Downregulation of Matrix Metalloproteinases and Reduction in Collagen Damage in the Failing Human Heart After Support With Left Ventriculat Assist Devices

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Background—Left ventricular assist device (LVAD) support of the failing heart induces salutary changes in myocardial structure and function. Matrix metalloproteinases (MMPs) are increased in the failing heart and are induced by stretch in cardiac cells in vitro. We hypothesized that mechanical unloading may affect LV plasticity by regulating MMPs and their substrates.

Methods and Results—LV samples were collected from patients with dilated cardiomyopathy (DCM, n=14) or ischemic cardiomyopathy (ICM, n=16) at the time of implantation of the LVAD and again during cardiac transplantation. MMP-1, -3, and -9 were measured by ELISA, MMP-2 and -9 gelatinolytic activity by gelatin zymography, and tissue inhibitors of metalloproteinases (TIMPs) by Western blot. Total soluble and insoluble collagens were separated by pepsin solubilization, and the contents were determined by quantification of hydroxyproline. The undenatured soluble collagen was measured by Sircol collagen assay. The results showed that MMP-1 and -9 were decreased, whereas TIMP-1 and -3 were increased, but there was no change in MMP-2 and -3 and TIMP-2 and -4 after LVAD support. The undenatured collagen was increased, with the ratio of undenatured to total soluble collagens increased in ICM and that of insoluble to total soluble collagens increased in DCM after LVAD support.

Conclusions—The reduced MMPs and increased TIMPs and ratios of undenatured to total soluble collagens and insoluble to total soluble collagens after LVAD support suggest that reduced MMP activity diminished damage to the matrix. These changes may contribute to the functional recovery and LV plasticity after LVAD support. (Circulation. 2001;104:1147-1152.)

Key Words: collagen ■ metalloproteinases ■ remodeling ■ heart-assist device ■ heart failure

Left ventricular assist devices (LVADs) provide mechanical support for the failing heart and serve as a bridge to cardiac transplantation, with the potential to be a destiny therapy for heart failure.1-3 Recent reports demonstrate that LVAD support may be associated with adaptive remodeling of the ventricular myocardium, including reduced LV mass, wall thickness, and myocyte diameter; changes in LV pressure-volume relationships; and reversal of LV chamber dilation and molecular remodeling of proteins involved in Ca2+ cycling.3-10 In addition, LVAD support has been associated with salutary changes in cardiomyocyte function. Indeed, a small subgroup of patients can be successfully weaned from LVAD after recovery of ventricular function.6,11-14 Although it has been suggested that these beneficial changes are attributed to chronic unloading of the ventricular myocardium, the molecular mechanisms that play a role in LVAD-induced myocardial plasticity and LV remodeling remain undefined.

We hypothesized that the myocardial remodeling that occurs with LVAD unloading might be attributable to alterations in the components of the extracellular matrix, and specifically in the activity of matrix metalloproteinases (MMPs) and the physical properties of collagens. The matrix collagens have been proposed to provide the support essential for maintaining alignment of myofibrils within the myocyte as well as for maintaining myocyte alignment within the myocardium.15 Extracellular matrix remodeling contributes to progressive LV remodeling, dilatation, and heart failure. MMPs are capable of degrading all the components of the extracellular matrix, including collagens, and may be responsible for the turnover of the extracellular matrix.16 We have demonstrated in an animal model of heart failure that MMP-2 and -9 are significantly increased, undenatured collagen is significantly decreased, and TIMP-1 and -3 are significantly increased after LVAD support. These findings suggest that mechanical unloading of the failing heart may be associated with decreased MMP activity, increased TIMP activity, and decreased collagen turnover.

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reduced, and total collagen is increased. Recent studies have also demonstrated that MMP-9 content and activity are increased in the failing human heart, and MMPs are implicated in the progression of ventricular dilation and the development of heart failure. Excessive exposure to active MMPs leads to denaturation of collagens, which may serve as the initial step in the complete degradation of collagens and contribute to maladaptive extracellular matrix remodeling and dysfunction of the failing heart. Therefore, modulation of MMP activity may be able to alter the extracellular matrix remodeling process and the dysfunction of the failing heart.

Because MMPs and membrane-type MMPs in cardiac cells are induced by stretch in vitro, the increased expression and activity of MMPs in the failing heart may be due to stretch of the heart originating from overload. Thus, we hypothesized that LVAD unloading might downregulate MMPs and induce beneficial changes in the extracellular matrix. To test this hypothesis, we assessed the expression and gelatinolytic activity of MMPs, the expression of tissue inhibitors of metalloproteinases (TIMPs), and undenatured soluble collagen as well as insoluble collagen (cross-linked collagen) in LV myocardium of patients with dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) both before and after LVAD support.

