Increased Matrix Metalloproteinase Activity and Selective Upregulation in LV Myocardium From Patients With End-Stage Dilated Cardiomyopathy

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Background—One of the hallmarks of dilated cardiomyopathy (DCM) is left ventricular (LV) remodeling. The matrix metalloproteinases (MMPs) are a family of enzymes that contribute to extracellular remodeling in several disease states. Additionally, a family of inhibitors called tissue inhibitors of MMPs (TIMPs) has been shown to exist and to tightly regulate MMP activity. However, the types of MMPs and TIMPs expressed within the normal and DCM LV myocardium and the relation to MMP activity remain unexplored.

Methods and Results—Relative LV myocardial MMP activity was determined in the normal (n=8) and idiopathic DCM (n=7) human LV myocardium by substrate zymography. Relative LV myocardial abundance of interstitial collagenase (MMP-1), stromelysin (MMP-3), 72 kD gelatinase (MMP-2), 92 kD gelatinase (MMP-9), TIMP-1, and TIMP-2 were measured with quantitative immunoblotting. LV myocardial MMP zymographic activity increased with DCM compared with normal (984±149 versus 413±64 pixels, P<.05). With DCM, LV myocardial abundance of MMP-1 decreased to 16±6% (P<.05), MMP-3 increased to 563±212% (P<.05), MMP-9 increased to 422±64% (P<.05), and MMP-2 was unchanged when compared with normal. LV myocardial abundance of TIMP-1 and TIMP-2 increased by >500% with DCM. A high-molecular-weight immunoreactive band for both TIMP-1 and TIMP-2, suggesting a TIMP/MMP complex, was increased >600% with DCM.

Conclusions—This study demonstrated increased LV myocardial MMP activity and evidence for independent regulatory mechanisms of MMP and TIMP expression with DCM. These findings suggest that selective inhibition of MMP species within the LV myocardium may provide a novel therapeutic target in patients with DCM. (Circulation. 1998;97:1708-1715.)

Key Words: cardiomyopathy • enzymes • myocardium

The development of DCM is accompanied by LV dilation and pump dysfunction.1-2 In patients with DCM, the progressive LV dilation is associated with an increased incidence of morbidity and mortality.1,3,4 These clinical observations as well as experimental studies suggest that LV remodeling is an important contributory event in the progression to end-stage DCM.5-9 However, the structural basis and contributory mechanisms for the changes in LV geometry that occur during the progression of DCM remain unclear. An important constituent of the LV myocardium is the fibrillar collagen matrix, which contributes to the maintenance of LV geometry and the structural alignment of adjoining myocytes.10,11 Alterations in collagen structure and composition have been reported to occur within the LV myocardium in several cardiac disease states, which in turn may influence LV geometry.10,11 MMPs belong to an endogenous family of enzymes responsible for extracellular collagen degradation and remodeling.12,13 A number of species of MMPs have been described with reported differences in substrate specificity and differing abundance in various tissues.12-14 For example, stromelysin, or MMP-3, has been shown to activate other MMPs as well as to have affinity for a number of extracellular matrix proteins.12-14 Changes in MMP activity have been demonstrated in several disease states, including rheumatoid arthritis and tumor metastasis.12-14 More importantly, increased myocardial MMP activity has been reported to occur in both clinical and experimental forms of DCM.13,15-17 However, the types of MMPs expressed within the normal and DCM human LV myocardium and the relation to MMP activity remain unexplored. MMP activity is tightly controlled in normal myocardium by a family of closely related inhibitors known as TIMPs.12-14 Previous studies suggest that TIMP activity exists in normal myocardium15,16; however, the abundance and types of TIMPs expressed in normal human LV myocardium and the balance between MMP and TIMP abundance in the setting of DCM have not been examined. Therefore, the goals of the present study were twofold. First, this study examined the relative MMP activity and species...
abundance in normal and DCM human LV myocardium. Second, this study examined the relative abundance of the TIMP species and the balance between TIMP and MMP expression in normal and DCM human LV myocardium.

