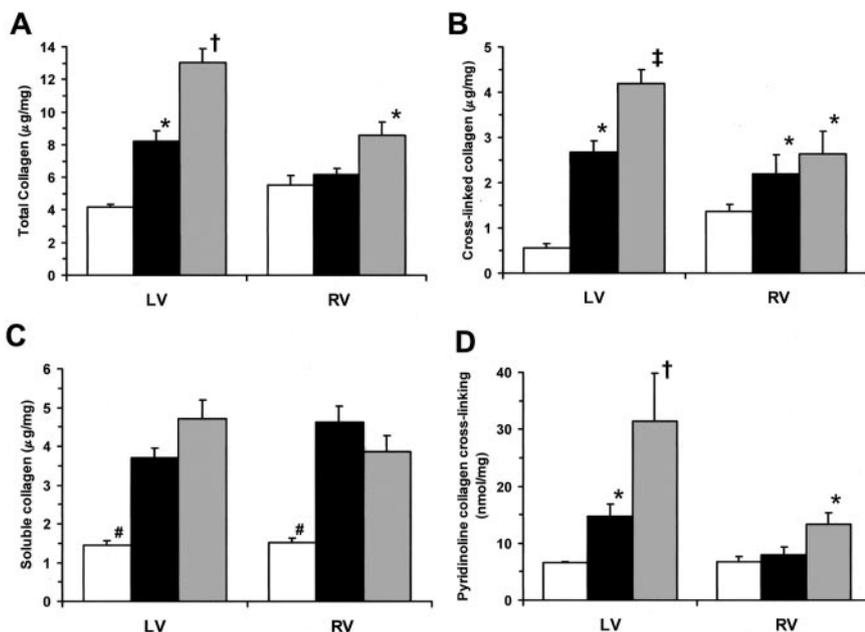
RV total myocardial collagen content increased after LVAD support ( $P < 0.05$  versus NF hearts), although not to the same extent as in the LV. RV soluble and cross-linked insoluble collagen increased in DCM compared with NF hearts ( $P < 0.05$ ) but did not change after LVAD support.

**Immunohistochemical Analysis of Type I and III Collagen and Their Ratio**

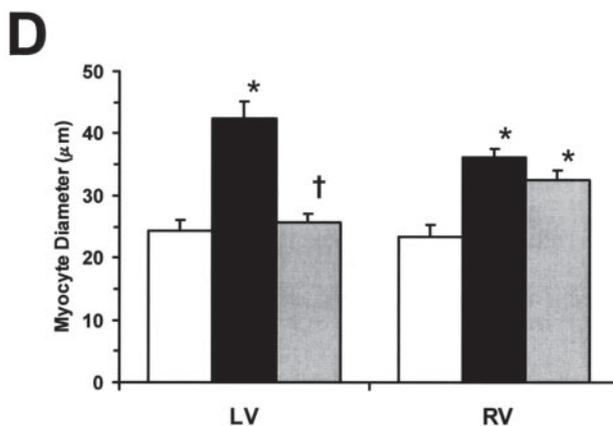
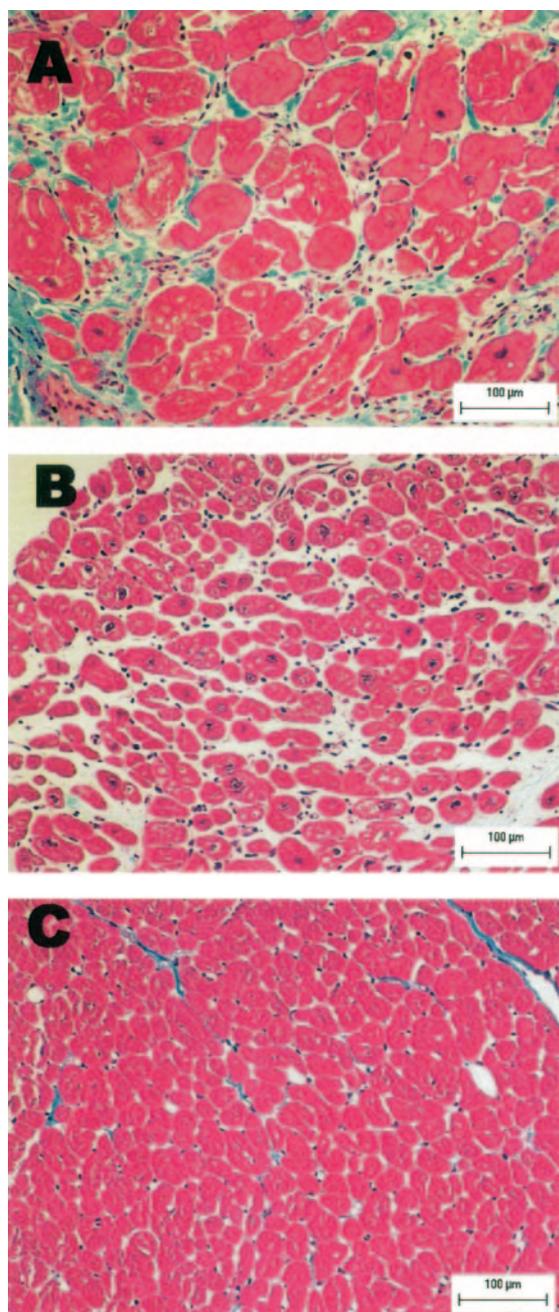
The proportion of total collagen staining positive for type I collagen was increased in the LV myocardium of patients after LVAD support compared with DCM hearts ( $15.8 \pm 7.4\%$  versus  $9.5 \pm 4.7\%$ ,  $P < 0.01$ ; NF hearts,  $0.9 \pm 0.7\%$ ). In RV tissue, the amount of type I collagen was similar in LVAD-supported and DCM hearts ( $8.3 \pm 4.2\%$  and  $7.1 \pm 3.1\%$ , respectively).

The stained area of type III collagen was increased in DCM compared with NF hearts ( $6.4 \pm 4.0\%$  versus  $0.3 \pm 0.2\%$ ;  $P < 0.001$ ) and increased further after LVAD support ( $8.4 \pm 3.6\%$ ;  $P < 0.05$  versus DCM). However, no change in the amount of type III collagen after LVAD support was evident in the RV compared with DCM hearts ( $5.4 \pm 1.6\%$  and  $5.7 \pm 2.3\%$ , respectively).

The ratio of collagen type I to III, determined by cyanogen bromide digestion, trended higher in patients with DCM compared with NF hearts ( $2.72 \pm 0.14$  versus  $2.65 \pm 0.55$ ;  $P = 0.08$ ). After LVAD support, the ratio of type I to III increased further because of a relative increase in type I and a relative decrease in type III collagen ( $3.45 \pm 0.57$ ;  $P < 0.05$  versus DCM and NF hearts). Significant changes between NF, DCM, and LVAD-supported



**Figure 6.** Collagen characterization in NF, DCM, and LVAD-supported hearts. A, Total myocardial collagen content is increased in DCM and increased further after LVAD support. B, Cross-linked collagen increased tremendously after LVAD support in LV samples. C, Soluble collagen was increased in DCM vs NF hearts but did not change significantly after LVAD support. D, Collagen cross-linking measured by pyridinoline assay. Similar to determination by hydroxyproline, cross-linking is increased in DCM and increased further after LVAD support. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs NF; † $P < 0.05$  vs DCM and  $P < 0.01$  vs NF; ‡ $P < 0.01$  vs DCM and NF; # $P < 0.01$  vs DCM and LVAD.



**Figure 7.** Trichrome-stained sections of LV free wall demonstrating myocyte diameter for DCM (A), DCM after LVAD support (B), and NF hearts (C) (original magnification  $\times 10$ ; scale bar =  $100 \mu\text{m}$ ). D, Normalization of myocyte diameter is evident after LVAD support only in LV. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.001$  vs NF; † $P < 0.001$  vs DCM.

hearts in the ratio of collagen type I to III were not evident in the RV.

### Myocyte Diameter

Myocyte diameter increased significantly in DCM hearts compared with NF hearts ( $P < 0.001$ ; Figure 7). After LVAD support, this finding could be almost completely reversed ( $P < 0.001$  versus DCM). However, the enlarged myocyte diameter in the RV in DCM patients could not be reversed to normal after LVAD support.

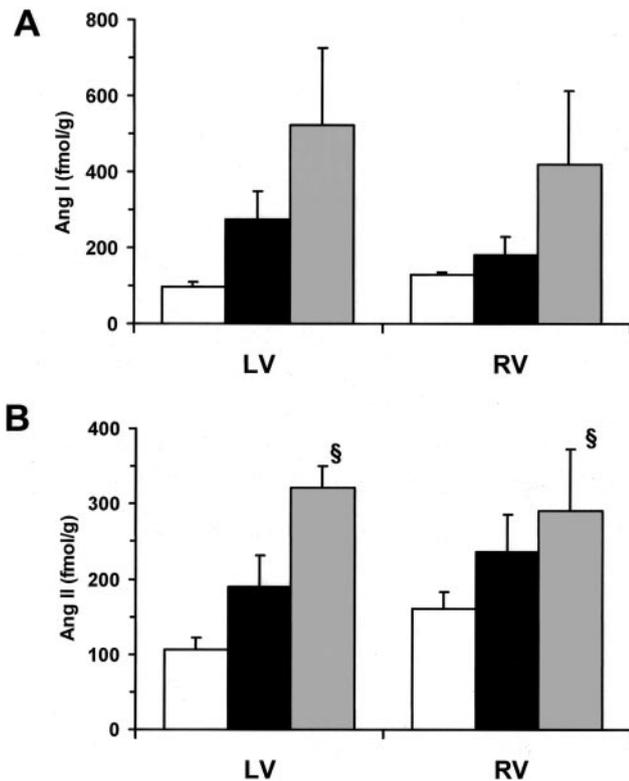
### Ang I and II Myocardial Tissue Levels

Compared with NF hearts, myocardial tissue levels of Ang I and II were elevated in DCM in LV and RV samples (Figure 8). After LVAD support, Ang I and II increased further in

both chambers (Ang II,  $P = 0.094$  versus NF hearts). Levels of Ang I and II in pre-LVAD core samples ( $244 \pm 137$  and  $136 \pm 39$  fmol/g) were similar to levels from the free wall in DCM hearts.