### Methods

#### Cardiac Tissue Sample Collection

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 boards of the University of Pittsburgh and the Cleveland Clinic Foundation. Consent was obtained from patients before tissue harvest. LV myocardial samples were obtained from 30 patients with advanced heart failure (12 from the University of Pittsburgh and 18 from the Cleveland Clinic Foundation) at the time of implantation of an LVAD (17 Novacor, 13 TCI Heartmate) and again when the heart was excised for cardiac transplantation (14 patients with DCM, average age 51.1 ± 11.7 years, duration of LVAD support 119.9 ± 67.8 days; 16 patients with ICM, average age 57.2 ± 6.4 years, duration of LVAD support 92.4 ± 79.7 days). Additional samples of the apex, middle free wall, and base of the LV were collected from 2 patients with DCM and 4 patients with ICM. Immediately after removal, the samples were immersed in St Thomas cardioplegia solution and transported to the laboratory, snap-frozen in liquid nitrogen, and kept at −70°C. Selected samples were also saved for pathologic analyses and immunohistochemical studies. Tissues without grossly apparent scar were used for analysis.

#### Gelatin Zymography

Gelatin zymography was performed with myocardial extracts isolated from frozen cardiac samples as described previously, except without the previous activation of MMPs. MMP gelatinolytic activity was quantified with ImageQuaNT software (Molecular Dynamics).

#### Measurement of Protein Expression of MMPs and TIMPs

MMP-1, -3, and -9 protein contents were measured with ELISA kits (Amersham Life Science). The MMP-1 assay detects both free MMP-1, and that complexed with TIMP-1. The MMP-3 assay detects total MMP-3, including pro-MMP-3, active MMP-3, and MMP-3/TIMP complexes. The MMP-9 assay detects both free and TIMP-1–complexed pro-MMP-9 but has only 2.7% cross-reactivity to active MMP-9.

Western blot analyses of TIMP-1, -2, -3, and -4 were performed as described previously. Positive controls for human TIMP-1, -2, and -3 were included each time a membrane was made. TIMP-3 protein was first probed with purified polyclonal antibodies (AB802 at 1:500, Chemicon). The reactions were developed with Supersignal West Dura Extended Duration Substrate (Pierce), and the images were visualized on film by exposure to x-rays. The membranes were then stripped with 1% SDS elution solution (Pierce) and probed with TIMP-2 antibody (AB801 at 1:3000, Chemicon), stripped again and probed with TIMP-1 monoclonal antibody at 1 μg/mL (Oncogene Research Products), and stripped the third time and probed with TIMP-4 polyclonal antibody (AB816 at 1:3000, Chemicon). After the final detection, the filters were stained with BLOT-FastStain (Chemicon). The films and filters were digitized and quantified with ImageQuaNT software. For TIMP-2 and -3, only the band that migrated with the positive controls was quantified. The relative protein levels of TIMPs were calculated by setting the pre-LVAD value as 0 after normalization to BLOT-FastStain–stained total protein bands at 60 to 30 kDa.

### Immunohistochemical Staining of Myocardial pro-MMP-9 and TIMP-1

Double immunofluorescent stainings of pro-MMP-9 (AB805, Chemicon) and CD-68 (macrophage marker, Dako) of failing myocardial sections and of TIMP-1 (IM32L, Calbiochem) and dystrophin (myocyte marker) of post-LVAD myocardial sections were performed by routine protocols at our laboratory to colocalize pro-MMP-9 and TIMP-1 with their potential producing cells. Nuclei were stained with Hoechst-33258.

### Measurement of Different Collagens

The myocardial collagens can be fractionated into pepsin-soluble and -insoluble collagens, with the latter being mostly cross-linked. The myocardial pepsin-soluble collagens were extracted overnight with 5 mg/mL pepsin in 0.5 mol/L acetic acid. The soluble and insoluble collagens were separated by centrifugation at 2100g for 6 minutes at 4°C. The soluble collagens can be further separated into undenatured and denatured collagens, with the undenatured collagen being quantified with a Sircol collagen assay kit and soluble type I collagen as a standard (Accurate Chemicals). The total soluble and insoluble collagens were hydrolyzed and contents measured by determination of hydroxyproline by a modified Stegemann method. 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 collagens was calculated and used as a measure of collagen cross-linking.