Methods

Tissue Source

Human left ventricular myocardium was obtained from explanted hearts from patients undergoing total orthotopic heart transplantation secondary to idiopathic DCM at this institution (Medical University of South Carolina). After induction of cardioplegic arrest, the hearts were removed and placed in a cold storage solution. Sections of the LV free wall were dissected, snap-frozen in liquid nitrogen, and stored at −70°C until use. In this study the underlying cause of DCM in all samples was identified as idiopathic. Ischemic hearts were excluded. Seven DCM hearts were included in this study, with ages ranging from 6 to 55 years and LV ejection fractions ranging from 20% to 40%. The homogenate was then centrifuged (4°C, 10 minutes, 800 g), and the supernatants were used for assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin (Sigma) under nonreducing conditions. The zymograms and immunoblotting samples were then purified and concentrated by size-fractionated banding pattern, which indicated MMP activity,

LV Myocardial MMP/TIMP Extraction

The LV myocardial samples were homogenized (3- to 30-second bursts) in 5 mL of an ice-cold extraction buffer (1:3 wt/vol) containing cacodylic acid (10 mmol/L), NaCl (0.15 mol/L), ZnCl2 (20 mmol/L), Na2N3 (1.5 mmol/L), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 minutes, 800g) and the supernatant was collected and used for assay. The zymograms and immunoblotting samples were then purified and concentrated by ultracentrifugation (4°C, 4.5 hours, 3000g, Centriplus-30, Amicon, Inc.).

Final protein concentration of the myocardial extracts was determined with a standard colorimetric assay (Bio-Rad Protein Assay). The extracted samples were then aliquoted and stored at −20°C.

Substrate Zymography

Extracts were thawed on ice, diluted to a final protein concentration of 400 μg/mL, and then incubated in activation buffer containing 0.005% Brij-35 and 1 mmol/L CaCl2 for 5 minutes at 37°C. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin (Sigma) under nonreducing conditions. The gels were run at 15 mA/gel through the stacking gel (4%) and at 20 mA/gel for the separating phase (10%), maintaining a running buffer temperature of 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 minutes each, rinsed in water, and incubated for 12 hours in a substrate buffer at 37°C (50 mmol/L Tris-HCl, 5 mmol/L CaCl2, and 0.02% NaN3, pH 7.5). After incubation, the gels were stained using 0.1% Amido Black, destained in water, analyzed, and dried for permanent record. Both nonstimulated and phorbol 12-myristate 13-acetate (PMA)-stimulated, conditioned media from an HT1080 fibrosarcoma cell line were used as a positive control (AG-770, Chemicon International, Inc.). For MMP-2 and MMP-9 a human epithelial cell line was used (AG-771, Chemicon International, Inc.). For MMP-3, TIMP-1, and TIMP-2 a human fibroblast cell line served a positive control (AG-770, Chemicon International, Inc.).

Data Analysis

The zymograms and the immunoblots were digitized with a Snapshot Photo Scanner 1 (Eastman Kodak Co). For the zymograms, the luminescent signal was detected by exposure to x-ray film (Amersham Life Science). The primary antisera were diluted in 1% powdered goat serum, 0.1% Tween-20, 0.08% BSA, 13% DME:F-12 cell culture media (Gibco Life Technologies), and 0.02% NaN3. For MMP-2 and MMP-9 a human epithelial cell line was used (AG-771, Chemicon International, Inc.). For MMP-3, TIMP-1, and TIMP-2 a human fibroblast cell line served a positive control (AG-770, Chemicon International, Inc.).

Statistical Analysis

MMP zymographic activity and relative abundance were compared between the normal and DCM samples with the Student’s t test. All
statistical procedures were performed with the BMDP statistical software package (BMDP Statistical Software Inc). Results are presented as mean ± SEM. Values of \( P < 0.05 \) were considered to be statistically significant.