### Myocardial Force Generation

To test whether myocardial force generation is different in the NF, DCM, and LVAD groups, we isolated muscle strips from the LV free wall and placed them in organ baths. The trabecular dimensions were similar among all groups. Interestingly, developed force at 1 Hz in NF, DCM, and LVAD-supported hearts was comparable between groups:  $10.5 \pm 1.0$  ( $n = 5$ ),  $9.2 \pm 1.7$  ( $n = 30$ ), and  $9.2 \pm 2.4$  ( $n = 16$ ) mN/mm<sup>2</sup>, respectively ( $P = \text{NS}$ ).



**Figure 8.** Myocardial tissue levels of Ang I and II in NF, DCM, and LVAD-supported hearts. Ang I and II levels were increased after LVAD support. Open bars indicate NF; solid bars, DCM; gray bars, LVAD.  $\$P=0.094$  vs NF.

## Discussion

Hemodynamic unloading during LVAD support leads to reverse structural, molecular, cellular, and functional remodeling.<sup>2–4,6,9,21,28,29</sup> In addition, collagen content increases after LVAD support, as shown in prior studies.<sup>3,30</sup> Similar findings were discovered after acute reduction in LV afterload in patients with aortic valve replacement, in pharmacological intervention in hypertensive heart disease, and in animal models of heterotopic transplantation.<sup>17–20</sup> Moreover, an increase in diastolic stiffness accompanied this increased collagen deposition in most of these studies.

In the present study, we confirmed that LVAD support leads to reverse structural remodeling of the LV, evidenced by a reduction in LV size, mass, and myocyte diameter and a leftward shift in the ex vivo EDPVR with near normalization of chamber stiffness. However, we extended these results from former studies by characterizing changes in collagen type and quantity by measuring MMP and determining myocardial stiffness.

Whereas chamber stiffness normalized after LVAD support, intrinsic myocardial stiffness increased above the already elevated levels seen in DCM hearts. This finding is compatible with the observed increase in cross-linked myocardial collagen content. A more detailed analysis of extracellular collagen matrix composition revealed that the absolute content of type I and III collagens and the ratio of collagen type I to III were all increased after LVAD support.

Furthermore, MMP-1 and MMP-9 levels and activity increased in the failing state compared with normal and

trended to decrease after LVAD support. In particular, TIMP-1 levels increased tremendously after LVAD support, leading to a normalization of the MMP-1-to-TIMP-1 ratio. This suggests that in end-stage heart failure there is a high rate of collagen breakdown that is reduced after LVAD support, resulting in the overall increase in collagen content that we observed.

Finally, we observed that myocardial tissue levels of Ang I and II, known regulators of myocardial collagen synthesis, showed trends toward further increased levels in the LV after LVAD support.

Additional novel results presented here on RV properties provide insight into mechanisms of the remodeling process during LVAD support. In contrast to the LV, the RV showed no signs of reverse structural remodeling. RV EDPVR, mass, size, and myocyte diameter in hearts supported with LVAD were similar to those of DCM hearts and did not normalize after LVAD support. Despite a small increase in total RV myocardial collagen content, cross-linked collagen, collagen types I and III, the ratio of collagen type I to III, and MMP protein expression and activity did not change compared with the RV of DCM hearts. The different effects on RV and LV properties suggest that the primary mechanism by which LVAD influences ECM properties relates to reduced mechanical stretch by mechanical unloading because LVADs pressure and volume unload the LV but do not reduce (and may even increase) the hemodynamic load on the RV.<sup>31,32</sup> The similar increase in tissue levels of Ang I and II in the LV and RV after LVAD support is not contrary to this proposition of LVAD-induced ECM remodeling but more specifically suggests that cardiac Ang generation depends more on extracardiac (ie, renal) than locally synthesized renin.<sup>33</sup>

The myocardial collagen matrix is considered to be an important determinant of ventricular passive diastolic properties and myocardial structural integrity.<sup>34,35</sup> Consisting mainly of collagen type I ( $\approx 85\%$ ), and collagen type III ( $\approx 11\%$ ), it has a high tensile strength, even though normally occupying only 4% of the extracellular space.<sup>36</sup> However, there is some controversy concerning how collagen content changes after LVAD support. One group reported a decrease in total collagen after mechanical unloading toward normal values,<sup>37,38</sup> whereas most studies showed an increase in collagen content in both humans and animal models,<sup>3,19,29,30,39</sup> which is consistent with our study. An explanation for these discrepancies could be that the former studies used immunohistochemical staining with picrosirius red, whereas the latter group of studies used biochemical assays for tissue collagen content, which in part could explain these discrepancies.<sup>40</sup>

It is believed that the absence of type I collagen leads to a reduction in myocardial contractile force and causes muscle fiber slippage and realignment with thinning of the myocardium, finally leading to chamber dilatation.<sup>35</sup> Conversely, in animal models, an increase in total collagen leads to enhanced myocardial stiffness, causing decreased ventricular elasticity.<sup>34</sup> New evidence demonstrates more specifically that increased cross-linked collagen and a greater amount of the stiffer type I collagen relative to the more elastic type III collagen plays an important role in enhanced myocardial stiffness and chamber remodeling.<sup>13,15,16,41,42</sup> Our study is the

first to examine the changes in collagen isotypes after LVAD support. We demonstrated that above and beyond the already known increase in the ratio of collagen type I to III in end-stage heart failure,<sup>43–45</sup> a further increase is present after LVAD support. Interestingly, the extreme increase in collagen deposition after LVAD support consists almost entirely of elevation of cross-linked collagen and is compatible with the increased myocardial stiffness calculated from the *ex vivo* pressure-volume relationship, chamber size, and myocardial mass. These findings were never documented in humans after LVAD support but are complementary to the findings of hemodynamic unloading after aortic valve replacement.<sup>17,18</sup> In the failing heart, it has previously been demonstrated that normal collagen is degraded by increased MMP activity and replaced by fibrous interstitial deposits of poorly cross-linked collagen, which allows ventricular dilatation, commonly referred to as remodeling.<sup>46</sup> The hypothesis is that these ECM changes occur as a result of increased mechanical stretch to cardiac fibroblasts.<sup>47</sup> Previously, Li et al<sup>10,30</sup> demonstrated that MMP-1 and MMP-9 protein expression is increased in heart failure and that LVAD support was associated with a significant reduction. However, this could be demonstrated only in ICM and not in DCM patients, whereas TIMP-1 and TIMP-3 levels were both significantly elevated after LVAD support in ICM and DCM patients. TIMP-2 and TIMP-4 levels showed no change after LVAD support. TIMPs are believed to bind MMPs in a stoichiometric 1:1 molar ratio.<sup>12</sup> We showed that LVAD support results in a stoichiometric imbalance favoring TIMP-1 activation and a normalization of the MMP-1-to-TIMP-1 ratio. We therefore hypothesize that increased collagen cross-linking after LVAD support could be attributed to decreased degradation of immature collagen, coupled with ongoing production of new collagen. The effect of LVAD support on RV changes of the ECM is different. Because changes in the extracellular collagen matrix and MMP and TIMP protein expression/activity are not evident in the RV after LVAD support, we can conclude that LVAD-induced hemodynamic unloading is the primary factor for these alterations.

Despite the fact that Ang II is a stimulator of fibrosis, it also stimulates myocardial cell growth and hypertrophy.<sup>48,49</sup> Concomitantly, the increased myocardial tissue levels of Ang II are consistent with the increase in collagen content after LVAD support. However, we see a reduction toward normal in myocyte diameter and regression of hypertrophy in patients after LVAD support. This suggests that the hemodynamic effect of unloading the LV overwhelms the effect of Ang II in terms of hypertrophy but not fibrosis. Evidence in mice supports our concept that Ang II could affect fibrosis without inducing hypertrophy.<sup>50</sup>

Although the present study has not examined the clinical implications of the current findings, it is of interest that although almost every other aspect of cardiac properties examined in prior studies returns toward normal during LVAD support, the ECM changes described here show progressive worsening. It is therefore tempting to question whether such changes could be a factor in the extremely low observed rate of full ventricular functional recovery during LVAD support and for the high rate of recurrent remodeling

(ie, progressive deterioration of pump function) that typically occurs after LVAD removal.<sup>28,51–53</sup> For example, could increased interstitial fibrosis after LVAD support interfere with myocyte contractile function as proposed<sup>54</sup> in other settings? Indeed, Rossman et al<sup>55</sup> noted at low rates of stimulation that peak developed force is lower after LVAD support than in the failing state. Although our findings are not identical, we did find no significant change in contractile force in LVAD-supported hearts. One prior study suggested that when studied in isolation, LVAD support significantly improved baseline myocyte contractile strength (ie, strength at low stimulation frequency, not subjected to  $\beta$ -adrenergic stimulation)<sup>5</sup>; it is conceivable that the increased ECM could be limiting the myocardial expression of improved baseline cellular function. Alternatively, LVAD support may not truly improve myocyte function *in vivo*, and alterations of ECM may not influence myocardial contractile strength. Neither the present study nor any prior study can sort out these alternatives.

Additionally, the question as to whether hearts really “redilate” after LVAD explantation remains controversial. Unfortunately, only small single-center experiences are available. Although one center describes redilation after LVAD explantation in selected patients,<sup>28,52</sup> another center reports only a deterioration in ejection fraction without dilation in patients who were successfully weaning but subsequently failed in the long-term follow-up.<sup>56,57</sup> In these reports, the hearts “redilate” to a relatively small amount ( $\approx 8$  to 11 mm) 3 to 39 months after LVAD explanation and did not show any difference between successful and failed long-term weaning. This suggests that increased collagen content and myocardial stiffness may prevent redilation of the ventricle once the heart is exposed to normal filling pressures after LVAD removal but did not prevent deterioration of cardiac function. Although difficult to evaluate, it is possible that the present results have important functional implications as they may relate to the frequency and permanence of LV recovery after LVAD support.