### Statistical Analysis

Changes in gelatinolytic activity of MMPs and the expression of TIMPs after LVAD support were presented as percentages, with the pre-LVAD value taken as 0. Paired t test was used to compare measurements of pre- and post-LVAD support samples, and independent t test was used to compare those in DCM and ICM with SPSS software. The quantitative results are presented as mean ± SEM. Statistical significance was considered to be a value of P < 0.05.

### Results

Gelatin zymography detected 2 major and 1 minor gelatinolytic bands in the myocardial extracts. The 2 major bands were generated by MMP-2 and -9. The identity of the 120-kDa band was not clear. Because its change was similar to pro-MMP-9 and its size was the sum of pro-MMP-9 and TIMP-1, it may be pro-MMP-9 complexed with TIMP-1. MMP-2 gelatinolytic bands did not change after LVAD support. By contrast, MMP-9 was decreased in 26 of the 30...
failing hearts (Figure 1AB). The downregulation of MMP-9 in post-LVAD samples was statistically significant compared with pre-LVAD samples, with no regard to the duration of LVAD support \((P < 0.05)\). The extent of changes in MMP-9 gelatinolytic activity was similar between DCM and ICM patients (Figure 1C).

Consistent with the results of gelatin zymography, the pro-MMP-9 protein content as measured by ELISA was also significantly reduced in the failing hearts after LVAD support (Figure 1D, \(P < 0.05\)). In addition, pro-MMP-9 was significantly higher in ICM than in DCM patients \((P < 0.05)\), and the overall changes in pro-MMP-9 content were predominantly due to ICM. MMP-1 showed downregulation similar to that for pro-MMP-9 (Figure 1E, \(P < 0.05\)), whereas MMP-3 was expressed at low levels and did not change after LVAD support.

The activity of MMPs is controlled by their physiological inhibitors, especially the TIMPs. To explore whether there is a coordinate change in the expression of TIMPs, we assessed the expression of TIMP-1, -2, -3, and -4 by Western blot analysis. The results showed differential regulation of these TIMPs in the failing heart after LVAD support. TIMP-1 and -3 showed significant upregulation, whereas TIMP-2 and -4 showed no change. The upregulation of TIMP-1 and -3 was even higher in ICM patients (Figure 2).

To further determine the potential source of pro-MMP-9 and TIMP-1 in the heart, we examined the failing myocardium by immunohistochemistry. pro-MMP-9 was colocalized with macrophages and cardiomyocytes, and TIMP-1 with cardiomyocytes (Figure 3).

It is known that excessive exposure of collagen to active MMPs may lead to its denaturation and degradation. Therefore, the decreased MMPs and increased TIMPs favor less MMP activity, which would lead to less damage to the collagen. The myocardial pepsin-soluble collagen as well as insoluble collagen contents in both pre- and post-LVAD samples were examined. Indeed, the undenatured soluble collagen was significantly increased in both DCM and ICM patients after LVAD support (Figure 4A, \(P < 0.05\)). By contrast, no change in total collagen and total soluble collagen levels was found in combined and ICM patients after LVAD support (Figure 4, B and C). Furthermore, the ratio of undenatured soluble collagen to total soluble collagen was also increased (Figure 4D), although the change was due largely to that of ICM patients. The ratio of insoluble to total soluble collagen (Figure 4E) was increased, whereas the ratio of total soluble to total collagen was decreased (Figure 4F), after LVAD support in combined and DCM patients.

Because the pre-LVAD (apex) and post-LVAD (LV free wall) tissues were taken from different regions of the heart, we examined whether there were regional differences in collagen measurements in patients with DCM \((n=2)\) or ICM \((n=4)\). The results showed no difference in undenatured...
collagen, total soluble collagen, and the ratio of undenatured to total soluble collagens and that of insoluble to total soluble collagens among the samples from the apex, middle free wall, and base of the LV (Figure 5).

Discussion

Historically, LVAD support had been used exclusively as a bridge to transplantation, although recent reports suggest that LVAD support may provide a “bridge to recovery.”1–3 Although prolonged LVAD support has been shown to effect salutary changes in myocyte function, myocardial remodeling, and LV geometry,4–9 the cellular and molecular mechanisms responsible for alterations in the heart failure phenotype remain undefined. It is known that exposure of collagens, the major components of extracellular matrix, to activated MMPs may lead to their denaturation, partial degradation, and eventual complete degradation. Furthermore, because extracellular matrix remodeling is a critical feature of the failing heart, and the regulatory components of the extracellular matrix, ie, MMPs and TIMPs, are regulated at least in vitro by stretch, we tested the hypothesis that cardiac unloading by LVAD support could modify the property of the extracellular matrix. Consistent with this hypothesis, we have demonstrated that MMP-1 and -9 were downregulated and TIMP-1 and -3 were upregulated after LVAD support, a reversal of the findings in the failing human heart we reported previously.20 The reduced MMPs and increased TIMPs favor less destruction of normal extracellular matrix. Indeed, the undenatured collagen and its ratio to total soluble collagen were significantly increased after LVAD support.
The ratio of insoluble to total soluble collagen, a measure of collagen cross-linking, was also increased (Figure 4).