Results

In the present study, normal and DCM LV myocardial extracts were treated and prepared in identical fashion for MMP zymography and immunoblotting.

Substrate Zymography

LV myocardial extracts were subjected to gelatin zymography in the basal state and after serine protease activation with trypsin. Representative zymograms for normal and DCM LV samples are shown in Figure 1. Abundant zymographic activity was observed in both normal and DCM samples, and this activity corresponded to molecular weights consistent with active species of MMPs. In the DCM samples, increased zymographic activity was observed in both the basal state and after serine protease activation. Representative densitometric profiles that were used to compute relative MMP activity are shown in Figure 2. The proteolytic activity quantitated from the gelatin zymograms was linear over different protein concentrations in both normal and DCM myocardium (Figure 3). In the DCM samples, total MMP zymographic activity was increased twofold in both the basal state and after serine protease activation. The individual values as well as the mean values obtained in the normal and DCM myocardial extracts with respect to MMP zymographic activity are shown in Figure 4.

With trypsin pretreatment, MMP zymographic activity was increased sixfold in both normal and DCM samples compared with the nonactivated state. To confirm that the trypsin pretreatment increased zymographic activity in myocardial extracts was due to serine protease activation of the MMPs, an additional series of studies was performed with the serine protease plasmin. Myocardial extracts were incubated at 37°C for 5 minutes in the presence and absence of plasmin (20 \( \mu \)g/mL, Porcine Plasmin, EC 3.4.21.7, Sigma) and then subjected to zymography. Increased proteolytic activity against the substrate gelatin was observed in both normal and DCM myocardial extracts treated with plasmin (Figure 5). Thus the increased zymographic activity that was obtained with either trypsin or plasmin pretreatment of myocardial extracts was likely due to serine protease activation of MMP. In an additional series of studies, MMP zymographic activity was examined in the presence of 10 mmol/L EDTA or 2 mmol/L of PMSF. Incubation with EDTA inhibited all zymographic activity consistent with past reports (not shown); however, in the presence of 2 mmol/L PMSF, a serine proteinase inhibitor, zymographic activity was unchanged, consistent with MMP activity.
MMP Immunoblotting
LV myocardial extracts were subjected to immunoblotting for specific species of MMPs: interstitial collagenase (MMP-1), 72 kD gelatinase (MMP-2), stromelysin (MMP-3), and 92 kD gelatinase (MMP-9). Representative immunoblots for normal and DCM samples are shown in Figure 6. MMP-1 was localized to the 57 kD region, MMP-2 to the 72 kD region, MMP-3 to the 59 and 45 kD regions, and MMP-9 to the 92 and 82 kD regions, corresponding to molecular weights consistent with these species of MMPs. The relative abundance of MMP-1 decreased in the DCM samples compared with normal (16 ± 6%, P < .05). In the DCM samples, the relative abundance of MMP-3 (563 ± 212%) and MMP-9 (422 ± 64%) were significantly increased (P < .05). The relative abundance of MMP-2 in the DCM samples was unchanged from normal (101 ± 21%, P > .50).

TIMP Immunoblotting
Representative immunoblots for TIMP-1 and TIMP-2 are shown in Figure 7. TIMP-1 localized to the 28 kD region and TIMP-2 to the 21 kD region, corresponding to molecular weights consistent with these species of TIMPs. The relative abundance of TIMP-1 and TIMP-2 were quantitated, and these results are summarized in the Table. TIMP-1 and TIMP-2 were increased over 5-fold in the DCM samples. A 6-fold increase in a 50 kD molecular weight band was observed for both TIMP-1 and TIMP-2 in the DCM samples. The relative ratio of TIMP-1 to MMP-1 increased > 60-fold, and the ratio of TIMP-2 to MMP-2 increased > 7-fold in the DCM samples compared with normal.