### Study Limitations

Hearts of patients with ischemic cardiomyopathy were specifically excluded from the present study. The reason was that the presence of multiple infarcts in varying degrees of healing would dramatically complicate an analysis of ECM properties and metabolism. The results should therefore not be extrapolated to ischemic cardiomyopathic hearts. ACE inhibitors are known to influence collagen metabolism, and 6 of our 16 patients with LVAD support were taking such drugs. However, analysis failed to demonstrate a difference between patients taking and not taking ACE inhibitors. Because of the relatively small sample size, subtle but potentially important effects could be present. Finally, we analyzed the effect of LVAD support only on MMP-1, MMP-9, and TIMP-1 protein expression/activity and did not include other known proteinases and MMP inhibitors, which may play an important role in ECM remodeling.

### Conclusions

LVAD-induced mechanical unloading of end-stage heart failure hearts is associated with a dramatic shift toward lower volumes of the LV pressure-volume relationship, in combi-

nation with reduced cardiomyocyte diameter, LV mass, and LV size. Changes in MMP-1-to-TIMP-1 protein expression ratio after LVAD support were accompanied by an increased ratio of collagen type I to III and an increase in the total amounts of type I and III collagen. In addition, total myocardial collagen content and importantly collagen cross-linking increased, which we propose as the mechanism of the observed increase in myocardial stiffness. These changes were not evident in the RV, suggesting that reduced myocardial stretch by LVAD-induced hemodynamic unloading is the primary factor from these alterations in the extracellular collagen matrix. Although most abnormal phenotype and genotype changes noted to occur in the setting of heart failure are normalized during LVAD support, these changes in ECM are not totally normal. It is possible that these abnormalities could contribute to the low rate of complete recovery of LV function during LVAD support and the high incidence of deterioration of LV function after LVAD removal. As such, the ECM may be an appropriate therapeutic target for enhancing the incidence of sustained ventricular recovery of the end-stage failed heart.

### Acknowledgment

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# Mechanical Unloading During Left Ventricular Assist Device Support Increases Left Ventricular Collagen Cross-Linking and Myocardial Stiffness

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**Background**—Left ventricular assist devices (LVADs) induce reverse remodeling of the failing heart except for the extracellular matrix, which exhibits additional pathophysiological changes, although their mechanisms and functional consequences are unknown.

**Methods and Results**—Hearts were obtained at transplant from patients with idiopathic dilated cardiomyopathy (DCM) not requiring LVAD support (n=30), patients requiring LVAD support (n=16; LVAD duration, 145±33 days), and 5 nonfailing hearts. Left (LV) and right ventricular (RV) ex vivo pressure-volume relationships were measured, and chamber and myocardial stiffness constants were determined. Myocardial tissue content of total and cross-linked collagen, collagen types I and III, MMP-1, MMP-9, TIMP-1, and angiotensin (Ang) I and II were measured. LV size, mass, and myocyte diameter decreased after LVAD compared with DCM without LVAD ( $P<0.05$ ). Total and cross-linked collagen and ratio of type I to III collagen increased in DCM compared with nonfailing hearts and increased further after LVAD ( $P<0.05$  versus DCM and nonfailing). Concomitantly, chamber and myocardial stiffness increased with LVAD. The ratio of MMP-1 to TIMP-1 increased in DCM and almost normalized after LVAD, favoring decreased collagen degradation. Tissue Ang I and II also increased during LVAD. There was no significant change in the RV of LVAD-supported heart compared with DCM.

**Conclusions**—LVAD support increases LV collagen cross-linking and the ratio of collagen type I to III, which is associated with increased myocardial stiffness. Decreased tissue MMP-1-to-TIMP-1 ratio (decreased degradation) and increased Ang levels (stimulants of synthesis) are likely mechanisms for these changes. Lack of effects on the RV indicate that hemodynamic unloading of the LV (not provided to the RV) might be the primary factor that regulates these extracellular matrix changes. LVAD-induced hemodynamic unloading induced reverse remodeling of the LV by normalizing diastolic pressure-volume relations, myocyte diameter, and mass. However, triggered by a normalized MMP-1-to-TIMP-1 ratio and increased Ang I and II tissue levels, total and cross-linked collagen content increased to values higher than in end-stage heart failure, which leads to a tremendous increase in myocardial stiffness. With the absence of these findings in the RV, LVAD-induced hemodynamic unloading plays the primary role for these alterations of the extracellular collagen matrix. (*Circulation*. 2005;112:&NA;-)

**Key Words:** cardiomyopathy ■ collagen ■ heart-assist devices ■ heart failure ■ metalloproteinases

Left ventricular assist devices (LVADs) provide mechanical support for the end-stage failing human heart and have been used as a bridge to cardiac transplantation. We have demonstrated that LVAD support is associated with normalization of diastolic chamber properties as indexed by the passive pressure-volume relation.<sup>1-3</sup> This normalization of diastolic properties results from a regression of myocyte hypertrophy, including reduced LV mass, wall thickness, and myocyte diameter.<sup>4,5</sup> In addition, LVAD support has been

associated with a trend toward normalization in cardiomyocyte function,<sup>6</sup> calcium cycling properties,<sup>7</sup> and various gene expressions.<sup>8</sup>

In addition to changes in intrinsic myocardial properties, LVAD use is associated with changes in the characteristics and metabolism of the extracellular matrix (ECM). However, unlike almost all other aspects of reverse remodeling of the myocardium and ventricular chamber, the ECM changes do not uniformly reflect a return toward normal conditions.<sup>9-11</sup>

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Changes in myocardial total collagen content, collagen subtypes, and collagen cross-linking are important features of ECM remodeling, and each has specific implications for changes in passive myocardial properties.<sup>12–14</sup> Responsible for fibrillar collagen denaturation and degradation are matrix metalloproteinases (MMPs), a family of functionally related enzymes that cleave matrix components. Therefore, MMPs and the tissue inhibitors of metalloproteinases (TIMPs) play an important role in regulating ECM turnover. Recent data in animal models of hypertension suggest that especially increased myocardial collagen cross-linking, rather than total collagen, contributes to enhanced stiffness.<sup>13,15,16</sup> The effect of LVAD-induced hemodynamic unloading on changes in the ECM properties, especially collagen turnover, has not been investigated. Studies in humans, focused on hemodynamic unloading, primarily used patients after aortic valve replacement,<sup>17,18</sup> heterotopic transplantation,<sup>19</sup> or pharmacological intervention in hypertensive heart disease.<sup>20</sup> In these studies, the unique finding was increased fibrosis and chamber stiffness after treatment. However, no studies specifically link changes in the ECM to alterations in passive properties of the human heart after LVAD support.

To test links between LVAD-induced reverse remodeling and changes in the ECM, passive pressure-volume relationships and changes in myocardial and chamber stiffness were correlated to changes in protein expression and activity of MMP-1, MMP-9, TIMP-1; total collagen; insoluble collagen (cross-linked collagen); and the ratio of collagen subtypes I and III in left (LV) and right ventricular (RV) myocardial samples of patients with dilated cardiomyopathy (DCM) with and without LVAD support. Furthermore, myocardial tissue levels of the profibrotic factors angiotensin (Ang) I and II were measured to assess correlations with changes in cardiac collagen deposition. Examination of these same factors in the RV (a chamber that is not unloaded and does not reverse remodel during LVAD support) was performed to test whether hemodynamic unloading might be the primary mechanism responsible for ECM changes during LVAD support. In addition, we suggest that these changes may contribute to the low incidence of global ventricular recovery and lack of permanence of recovery when it is observed.

## Methods

This study was performed according to the guidelines of the Declaration of Helsinki. All procedures involving human tissue use were approved by the institutional review board of the New York Presbyterian Medical Center. Hearts were obtained at the time of cardiac transplantation from 30 patients with idiopathic DCM without LVAD support and from 16 transplant patients with DCM after LVAD support for a minimum of 30 days (average duration of LVAD support, 144.8±33.1 days with a HeartMate VE LVAD, Thoratec Corp). Five nonfailing (NF) hearts unsuitable for transplantation were also available. In addition, in 11 of the 16 LVAD patients, we were able to obtain LV myocardial tissue samples just before LVAD implantation (LVAD core). The demographic data for all groups are presented in the Table.

### Passive Pressure-Volume Relationship (End-Diastolic Pressure-Volume Relationship) and Estimation of Chamber and Myocardial Stiffness

All hearts were perfused with cold, hypocalcemic, hyperkalemic cardioplegic solution at explantation. The passive LV and RV

### Demographic Data and Medication Before Cardiac Transplantation

	LVAD (n=16)	DCM (n=30)	P
Age, y	46±12	49±12	NS
Male/female, %	81.3	66.7	NS
LVAD duration, d	145±33	...	...
Medication at transplantation, n (%)			
Inotropic support	2 (13)	25 (83)	<0.01
β-Blocker	4 (25)	9 (30)	NS
ACE inhibitor	6 (38)	23 (77)	<0.05
Digitalis	4 (25)	22 (73)	<0.05
Diuretics	6 (38)	26 (87)	<0.01

pressure-volume relationships of each arrested heart was measured as described previously.<sup>21</sup> In brief, the aortic root, pulmonary artery, and in the case of LVAD-supported hearts, the LVAD inflow cannula were clamp occluded. A metal adapter was attached to the mitral/tricuspid annulus, and a compliant water-filled latex balloon was placed within the LV and RV chambers. Pressure within each balloon was measured with a high-fidelity micromanometer as volume was progressively increased. Pressure was then plotted as a function of volume at each step, resulting in a passive pressure-volume relationship, equivalent to the end-diastolic pressure-volume relationship (EDPVR) of the beating heart. While this relationship was measured in a given chamber (ie, LV or RV), the contralateral chamber was emptied. The size of each chamber was indexed by the volume at which pressure within the ventricle reached 30 mm Hg (LVV<sub>30</sub> and RVV<sub>30</sub>).