In addition to a direct role of myocardial unloading/removal of stretch, several other potential mechanisms could contribute to the regulation of MMPs and TIMPs in LVAD-supported hearts: (1) reduced cellular infiltrates, (2) reduced stimulation by proinflammatory cytokines, and (3) changes in ischemia.31 Infiltrating cells together with cardiomyocytes are the major source of pro-MMP-9 in the myocardium (Figure 3). Thus, reduced MMP-9 may be a result of decreased infiltrating cells and inflammation. TIMP-1 was produced by cardiomyocytes and localized around cardiomyocytes, which may have a role in the protection of those cells. Ischemia induces MMPs and suppresses TIMP-1 in the myocardium.31 The reversed changes in MMPs and TIMPs in LVAD-supported heart may imply improved perfusion of the myocardium. In addition, the patient’s age, medications, and comorbidities, such as diabetes, may also affect the expression of MMPs or TIMPs. Age and diabetes may have an effect through ischemia. There was no difference, however, in the levels of MMP-9 and TIMP-1 in patients treated with and those not treated with ACE inhibitors (captopril, enalapril, or lisinopril) or catecholamines (dobutamine or dopamine) in the present study (data not shown).

Other studies have investigated the effects of LVAD support on fibrosis; however, those studies have shown disparate results. Quantifying interstitial fibrosis by available techniques does not reliably predict the outcomes of LVAD support.2 Fibrosis has been shown to increase after LVAD placement in some patients, presumably as a result of ongoing cell death and replacement fibrosis.1 By contrast, decreased fibrosis has been demonstrated in patients who showed signs of recovery after LVAD support.1,4,32 One explanation for these disparities is that collagen content alone does not reliably measure changes in the extracellular matrix. Indeed, collagen cross-linking rather than collagen content is associated with ventricular dilatation in rat models.39 Alternatively, differences in treatment regimens, time of LVAD support, aggressiveness of weaning protocols, and the small sample size of those studies may have affected study outcome. Regardless, in our relatively large population of pre- and post-LVAD samples, LVAD support is clearly associated with alterations in MMPs, TIMPs, and collagen quality. The increased un-denatured collagen, its ratio to total soluble collagen, and collagen cross-linking rather than total collagen content, as demonstrated in the present study, may better serve as indicators of extracellular matrix remodeling as well as factors responsible for myocardial plasticity.

Previous reports have shown a reversal of ventricular dilation and an improvement in the end-diastolic pressure-volume relation of the failing heart after extended unloading with LVAD.4,9 The mechanisms by which mechanical unloading alters LV plasticity, however, were not defined. Furthermore, the long-term benefits of LVAD support have not been evaluated. Because the extracellular matrix is the primary support for cardiomyocytes, collagen denaturation and loss of function may play a significant role in the dilation process of the failing heart. Presumably, changes in the ratios of un-denatured and cross-linked collagens to total soluble collagens after LVAD support may reverse the dilation process. This hypothesis, however, presupposes that the expression of new collagens would allow for the reconstitution of the original architecture and an ability of myocytes to “slide back” to prefailure geometry.

In conclusion, the present study demonstrates that MMP-1 and -9 were significantly downregulated and TIMP-1 and -3 were significantly upregulated in the failing human heart after LVAD support. These changes were associated with increased un-denatured collagen, suggesting that decreased MMP diminished damage to the matrix collagen. The results suggest that the failing heart may benefit from LVAD support by changes at both the hemodynamic and molecular levels and that MMPs may play a significant role in LV plasticity in heart failure.

Acknowledgments

This study was supported by National Institutes of Health grants HL-66949-01 to Drs Li, McTiernan, and Feldman and HL-60032-01 to Drs McTiernan and Feldman. We thank the members of the Division of Cardiothoracic Surgery, University of Pittsburgh Medical Center, and the Department of Thoracic and Cardiovascular Surgery, Cleveland Clinic Foundation, for their assistance in the collection of cardiac specimens, and Romesh Draviam for technical assistance.

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_Circulation_. 2001;104:1147-1152
doi: 10.1161/hc3501.095215
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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