Discussion
Clinical observations as well as experimental studies have demonstrated that LV remodeling and dilation occur with the progression to end-stage LV failure. In addition, changes in...
the LV myocardial collagen matrix accompany the LV dilation observed with DCM. MMPs and TIMPs have been shown to exist in various tissues, including myocardium, and to be involved in collagen remodeling. More importantly, MMPs have been shown to be upregulated in various disease states. The relation between MMP activity and MMP and TIMP species abundance in LV myocardium in the setting of idiopathic DCM remained unexplored. Accordingly, this study examined MMP activity and MMP and TIMP abundance in normal and DCM human LV myocardium. The significant and unique findings of this study were threefold. First, this study demonstrated increased MMP zymographic activity in DCM myocardium. Second, alterations in the relative abundance of MMP species were observed with DCM. Specifically, interstitial collagenase (MMP-1) was reduced, stromelysin (MMP-3) and 92 kD gelatinase (MMP-9) were increased, whereas 72 kD gelatinase (MMP-2) was unchanged. Third, increased abundance of TIMP-1 and TIMP-2 occurred with DCM. Taken together, these findings suggest that selective upregulation of MMPs occurred in DCM and may contribute to the LV myocardial remodeling, which occurs in this disease process.

Alterations in the structure and composition of the LV myocardial collagen matrix have been reported to occur in several cardiac disease states. For example, Weber et al reported alterations in the myocardial fibrillar collagen architecture in patients with DCM. Tyagi et al reported increased collagen degradation products in DCM myocardial samples, providing evidence for increased collagen degradation. More recently, several studies have demonstrated increased myocardial MMP zymographic activity in patients with DCM. Furthermore, experimental models of LV dilation and failure reported increased MMP zymographic activity with the development of severe LV pump dysfunction. In the present study, increased LV myocardial MMP zymographic activity was observed with human end-stage DCM.

**Figure 5.** To more carefully determine that the increased zymographic activity in myocardial extracts with trypsin pretreatment was due to serine protease activation of the MMPs, an additional series of studies was performed with the serine protease plasmin. Myocardial extracts were incubated in the absence or presence of plasmin (PLN) or trypsin (TRN) and then subjected to zymography. The proteolytic zone obtained at the low molecular weight in the plasmin lane reflects the active light chain of plasmin (~28 kD). Increased proteolytic activity against the substrate gelatin was observed in both normal and DCM myocardial extracts pretreated with either plasmin or trypsin. Thus the increased zymographic activity that was obtained with either trypsin or plasmin pretreatment of myocardial extracts probably was due to serine protease activation of MMP.

**Figure 6.** Representative immunoblots for (from top to bottom, respectively) MMP-1 (57 kD), MMP-2 (72 kD), MMP-3 (59 and 45 kD), and MMP-9 (92 and 83 kD) in normal (N) and DCM (D) LV myocardial extracts. Approximate molecular weight reference is indicated to the left. For MMP-1, a diminished immunoreactive signal was observed in the DCM samples. For MMP-3 and MMP-9, the immunoreactive signal appeared increased in DCM samples. For MMP-2, there was no observable difference between normal and DCM. A positive control (+) was included in each immunoblot. For MMP-1, the positive control was cell culture media from a human fibrosarcoma cell line. For MMP-2 and MMP-9 a human epithelial cell line was used (AG-771, Chemicon International, Inc). For MMP-3, a human fibroblast cell line served as positive control (AG-770, Chemicon International, Inc). Relative LV myocardial abundance for each MMP species is summarized in the “Results” section.