LV and RV ventricular chamber stiffness ( $\alpha$ ) was calculated from the ex vivo EDPVRs according to the following equation as described by Mirsky and Pasipoularides<sup>22</sup>:  $P = \beta e[\alpha(V/V_w)]$ , where P is pressure, V is volume,  $V_w$  is myocardial wall volume (calculated from measured LV mass and assumed density of 1.05 g/mL),  $\alpha$  is the chamber stiffness, and  $\beta$  is a stiffness constant.

LV myocardial stiffness ( $\kappa$ ) is the slope of the ln(stress) versus ln(strain) relationship for which myocardial stress and strain are estimated assuming a spherical geometry of the heart with an internal radius (a) and an external radius (b).<sup>22</sup> Midwall stress ( $\sigma_m$ ) and midwall strain ( $\epsilon_m$ ) were calculated according to the following equations:  $\sigma_m = 2.03PV/V_w \{2(b/a)/[1+(b/a)]\}^3$ ,  $\epsilon_m = (1/2)[(3/4\pi V)]^{1/3} (1+b/a)$ , and  $(b/a) = (1+V_w/V)^{1/3}$ . Because there is no accepted, simple analytical approach for estimating RV stresses and strains, no attempt was made to estimate RV myocardial stiffness.

All calculations were performed with commercially available software (Igor Pro 4.01, WaveMetrics Inc).

### Cardiac Tissue Sample Collection

After measuring the ex vivo passive pressure-volume relationship, we collected myocardial samples from the LV free wall, LV apex, and RV free wall of each heart. The samples were snap-frozen in liquid nitrogen and kept at  $-80^\circ\text{C}$  for further analysis. Heart samples were also saved for immunohistochemical studies described below. In addition, LVAD core samples from the LV apex were obtained just before LVAD implantation in 11 of the 16 LAVD patients.

### MMP-1, MMP-9, and TIMP-1 Protein Content

Heart samples were homogenized in 1 mL of lysis buffer (0.05 mol/L Tris-Cl, 150 mmol/L NaCl, 1% Triton, pH 8.0). The homogenate was centrifuged at 3000g for 20 minutes at  $4^\circ\text{C}$ , and the supernatant was collected. The supernatant (100 mL) was tested in duplicate for total MMP-1 (free and MMP-1/TIMP-1 complexed), MMP-9 (free and TIMP-1 complexed pro-MMP-9), and TIMP-1 with ELISA kits (Amersham Biotrak) following the manufacturer's instructions. Values of MMP-1, MMP-9, and TIMP-1 are standardized per 1 mg of heart tissue protein.

### Gelatin Zymography and MMP-1 Activity

Gelatin zymography was performed with myocardial extracts isolated from frozen cardiac samples. Equal amounts of homogenized heart protein were loaded per lane and subjected to 10.0% SDA-PAGE (Bio-Rad apparatus) with 1.0% gelatin (Sigma) in nondenaturing, nonreducing conditions. Gels were washed 3 times in 2.5% Triton X-100 for 20 minutes each time and incubated for 24 hours in 100 mM Tris, pH 7.4, and 1.5 mmol/L CaCl<sub>2</sub> at 37°C, stained with 0.1% Coomassie blue G-250 (Bio-Rad) for 30 minutes in 40% methanol, and then destained in 5% acetic acid until clear gelatinolytic bands were visualized. Band densitometry using Kodak ImageWare software was used to quantify the zymographic results. The zymogram studies were repeated at least 2 times, and the separately obtained results were combined. All densitometric data are expressed as relative units by dividing the signal intensity by pixel area.

For MMP-1 activity, myocardial samples were pulverized, homogenized in 10 volumes of protein extraction buffer (50 mmol/L Tris, pH 7.4, 1.5 mmol/L CaCl<sub>2</sub>, Triton X-100), and centrifuged for 10 minutes at 2000g to remove cellular debris. The supernatant was assayed for total protein content, and 100  $\mu$ L of each sample was assayed for MMP-1 activity with the MMP-1 Biotrak Activity Assay Kit (Amersham Biosciences) according to the manufacturer's instructions. All measurements were run in duplicate. P-aminophenylmercuric acetate activation was not performed. The levels of activity are reported as nanogram per milliliter per milligram of tissue protein.

### Collagen Characterization

Myocardial collagen can be fractionated into pepsin-soluble and pepsin-insoluble collagens, with the latter reflecting primarily cross-linked collagen. The myocardial pepsin-soluble collagens were extracted overnight with 5 mg/mL pepsin in 0.5 mol/L acetic acid.<sup>23</sup> The soluble and insoluble collagens were separated by centrifugation at 3000g for 10 minutes at 4°C. The soluble collagens included both denatured and undenatured collagens, with the latter quantified by the Sircol collagen assay kit with soluble type I collagen as the standard (Accurate Chemicals).

Total myocardial collagen content was assessed by measuring hydroxyproline content using a modified Stegemann method in hydrolyzed total soluble and insoluble collagens.<sup>24</sup> The extent of collagen denaturation was assessed by the content of undenatured collagen and the ratio of undenatured to total soluble collagens. The ratio of insoluble to total soluble collagen was calculated and used as a measure of cross-linking.

Because the measurement of cross-linked collagen by hydroxyproline is indirect, additional levels of pyridinoline cross-linking were measured in the same samples using Metra Serum PYD EIA kits (Quidel). All samples were diluted 1:10 and 1:20 in assay buffer and then measured following the company's protocol expressed as nanomole pyridinoline per 1  $\mu$ g tissue.

### Ratio of Collagen Type I to III

Heart samples were homogenized in an SDS buffer and centrifuged to pellet the collagen and elastin. Purified lyophilized type I and III standards (2 mg) were added to separate 1-mL aliquots of 70% formic acid, and the sample mixtures were heated. Cyanogen bromide (Sigma-Aldrich Corp) was added, and the samples were deoxygenated by bubbling nitrogen gas through them. After incubation, 1-mL samples were placed individually into 10 000 molecular-weight cutoff Slide-A-Lyzer dialysis cassettes (Pierce Biotechnology Inc) and dialyzed twice against PBS for 12 hours each time. The supernatant samples were concentrated 10-fold with YM-10 Centricon filters (Millipore). Samples mixed 1:1 with 2-SDS loading buffer were loaded onto a 15% acrylamide gel (40  $\mu$ L/well) and run for 2 hours at 130 V. The gel was stained with silver (Bio-Rad). The peptides chosen for quantification were  $\alpha_1$ (III)-CB5 for type III collagen and  $\alpha_1$ (I)-CB8 for type I collagen.<sup>25</sup> Densitometric analysis was conducted with Bio-Rad software, and the data are reported as the ratio of type I to type III collagen.

### Immunohistochemical Staining of Myocardial Type I and III Collagen and Myocyte Diameter

Freshly collected LV and RV myocardial samples were immediately fixed in buffered formalin (10%), embedded in paraffin, and mounted on glass slides. Double immunofluorescent staining of type I and type III collagen (Rockland) was performed by routine protocols in our laboratory. A methodical negative control went through every step of the procedure except for incubation with the primary antibody. Images were viewed on a Nikon Optiphot-2 microscope (Nikon Corp) with an MTI 3CCD digital camera (Dage-MTI Inc). Digitally acquired images were analyzed with Image Pro Plus V3.0 analysis software (Media Cybernetics) using color-cube-based selection criteria for positive staining. On each section, a density range was set to resemble collagen content. The number of pixels included in this range was divided by the total number of pixels in this field. Ten fields at  $\times 20$  magnification per sample were analyzed, and results were then averaged.

To quantify myocyte diameter, samples were prepared with Masson's trichrome stain. At  $\times 20$  magnification, 2 orthogonal diameters were obtained per myocyte and then averaged. On average, we analyzed 6 fields per slide per patient. In each field,  $\approx 10$  myocyte diameters were determined. Only sections cut in cross section were analyzed.

### Ang I and II Myocardial Tissue Levels

Tissue Ang I and II levels were measured by radioimmunoassay after SepPak extraction and high-performance liquid chromatography separation.<sup>26</sup> A known amount of <sup>125</sup>I-Ang I was added as an internal standard before the extraction procedure, and the recovery of <sup>125</sup>I-Ang I after high-performance liquid chromatography separation was used to correct for losses (maximally 20% to 30%) that occurred during extraction and separation.<sup>27</sup>

### Myocardial Force Generation

Myocardial force data were obtained from LV free wall trabeculae as described previously.<sup>6,21</sup> In brief, immediately after cardiectomy, trabeculae <1 mm in diameter were excised and immersed in oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) ice-cold Krebs-Ringer solution with 30 mmol/L 2,3-butanedione monoxime. For the mechanical measurements, muscle strips were mounted in a bath, with one end connected to a force transducer and the other connected to an adjustable-length micrometer gauge using fine steel hooks. During superfusion with 37°C oxygenated Krebs-Henseleit buffer (rate, 1 mL/L; bath volume, 1 mL), trabeculae were stimulated at 1 Hz and allowed to equilibrate for at least 1 hour at slack length. After the resting period, isometric twitches were evoked with stimulation voltage 20% above threshold (duration, 5 ms). The trabeculae were then progressively stretched to the length of maximal force generation (L<sub>max</sub>) at 1-Hz frequency. Adequate muscle oxygenation was confirmed as detailed previously.<sup>6</sup> Myocardial force generation was calculated after normalization to cross-sectional area.

