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.

(Key Words: cardiomyopathy • enzymes • myocardium)
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 <20%. The medications of the DCM patients included digoxin (seven of seven), diuretics (seven of seven), ACE inhibition (six of seven), and anticoagulation with coumarin (five of seven). Eight normal LV myocardium samples were obtained from donor hearts used for valve harvest (Cryolife, Inc). For all normal hearts there was no history of cardiac disease, and ages ranged from 16 to 22 years. Patient consent for the use of myocardial samples was obtained in all cases.

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/mL), NaN3 (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 decanted and saved on ice. The zymography and immunoblotting samples were then purified and concentrated by ultrafiltration (4°C, 4.5 hours, 3000g, Centricon-30, Amicon, Inc). Final protein concentration of the myocardial extracts was determined with a standardized colorimetric assay (Bio-Rad Protein Assay). The LV myocardial extracts were diluted to the appropriate loading concentration with sample buffer (0.1 mol/L Tris-HCl, 0.2 mol/L dithiothreitol, pH 6.8, containing 4% SDS and 0.01% bromophenol blue). LV extracts (4.0 μg) were loaded onto an 8% SDS-polyacrylamide gel and separated at 40 mA in 0.02 mol/L tris-base, 0.2 mol/L glycine, pH 6.8, containing 0.1% SDS. The separated proteins were transferred at 100 V to a nitrocellulose membrane (Trans-blot transfer medium, 0.45 μm, Bio-Rad Laboratories) in 0.025 mol/L tris-base, 0.2 mol/L glycine, pH 8.2, containing 20% methanol (vol/vol). Membranes were blocked with 0.2 mol/L tris-base, 1.4 mol/L NaCl, pH 7.6, containing 5% powdered goat milk, 0.1% Tween-20, and 0.02% NaN3. After washing with 0.2 mol/L tris-base, 1.4 mol/L NaCl, pH 7.6, containing 0.1% Tween-20, membranes were incubated overnight at 4°C in specific monoclonal antibodies corresponding to each MMP or TIMP species (1.0 μg/mL, Oncogene Research Products). The primary antisera were diluted in 0.2 mol/L tris-base, 1.4 mol/L NaCl, pH 7.6, containing 1% powdered goat milk, 0.1% Tween-20, 0.08% BSA, 13% DME/F-12 cell culture media (Gibco Life Technologies), and 0.02% NaN3. After stringent washing, the membranes were incubated for 1 hour in horseradish peroxidase–conjugated secondary antibody (1:5000 dilution, Bio-Rad Laboratories). The membranes were washed again and the horseradish peroxidase–conjugated secondary antibody was activated with peracid and luminol (ECL Western blotting detection reagents, Amershams Life Science). The luminescent signal was detected by exposure to x-ray film (Amershams Life Science) for exactly 5 minutes. A positive control was included in each immunoblot. For MMP-1, the positive control was cell culture media from PMA-stimulated HT-1080 fibrosarcoma cell line.13,24 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). Prestained molecular weight markers (Bio-Rad Laboratories) were used to ensure adequate protein separation and transfer.

Data Analysis

The zymograms and the immunoblots were digitized with a Snapshot Photo Scanner 1 (Eastman Kodak Co). For the zymograms, a single linear array (5 pixel width) was placed over each of the lysis areas and two-dimensional size-fractionated banding pattern, which indicated MMP activity, was determined by quantitated image analysis (Gel Pro Analyzer, Media Cybernetics). A fixed area of interest (AOI; 0.5×0.5 mm) was then placed over each of the lysis areas and two-dimensional integrated optical density (IOD) was computed as ΣOD(x, y) = ∑l/(−log(Intensity(x, y))−Black Reference)/Incident Light−Black Reference). For the immunoblots, a single linear array (5 pixel width) was placed over the center of each lane and the IOD was computed for each molecular weight species. The IOD was normalized to normal samples and assumed to be 100%.

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 $P < .05$ 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 with trypsin. 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 μg/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. The relative abundance of TIMP-1 and TIMP-2 was increased over 5-fold in the DCM samples. A 6-fold increase in a 50 kD molecular weight band was observed for both the normal and DCM groups. However, in the DCM group, MMP zymographic activity was approximately threefold higher than normal values (P < .05 vs 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. 

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. The approximate molecular weight reference is indicated to the left. Data for relative LV myocardial abundance of 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. The 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. 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 chymase-like enzymatic activity. Thus increased serine protease activity such as chymase in DCM myocardium may result in heightened MMP activity. Significant recruitable MMP activity was observed in both normal and DCM myocardial extracts. More importantly, based on the findings of the current study, the specific MMP activational 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 ∼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 activational 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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