**Figure 7.** Representative immunoblots of TIMP-1 (28 kD, left) and TIMP-2 (21 kD, right) in normal (N) and DCM (D) LV myocardial extracts. Approximate molecular weight reference is indicated to the left. For TIMP-1 and TIMP-2 the immunoreactive signal appeared increased with DCM. Additionally, a high-molecular-weight immunoreactive band was observed at 50 kD. Cell culture media from a human fibroblast cell line was included in each immunoblot as a positive control (+). Data for relative LV myocardial abundance of TIMP-1 and TIMP-2 are shown in the Table.
MMPs are synthesized and released into the extracellular space as inactive proenzymes. Subsequent activation requires the cleavage of an N-terminal amino acid sequence to induce a conformational change and expose the catalytic domain. The current study activated LV myocardial extracts with the serine protease trypsin before zymographic analysis. Activation of MMPs from the latent state can occur in a stepwise fashion with the production of several active intermediate forms during the transition to full activation. In the current study, the proteolytic activity that was observed in LV myocardial extracts after trypsin activation may have been due to the induction of intermediate active forms of MMPs, the unfolding and subsequent activation of specific MMP species, or a combination of both of these factors. Thus the zymographic activity that was obtained with trypsin activation in the present study was referred to as “recruitable” MMP activity. Significant recruitable MMP activity was observed in both normal and DCM myocardial extracts. More importantly, through this approach, significantly increased LV myocardial recruitable MMP activity was demonstrated with human end-stage DCM. These results suggest that in addition to increased basal MMP activity, increased recruitable MMP activity exists in DCM myocardium. Although the precise mechanism(s) for the in vivo activation of MMPs remain unclear, MMPs can be activated in vitro by serine proteases such as trypsin and plasmin. Furthermore, it has been demonstrated previously that mast cells within the myocardium synthesize a number of serine proteases such as chymase. Chymase activity has been demonstrated to be increased in the myocardium after both pressure- and volume-overload states. For example, Dell’Italia et al reported that the LV dilation caused by chronic mitral regurgitation in dogs was accompanied by an approximately twofold increase in myocardial chymaselike enzymatic activity. Thus increased serine protease activity such as chymase in DCM myocardium may result in heightened MMP activation. In light of the findings of the current study, the specific MMP activation cascade and the potential role of endogenous serine protease activity in DCM myocardium warrants further investigation.

In our study, the relative abundance of MMP species was determined with specific monoclonal antisera and high sensitivity chemiluminescence detection. The presence of MMP-1 was detected in both normal and DCM LV myocardial samples. However, the relative abundance of MMP-1 was significantly reduced with DCM. In a past report, Gunja-Smith et al demonstrated an immunoreactive band for MMP-1 in normal myocardium samples that was not detected with DCM. Taken together, the results of this study and the past report suggest that MMP-1 expression is significantly reduced with the development of end-stage DCM. Stromelysin, or MMP-3, is an important constituent of the MMP family because of its ability to degrade a wide range of extracellular matrix components and to activate other latent MMPs. In the present study, MMP-3 was detected in both normal and DCM LV myocardial samples. To our knowledge, this is the first study that has demonstrated that MMP-3 exists in human LV myocardium. More importantly and in contrast to what was observed with MMP-1, the relative abundance of MMP-3 was significantly increased with DCM. The increased abundance of MMP-3 in DCM myocardium may have contributed to overall LV zymographic activity as well as participated in the activation of other endogenous MMPs. The relative abundance of MMP-2 was similar in normal and DCM myocardial samples, whereas the relative abundance of MMP-9 was increased with DCM. In a canine model of pacing-induced heart failure, Armstrong et al reported that zymographic activity appeared to be increased for MMP-9, with no change for MMP-2. Taken together, these results suggest that the increased abundance of MMP-9 observed in the DCM myocardial samples probably contributed to the increased myocardial zymographic activity.

Previous studies have reported that species of MMPs are the products of separate genes and appear to be independently regulated. In this study, the relative abundance of MMP-1 was reduced by 80%, MMP-3 and MMP-9 were increased more than fourfold, and MMP-2 abundance remained unchanged with DCM. These differences in MMP relative abundance suggest that selective changes in MMP expression may have occurred within the DCM myocardium. Past studies have demonstrated that MMP expression can be influenced by a variety of cytokines and bioactive peptides. MacNaul et al using synovial fibroblasts, reported that interleukin-1 and tumor necrosis factor caused a sustained increase in MMP-3 expression, whereas MMP-1 expression normalized over time. Recent studies have demonstrated that the development of DCM is accompanied by increased circulating levels of these specific cytokines. Thus increased levels of specific cytokines with the development of DCM may modulate MMP species abundance. The MMPs are a family of metalloproteinases in which catalytic activity is influenced by substrate product stoichiometry. Thus alterations in the myocardial collagen matrix and MMP substrate availability may modulate MMP activity and expression with DCM. However, the present study only identified differences in steady-state MMP levels, thus whether the...
differences in MMP abundance with DCM are due to transcriptional, translational, or posttranslational processes remains to be defined.