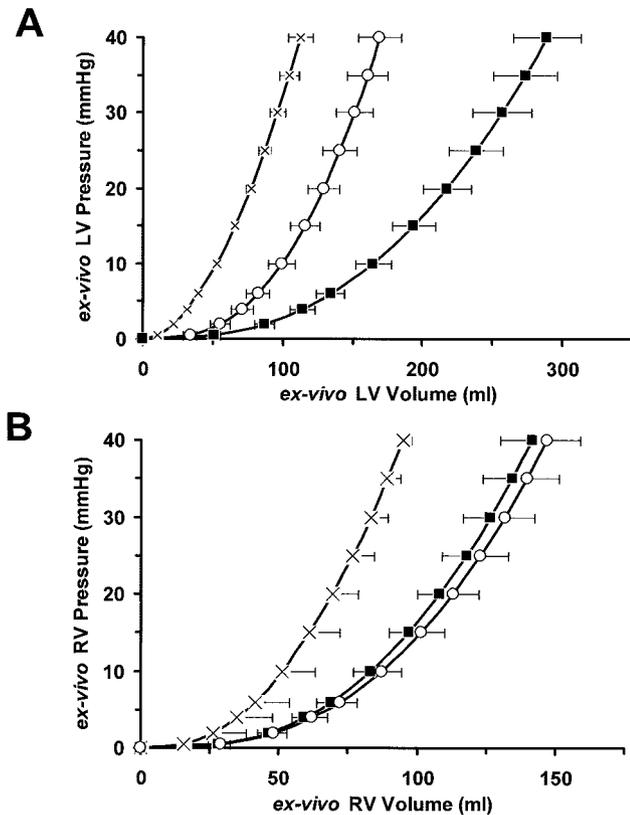
### Statistical Analysis

Results are presented as mean  $\pm$  SEM. One-way ANOVA was used to select differences between groups, followed by Tukey-Kramer post-hoc testing. A paired *t* test was used to compare paired pre- and post-LVAD samples. The statistical analysis was conducted with a commercially available statistical software package (SPSS 11.5). Values of *P* < 0.05 were considered statistically significant.

## Results

### LV and RV Ex Vivo Passive Pressure-Volume Relationships and Mass

Ex vivo EDPVRs are shown in Figure 1. A marked difference between NF and DCM hearts is evident in the LV, with the average curve derived from LVAD-supported hearts close to that of NF hearts. Ventricular size, indexed by V<sub>30</sub>, is summarized in Figure 2A. The mean LVV<sub>30</sub> of hearts sup-



**Figure 1.** A, LV passive EDPVR for NF (×), DCM (■), and LVAD-supported (○) hearts. After LVAD support, EDPVR is shifted left toward NF hearts. B, RV EDPVR. No change in EDPVR is evident between DCM and LVAD-supported hearts.

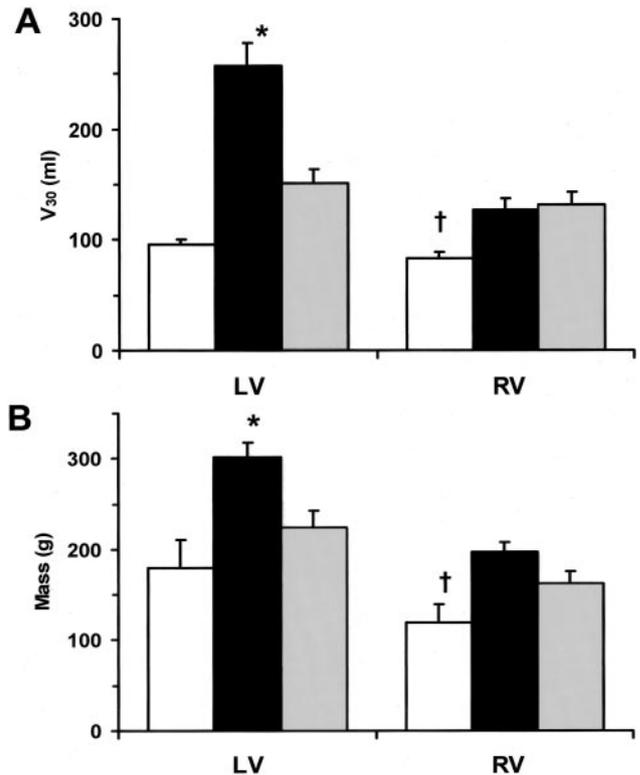
ported by LVADs for >30 days was significantly smaller than that of DCM hearts without LVAD support ( $P<0.05$ ) and reached values close to those of the NF hearts. In contrast, there was no significant difference between DCM and LVAD-supported hearts with respect to the RV EDPVR and mean  $RVV_{30}$ , indicating that LV unloading is the main trigger (Figures 1 and 2A).

After LVAD support, LV mass was significantly reduced compared with nonsupported DCM hearts ( $P<0.05$ ; Figure 2B) and reached values similar to NF hearts. RV mass was increased in DCM hearts ( $P<0.05$  versus NF) but showed no significant reduction after LVAD support.

### Chamber and Myocardial Stiffness

The LV dimensionless chamber stiffness constant ( $\alpha$ ) was significantly increased after LVAD support compared with DCM ( $P<0.05$ ; Figure 3A) and reached an almost normal value. In contrast, RV chamber stiffness constant was decreased significantly in LVAD-supported hearts compared with NF and DCM hearts ( $P<0.01$ ).

LV myocardial stiffness constant ( $\kappa$ ) increased significantly in DCM compared with NF hearts ( $P<0.05$ ; Figure 3B). However, after LVAD support, myocardial stiffness increased further ( $P<0.05$  versus DCM and  $P<0.01$  versus NF hearts).



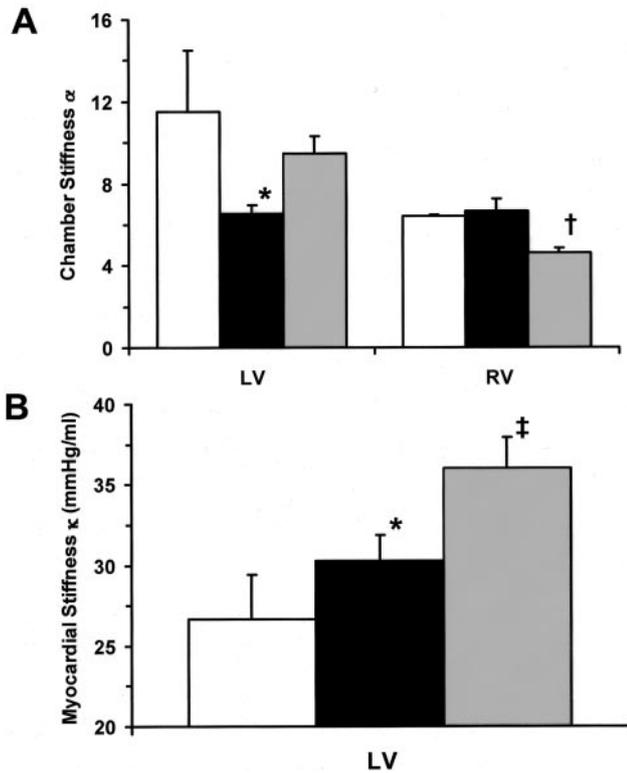
**Figure 2.** A,  $V_{30}$ , ex vivo volume providing pressure of 30 mm Hg, is index of ventricular size.  $LVV_{30}$  was decreased after LVAD support for >30 days and reached values close to NF hearts.  $RVV_{30}$  did not change significantly after LVAD support. B, Significant decrease in mass was evident only in LV. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P<0.05$  vs LVAD and NF; † $P<0.05$  vs LVAD and DCM.

### Protein Expression of MMP-1, MMP-9, and TIMP-1

MMP-1 protein expression increased significantly in the DCM group versus NF hearts in both the LV and RV (Figure 4A;  $P<0.05$ ). After LVAD support, MMP-1 protein expression trended toward lower levels in the LV but did not change in the RV. In contrast, TIMP-1 expression, which was reduced in DCM hearts, increased tremendously during LVAD support, reaching almost normal levels in the RV and levels higher than normal in the LV (Figure 4B;  $P<0.05$  versus DCM). Hence, the MMP-1-to-TIMP-1 ratio was normalized in the LV ( $P<0.05$  versus DCM) and trended toward normal in the RV (Figure 4C). MMP-9 protein expression was elevated in end-stage heart failure in both the LV and RV ( $P<0.05$  versus NF hearts) and trended to be downregulated in the LV but not RV after LVAD support (Figure 4D).

### Gelatin Zymography and MMP-1 Activity

Gelatin zymography detected 2 major gelatinolytic bands in the myocardial extracts that were generated by MMP-2 and MMP-9 (Figure 5A). MMP-2 gelatinolytic activity was increased in pre-LVAD samples, but did not change after LVAD support in both the LV and RV. In contrast, MMP-9 gelatinolytic activity was significantly decreased in LV samples after LVAD support to almost normal activity ( $P<0.05$ ).



**Figure 3.** A, Dimensionless chamber stiffness constant ( $\alpha$ ) was almost normalized after LVAD support in LV and reduced in RV. B, LV myocardial stiffness constant ( $\kappa$ ) was increased in DCM and increased further after LVAD support in LV. RV myocardial stiffness was not calculated because there is no accepted, simple analytic approach for estimating RV stresses and strains. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs NF and LVAD; † $P < 0.01$  vs NF and DCM; ‡ $P < 0.05$  vs DCM;  $P < 0.01$  vs NF.

versus DCM) but did not change in RV samples compared with DCM (Figure 5B). Results of densitometric analysis provided values (in relative, unitless values) for MMP-9 of 34 for NF hearts, 59 for DCM hearts, and 31 for LVAD hearts.

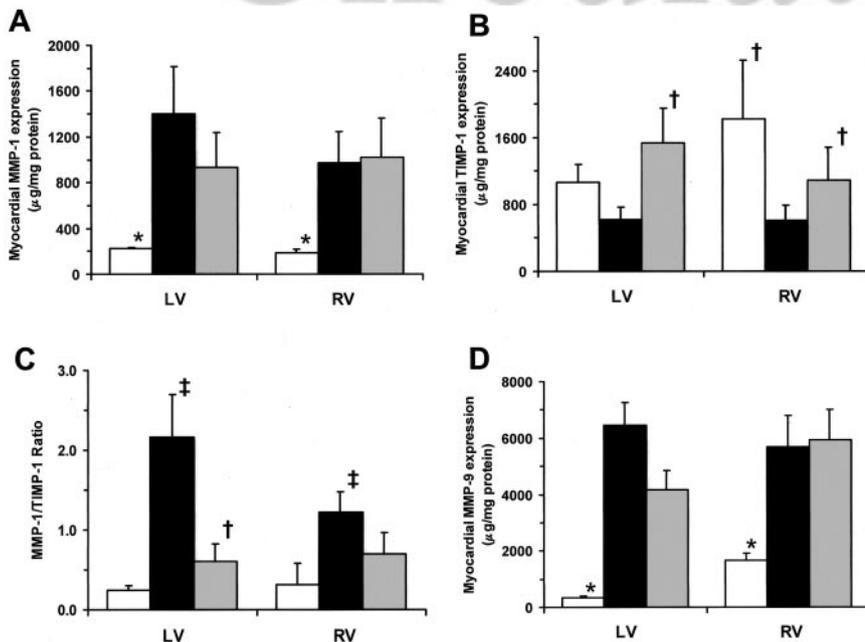
For MMP-2, the densitometric relative values are 40, 66, and 35, respectively.

To measure the levels of MMP-1 activity in addition to the MMP-1 protein expression, we used a commercially available kit to quantify MMP-1 activity in paired pre- and post-LVAD samples. In pre-LVAD core samples, MMP-1 activity was elevated, similar to the elevation seen in total MMP-1 content (Figures 4A and 5C). After LVAD support, MMP-1 activity trended to be downregulated in the LV ( $P = 0.08$ ).

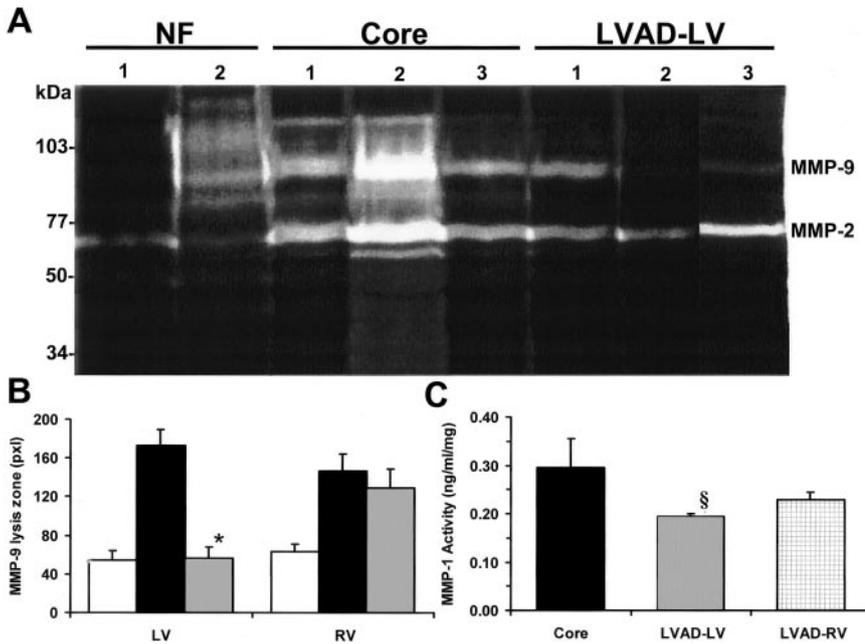
**Collagen Content and Cross-Linking**

The goal of this study is to compare ECM components and stiffness in the LV and RV after LVAD support. State-of-the-art collagen measurements should be performed in paired samples from the same patient before and after LVAD implantation. However, because EDPVR measurements and stiffness estimation are possible only from whole hearts and because RV myocardial tissue is not routinely available from pre-LVAD patients, we performed a subanalysis to demonstrate that collagen content in pre-LVAD samples from the LV apex is similar to the DCM apex and free wall samples. In 11 of the 16 patients from the LVAD group, paired samples (pre- and post-LVAD implantation) were available. In 9 of these 11 patients, total and cross-linked collagen increased after LVAD support. There were no significant differences between pre-LVAD samples from the LVAD group and samples from the DCM group (total collagen,  $9.72 \pm 0.40$  versus  $8.73 \pm 0.72$   $\mu\text{g}/\text{mg}$ ; cross-linked collagen,  $2.69 \pm 0.42$  versus  $2.68 \pm 0.25$   $\mu\text{g}/\text{mg}$ , respectively; both  $P = \text{NS}$ ). In addition, total and cross-linked collagen was the same in tissue samples obtained from LV apex and LV free wall in DCM patients (total collagen,  $9.15 \pm 0.59$  versus  $8.47 \pm 0.19$   $\mu\text{g}/\text{mg}$ ; cross-linked collagen,  $2.32 \pm 0.54$  versus  $2.85 \pm 0.40$   $\mu\text{g}/\text{mg}$ , respectively; both  $P = \text{NS}$ ).

Total LV myocardial collagen content (assessed by hydroxyproline) was increased in patients with DCM compared with those with NF hearts ( $P < 0.05$ ; Figure 6A). However,



**Figure 4.** MMP-1, MMP-9, and TIMP-1 protein expression in NF, DCM, and LVAD-supported hearts. A, MMP-1 expression is significantly increased in DCM hearts, and is slightly decreased after LVAD support in LV, but does not change in RV. B, Increase in TIMP-1 protein expression in LVAD-supported hearts in LV and RV. C, Normalization of ratio of MMP-1 to TIMP-1 after LVAD support in LV. D, MMP-9 protein expression trended to be downregulated after LVAD support in LV but not RV samples. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.05$  vs DCM and LVAD; † $P < 0.05$  vs DCM; ‡ $P < 0.05$  vs non-ailing.



**Figure 5.** Reduced MMP activity in LVAD-supported failing hearts. A, Representative gelatin zymography for MMP gelatinolytic activity in NF, before (core), and after LVAD support (LVAD). MMP-2 did not change, but MMP-9 decreased after LVAD support. B, MMP-9 activity was significantly reduced in LV samples after support but not RV samples. C, MMP-1 activity in paired samples before and after LVAD support for LV (n=15 per group) and RV (n=10). After LVAD support, MMP-1 activity trended to be reduced in LV. \* $P<0.05$  vs DCM; § $P=0.08$  vs core.

total collagen content increased further after LVAD support ( $P<0.05$  versus DCM,  $P<0.01$  versus NF hearts). In particular, cross-linked collagen showed a tremendous increase after LVAD support compared with DCM and NF hearts ( $P<0.01$  versus DCM and NF hearts; Figure 6B). There was no change in soluble collagen between DCM and LVAD-supported hearts (Figure 6C). To avoid errors resulting from the indirect measurement of cross-linked collagen by the hydroxyproline assay, we also determined collagen cross-links by direct measurement of pyridinoline. Similar to the hydroxyproline measurement, LV pyridinoline cross-linking was increased in DCM compared with NF hearts ( $P<0.05$ ) and increased significantly after LVAD support ( $P<0.05$  versus DCM,  $P<0.01$  versus NF hearts; Figure 6D).

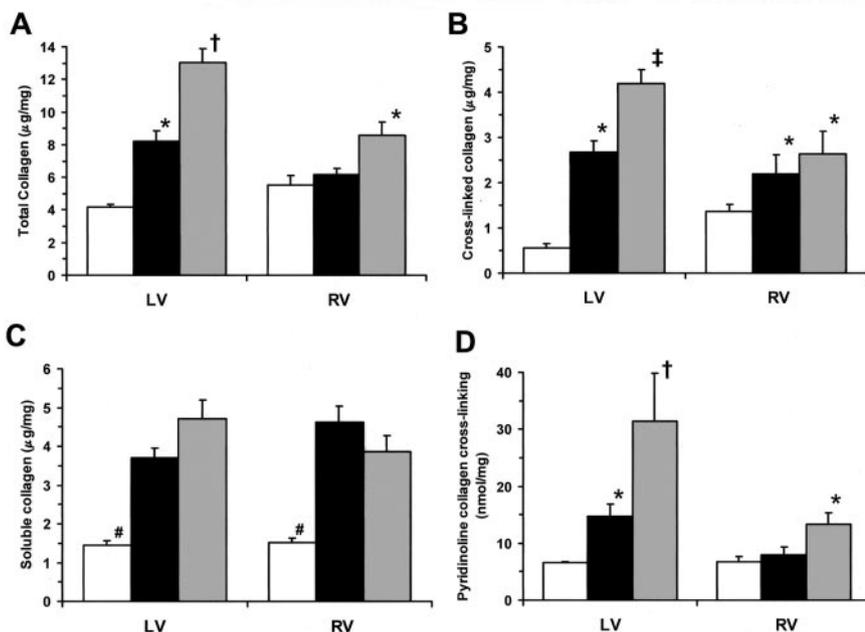
RV total myocardial collagen content increased after LVAD support ( $P<0.05$  versus NF hearts), although not to

the same extent as in the LV. RV soluble and cross-linked insoluble collagen increased in DCM compared with NF hearts ( $P<0.05$ ) but did not change after LVAD support.