The TIMPs are part of an endogenous system for the deactivation of MMPs and provide posttranslational regulation of MMP activity in various tissues including the myocardium. In this study, TIMP-1 and TIMP-2 were detected by immunoblotting in both normal and DCM myocardial samples. The relative abundance of both TIMP-1 and TIMP-2 was increased in DCM myocardium. Additionally, an \( \approx 50 \) kD molecular weight band was observed for TIMP-1 and TIMP-2 in both normal and DCM myocardium. Previous studies have reported that TIMP-1 forms a complex with MMP-1 and TIMP-2 forms a complex with MMP-2. The MMP/TIMP complex forms a stable noncovalent moiety that is not completely dissociated after SDS-PAGE or through preactivation by the phorbol ester PMA. Proteolytic treatment of an MMP/TIMP complex has been demonstrated to produce a partially active complex when analyzed by zymography. Furthermore, the monoclonal antiserum used in the present study recognizes the TIMPs in the unbound form as well as when complexed to MMP. Thus the 50 kD molecular weight band observed in the TIMP immunoblots may represent a TIMP/MMP complex. Previous studies have reported that TIMP expression is regulated independent of MMP expression. In the present study, the ratios of TIMP-1/MMP-1 and TIMP-2/MMP-2 were significantly increased in the DCM samples. These results suggest that TIMP-1 expression may be upregulated in DCM myocardium independent of MMP-1 expression. The degree of MMP inhibition that is achieved through the formation of MMP/TIMP complexes appears to occur in a stoichiometric fashion. However, whether and to what degree the changes in TIMP levels may influence MMP activity and activation states within the intact DCM myocardium remain to be established. It has been demonstrated that local MMP activity can be determined by in situ zymography. Thus future studies with in situ zymography may more clearly identify whether endogenous MMP activity is increased in LV myocardium.

There are limitations to this study that must be recognized. First, to maintain a uniform group of samples, only end-stage idiopathic DCM LVs were studied. Whether changes in MMP abundance and activity occur in inflammatory and ischemic forms of DCM warrant further investigation. Second, samples were obtained from patients in end-stage DCM treated with chronic ACE inhibition. It is likely that specific pharmacologic interventions may influence myocardial MMP abundance and/or MMP activity. For instance, Brilla et al recently reported that angiotensin II can influence MMP-1 activity in vitro. Thus future studies that use animal models of LV dilation and failure may provide insight into the mechanism(s) that regulate MMP expression and activity. For example, chronic rapid pacing in animals has been demonstrated to cause LV dilation and pump dysfunction and increased MMP activity. Finally, although our study demonstrated increased MMP abundance and activity in DCM myocardium, the cell types responsible for MMP production remain unknown. Nevertheless, the results of our study demonstrated increased LV myocardial abundance of MMP-3 and MMP-9 and increased MMP activity in DCM myocardium. Thus increased MMP activity may contribute to the LV remodeling that occurs in this disease process. Several recent studies have examined the use of synthetic MMP inhibitors in various disease states. For example, Wang et al recently reported that MMP inhibitor treatment reduced adenocarcinoma growth, spread, and metastasis in mice. Thus pharmacologic inhibition of MMP activity may directly influence extracellular remodeling in several disease states. While remaining speculative, the results of the present study suggest the intriguing possibility that MMP inhibition, particularly that targeted for MMP-3 and MMP-9, may be a novel therapeutic target in the setting of idiopathic DCM.

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