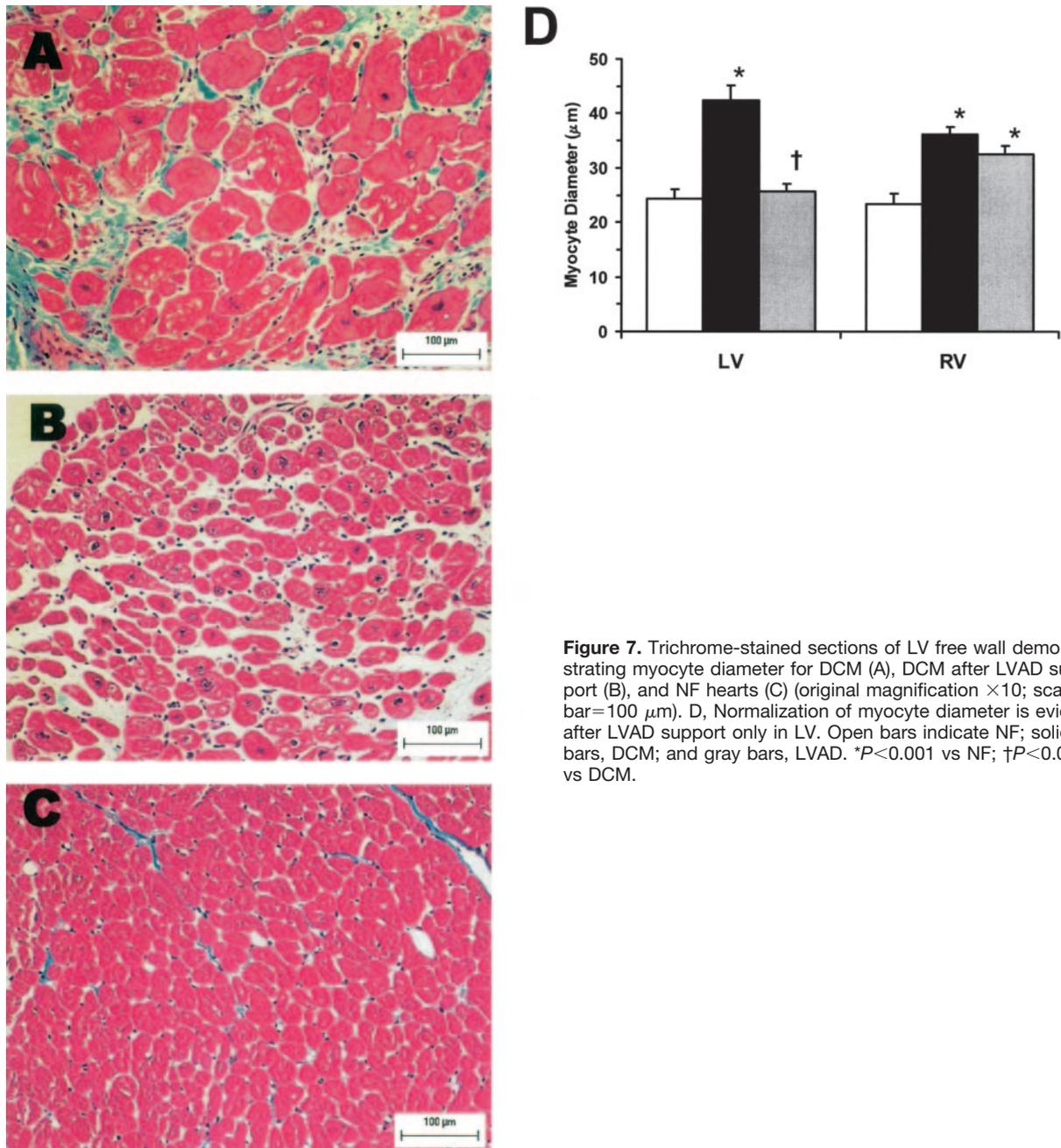
### Immunohistochemical Analysis of Type I and III Collagen and Their Ratio

The proportion of total collagen staining positive for type I collagen was increased in the LV myocardium of patients after LVAD support compared with DCM hearts ( $15.8\pm7.4\%$  versus  $9.5\pm4.7\%$ ,  $P<0.01$ ; NF hearts,  $0.9\pm0.7\%$ ). In RV tissue, the amount of type I collagen was similar in LVAD-supported and DCM hearts ( $8.3\pm4.2\%$  and  $7.1\pm3.1\%$ , respectively).

The stained area of type III collagen was increased in DCM compared with NF hearts ( $6.4\pm4.0\%$  versus  $0.3\pm0.2\%$ ;  $P<0.001$ ) and increased further after LVAD



**Figure 6.** Collagen characterization in NF, DCM, and LVAD-supported hearts. A, Total myocardial collagen content is increased in DCM and increased further after LVAD support. B, Cross-linked collagen increased tremendously after LVAD support in LV samples. C, Soluble collagen was increased in DCM vs NF hearts but did not change significantly after LVAD support. D, Collagen cross-linking measured by pyridinoline assay. Similar to determination by hydroxyproline, cross-linking is increased in DCM and increased further after LVAD support. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P<0.05$  vs NF; † $P<0.05$  vs DCM and  $P<0.01$  vs NF; ‡ $P<0.01$  vs DCM and NF; # $P<0.01$  vs DCM and LVAD.



**Figure 7.** Trichrome-stained sections of LV free wall demonstrating myocyte diameter for DCM (A), DCM after LVAD support (B), and NF hearts (C) (original magnification  $\times 10$ ; scale bar =  $100 \mu\text{m}$ ). D, Normalization of myocyte diameter is evident after LVAD support only in LV. Open bars indicate NF; solid bars, DCM; and gray bars, LVAD. \* $P < 0.001$  vs NF; † $P < 0.001$  vs DCM.

support ( $8.4 \pm 3.6\%$ ;  $P < 0.05$  versus DCM). However, no change in the amount of type III collagen after LVAD support was evident in the RV compared with DCM hearts ( $5.4 \pm 1.6\%$  and  $5.7 \pm 2.3\%$ , respectively).

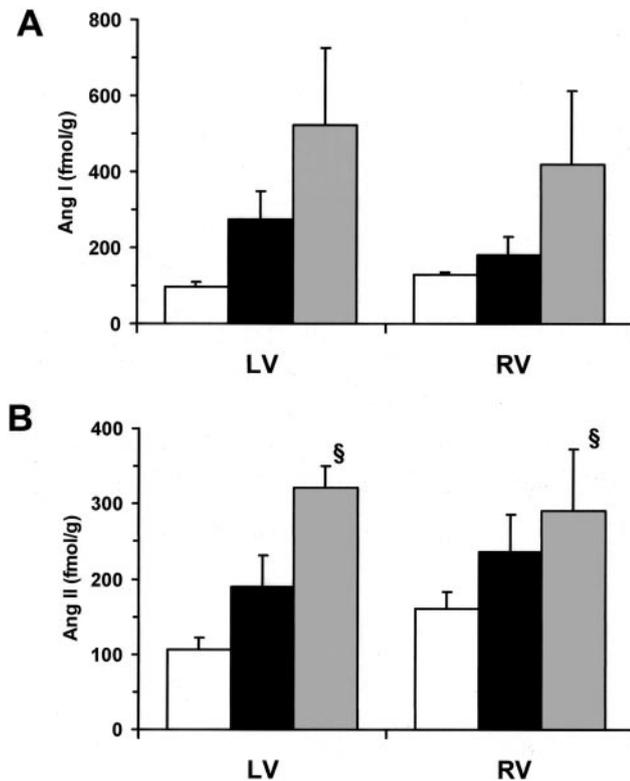
The ratio of collagen type I to III, determined by cyanogen bromide digestion, trended higher in patients with DCM compared with NF hearts ( $2.72 \pm 0.14$  versus  $2.65 \pm 0.55$ ;  $P = 0.08$ ). After LVAD support, the ratio of type I to III increased further because of a relative increase in type I and a relative decrease in type III collagen ( $3.45 \pm 0.57$ ;  $P < 0.05$  versus DCM and NF hearts). Significant changes between NF, DCM, and LVAD-supported hearts in the ratio of collagen type I to III were not evident in the RV.

### Myocyte Diameter

Myocyte diameter increased significantly in DCM hearts compared with NF hearts ( $P < 0.001$ ; Figure 7). After LVAD support, this finding could be almost completely reversed ( $P < 0.001$  versus DCM). However, the enlarged myocyte diameter in the RV in DCM patients could not be reversed to normal after LVAD support.

### Ang I and II Myocardial Tissue Levels

Compared with NF hearts, myocardial tissue levels of Ang I and II were elevated in DCM in LV and RV samples (Figure 8). After LVAD support, Ang I and II increased further in both chambers (Ang II,  $P = 0.094$  versus NF hearts). Levels of Ang I and II in pre-LVAD core samples ( $244 \pm 137$  and



**Figure 8.** Myocardial tissue levels of Ang I and II in NF, DCM, and LVAD-supported hearts. Ang I and II levels were increased after LVAD support. Open bars indicate NF; solid bars, DCM; gray bars, LVAD.  $\$P=0.094$  vs NF.

$136 \pm 39$  fmol/g) were similar to levels from the free wall in DCM hearts.

### Myocardial Force Generation

To test whether myocardial force generation is different in the NF, DCM, and LVAD groups, we isolated muscle strips from the LV free wall and placed them in organ baths. The trabecular dimensions were similar among all groups. Interestingly, developed force at 1 Hz in NF, DCM, and LVAD-supported hearts was comparable between groups:  $10.5 \pm 1.0$  (n=5),  $9.2 \pm 1.7$  (n=30), and  $9.2 \pm 2.4$  (n=16) mN/mm<sup>2</sup>, respectively ( $P=NS$ ).

### Discussion

Hemodynamic unloading during LVAD support leads to reverse structural, molecular, cellular, and functional remodeling.<sup>2-4,6,9,21,28,29</sup> In addition, collagen content increases after LVAD support, as shown in prior studies.<sup>3,30</sup> Similar findings were discovered after acute reduction in LV afterload in patients with aortic valve replacement, in pharmacological intervention in hypertensive heart disease, and in animal models of heterotopic transplantation.<sup>17-20</sup> Moreover, an increase in diastolic stiffness accompanied this increased collagen deposition in most of these studies.

In the present study, we confirmed that LVAD support leads to reverse structural remodeling of the LV, evidenced by a reduction in LV size, mass, and myocyte diameter and a leftward shift in the ex vivo EDPVR with near normalization of chamber stiffness. However, we extended these results

from former studies by characterizing changes in collagen type and quantity by measuring MMP and determining myocardial stiffness.

Whereas chamber stiffness normalized after LVAD support, intrinsic myocardial stiffness increased above the already elevated levels seen in DCM hearts. This finding is compatible with the observed increase in cross-linked myocardial collagen content. A more detailed analysis of extracellular collagen matrix composition revealed that the absolute content of type I and III collagens and the ratio of collagen type I to III were all increased after LVAD support.

Furthermore, MMP-1 and MMP-9 levels and activity increased in the failing state compared with normal and trended to decrease after LVAD support. In particular, TIMP-1 levels increased tremendously after LVAD support, leading to a normalization of the MMP-1-to-TIMP-1 ratio. This suggests that in end-stage heart failure there is a high rate of collagen breakdown that is reduced after LVAD support, resulting in the overall increase in collagen content that we observed.

Finally, we observed that myocardial tissue levels of Ang I and II, known regulators of myocardial collagen synthesis, showed trends toward further increased levels in the LV after LVAD support.

Additional novel results presented here on RV properties provide insight into the remodeling process during LVAD support. In contrast to the LV, the RV showed no signs of reverse structural remodeling. RV EDPVR, mass, size, and myocyte diameter in hearts supported with LVAD were similar to those of DCM hearts and did not normalize after LVAD support. Despite a small increase in total RV myocardial collagen content, cross-linked collagen, collagen types I and III, the ratio of collagen type I to III, and MMP protein expression and activity did not change compared with the RV of DCM hearts. The different effects on RV and LV properties suggest that the primary mechanism by which LVAD influences ECM properties relates to reduced mechanical stretch by mechanical unloading because LVADs pressure and volume unload the LV but do not reduce (and may even increase) the hemodynamic load on the RV.<sup>31,32</sup> The similar increase in tissue levels of Ang I and II in the LV and RV after LVAD support is not contrary to this proposition of LVAD-induced ECM remodeling but more specifically suggests that cardiac Ang generation depends more on extracardiac (ie, renal) than locally synthesized renin.<sup>33</sup>

The myocardial collagen matrix is considered to be an important determinant of ventricular passive diastolic properties and myocardial structural integrity.<sup>34,35</sup> Consisting mainly of collagen type I ( $\approx 85\%$ ), and collagen type III ( $\approx 11\%$ ), it has a high tensile strength, even though normally occupying only 4% of the extracellular space.<sup>36</sup> However, there is some controversy concerning how collagen content changes after LVAD support. One group reported a decrease in total collagen after mechanical unloading toward normal values,<sup>37,38</sup> whereas most studies showed an increase in collagen content in both humans and animal models,<sup>3,19,29,30,39</sup> which is consistent with our study. An explanation for these discrepancies could be that the former studies used immunohistochemical staining with picrosirius red, whereas the latter

group of studies used biochemical assays for tissue collagen content, which in part could explain these discrepancies.<sup>40</sup>

It is believed that the absence of type I collagen leads to a reduction in myocardial contractile force and causes muscle fiber slippage and realignment with thinning of the myocardium, finally leading to chamber dilatation.<sup>35</sup> Conversely, in animal models, an increase in total collagen leads to enhanced myocardial stiffness, causing decreased ventricular elasticity.<sup>34</sup> New evidence demonstrates more specifically that increased cross-linked collagen and a greater amount of the stiffer type I collagen relative to the more elastic type III collagen plays an important role in enhanced myocardial stiffness and chamber remodeling.<sup>13,15,16,41,42</sup> Our study is the first to examine the changes in collagen isotypes after LVAD support. We demonstrated that above and beyond the already known increase in the ratio of collagen type I to III in end-stage heart failure,<sup>43–45</sup> a further increase is present after LVAD support. Interestingly, the extreme increase in collagen deposition after LVAD support consists almost entirely of elevation of cross-linked collagen and is compatible with the increased myocardial stiffness calculated from the *ex vivo* pressure-volume relationship, chamber size, and myocardial mass. These findings were never documented in humans after LVAD support but are complementary to the findings of hemodynamic unloading after aortic valve replacement.<sup>17,18</sup> In the failing heart, it has previously been demonstrated that normal collagen is degraded by increased MMP activity and replaced by fibrous interstitial deposits of poorly cross-linked collagen, which allows ventricular dilatation, commonly referred to as remodeling.<sup>46</sup> The hypothesis is that these ECM changes occur as a result of increased mechanical stretch to cardiac fibroblasts.<sup>47</sup> Previously, Li et al<sup>10,30</sup> demonstrated that MMP-1 and MMP-9 protein expression is increased in heart failure and that LVAD support was associated with a significant reduction. However, this could be demonstrated only in ICM and not in DCM patients, whereas TIMP-1 and TIMP-3 levels were both significantly elevated after LVAD support in ICM and DCM patients. TIMP-2 and TIMP-4 levels showed no change after LVAD support. TIMPs are believed to bind MMPs in a stoichiometric 1:1 molar ratio.<sup>12</sup> We showed that LVAD support results in a stoichiometric imbalance favoring TIMP-1 activation and a normalization of the MMP-1-to-TIMP-1 ratio. We therefore hypothesize that increased collagen cross-linking after LVAD support could be attributed to decreased degradation of immature collagen, coupled with ongoing production of new collagen. The effect of LVAD support on RV changes of the ECM is different. Because changes in the extracellular collagen matrix and MMP and TIMP protein expression/activity are not evident in the RV after LVAD support, we can conclude that LVAD-induced hemodynamic unloading is the primary factor for these alterations.

Despite the fact that Ang II is a stimulator of fibrosis, it also stimulates myocardial cell growth and hypertrophy.<sup>48,49</sup> Concomitantly, the increased myocardial tissue levels of Ang II are consistent with the increase in collagen content after LVAD support. However, we see a reduction toward normal in myocyte diameter and regression of hypertrophy in patients after LVAD support. This suggests that the hemody-

amic effect of unloading the LV overwhelms the effect of Ang II in terms of hypertrophy but not fibrosis. Evidence in mice supports our concept that Ang II could affect fibrosis without inducing hypertrophy.<sup>50</sup>

Although the present study has not examined the clinical implications of the current findings, it is of interest that although almost every other aspect of cardiac properties examined in prior studies returns toward normal during LVAD support, the ECM changes described here show progressive worsening. It is therefore tempting to question whether such changes could be a factor in the extremely low observed rate of full ventricular functional recovery during LVAD support and for the high rate of recurrent remodeling (ie, progressive deterioration of pump function) that typically occurs after LVAD removal.<sup>28,51–53</sup> For example, could increased interstitial fibrosis after LVAD support interfere with myocyte contractile function as proposed<sup>54</sup> in other settings? Indeed, Rossman et al<sup>55</sup> noted at low rates of stimulation that peak developed force is lower after LVAD support than in the failing state. Although our findings are not identical, we did find no significant change in contractile force in LVAD-supported hearts. One prior study suggested that when studied in isolation, LVAD support significantly improved baseline myocyte contractile strength (ie, strength at low stimulation frequency, not subjected to  $\beta$ -adrenergic stimulation)<sup>5</sup>; it is conceivable that the increased ECM could be limiting the myocardial expression of improved baseline cellular function. Alternatively, LVAD support may not truly improve myocyte function *in vivo*, and alterations of ECM may not influence myocardial contractile strength. Neither the present study nor any prior study that can sort out these alternatives has been conducted.

Additionally, the question as to whether hearts really “redilate” after LVAD explantation remains controversial. Unfortunately, only small single-center experiences are available. Although one center describes redilation after LVAD explantation in selected patients,<sup>28,52</sup> another center reports only a deterioration in ejection fraction without dilation in patients who were successfully weaning but subsequently failed in the long-term follow-up.<sup>56,57</sup> In these reports, the hearts “redilate” to only a small amount ( $\approx$ 8 to 11 mm) 3 to 39 months after LVAD explantation and did not show any difference between successful and failed long-term weaning. This suggests that increased collagen content and myocardial stiffness may prevent redilation of the ventricle once the heart is exposed to normal filling pressures after LVAD removal but did not prevent deterioration of cardiac function. Although difficult to evaluate, it is possible that the present results have important functional implications as they may relate to the frequency and permanence of LV recovery after LVAD support.

### Study Limitations

Hearts of patients with ischemic cardiomyopathy were specifically excluded from the present study. The reason was that the presence of multiple infarcts in varying degrees of healing would dramatically complicate an analysis of ECM properties and metabolism. The results should therefore not be extrapolated to ischemic cardiomyopathic hearts. ACE inhibitors

are known to influence collagen metabolism, and 6 of our 16 patients with LVAD support were taking such drugs. However, analysis failed to demonstrate a difference between patients taking and not taking ACE inhibitors. Because of the relatively small sample size, subtle but potentially important effects could be present. Finally, we analyzed the effect of LVAD support only on MMP-1, MMP-9, and TIMP-1 protein expression/activity and did not include other known proteinases and MMP inhibitors, which may play an important role in ECM remodeling.

### Conclusions

LVAD-induced mechanical unloading of end-stage heart failure hearts is associated with a dramatic shift toward lower volumes of the LV pressure-volume relationship, in combination with reduced cardiomyocyte diameter, LV mass, and LV size. Changes in MMP-1-to-TIMP-1 protein expression ratio after LVAD support were accompanied by an increased ratio of collagen type I to III and an increase in the total amount of type I and III collagen. In addition, total myocardial collagen content and importantly collagen cross-linking increased, which we propose as the mechanism of the observed increase in myocardial stiffness. These changes were not evident in the RV, suggesting that reduced myocardial stretch by LVAD-induced hemodynamic unloading is the primary factor from these alterations in the extracellular collagen matrix. Although most abnormal phenotype and genotype changes noted to occur in the setting of heart failure are normalized during LVAD support, these changes in ECM are not totally normal. It is possible that these abnormalities could contribute to the low rate of complete recovery of LV function during LVAD support and the high incidence of deterioration of LV function after LVAD removal. As such, the ECM may be an appropriate therapeutic target for enhancing the incidence of sustained ventricular recovery of the end-stage failed heart.

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