A Matrix Metalloproteinase Induction/Activation System Exists in the Human Left Ventricular Myocardium and Is Upregulated in Heart Failure

Francis G. Spinale, MD, PhD; Mytsi L. Coker, BA; Lena J. Heung, BS; Brian R. Bond, PhD; Himali R. Gunasinghe, BS; Takuma Etoh, MD, PhD; Aron T. Goldberg, MD; James L. Zellner, MD; A. Jackson Crumbley, MD

Background—Matrix metalloproteinases (MMPs) contribute to matrix remodeling in disease states such as tumor metastases. Extracellular matrix metalloproteinase inducer (EMMPRIN) has been reported to increase MMP expression, and membrane-type MMP or MT1-MMP has been implicated to activate MMPs. The present study examined whether and to what degree EMMPRIN and MT1-MMP were expressed in human left ventricular (LV) myocardium as well as the association with MMP activity and expression in dilated cardiomyopathy (DCM).

Methods and Results—LV myocardial zymographic MMP activity increased by 2-fold with both nonischemic DCM (n = 21) and ischemic DCM (n = 16) compared with normal (n = 13). LV myocardial abundance of MMP-9 was increased with both forms of DCM. MMP-2 and MMP-3 were increased with nonischemic DCM. MMP-1 levels were decreased with both forms of DCM. EMMPRIN increased by 250% and MT1-MMP increased by 1000% with both forms of DCM.

Conclusions—Increased LV myocardial MMP activity and selective upregulation of MMPs with nonischemic and ischemic forms of DCM occurred. Moreover, a local MMP induction/activation system was identified in isolated normal human LV myocytes that was upregulated with DCM. The control of MMP activation and expression in the failing human LV myocardium represents a new and potentially significant therapeutic target for this disease process. (Circulation. 2000;102:1944-1949.)

Key Words: myocardium remodeling metalloproteinases cardiomyopathy

Left ventricular (LV) remodeling is an important contributory event in the progression to end-stage congestive heart failure (CHF). However, the underlying cellular and molecular basis for LV myocardial remodeling in patients with severe CHF remains poorly understood. As in most tissue remodeling processes, LV myocardial remodeling with CHF is accompanied by changes in the structure and composition of the collagen matrix.1-4 Matrix metalloproteinases (MMPs) are an endogenous family of zinc-dependent enzymes that have been identified to be responsible for matrix remodeling in several disease states.

See p 1874

Dilated cardiomyopathy (DCM) is a common cause of CHF in which a primary determinant of the disease process is LV remodeling. Although the underlying causes are diverse, DCM can be partitioned into nonischemic or ischemic origin. The first objective of this study was to quantify MMP activity and expression in normal myocardium as well as in that of nonischemic and ischemic DCM. The second objective was to identify a potential basis for alterations in MMP activity and abundance that occur in patients with end-stage DCM with a nonischemic or an ischemic cause.

Methods

Patients

Human LV myocardium was obtained from explanted hearts of patients (age 35 to 64 years) undergoing total orthotopic heart transplantation secondary to nonischemic (idiopathic; n = 21) or ischemic (n = 16) DCM. Sections of the LV free wall were snap-frozen in liquid nitrogen and stored at −70°C until use. Nonischemic DCM was identified by preoperative presentation and ischemic DCM confirmed by coronary catheterization studies. Patients with confirmed myocarditis were excluded from this study. In the patients with DCM, medications included digoxin (95%), diuretics (98%), ACE inhibitors (78%), β-adrenergic antagonists (9%), and calcium channel blockers (4%). The treatment penetration was equivalent in both forms of DCM (χ² = 2.97, P = 0.226). Normal LV myocardial samples (n = 13; age 16 to 22 years) were obtained from donor hearts not matched for transplantation or used for valve harvest (Cryolife, Inc). For the isolated myocyte studies, myocardial biopsies were obtained from 6 patients with normal LV ejection fraction (>60%) undergoing elective coronary artery bypass surgery as described previously.5 Patient consent was obtained for all myocardial samples used in the study, and protocols were approved by the Medical
Proteases such as trypsin or plasmin. In the present study, trypsin was used to activate myocardial extracts with trypsin. It has been demonstrated previously that the stepwise activation of MMPs can be elicited by serine proteases such as trypsin or plasmin.1–4,6,7

Gelatinase activity was assessed similarly after preactivation of LV myocardial samples with trypsin.1–3,6 A positive control was included in all immunoblots (MMP-13: CC068, MT1-MMP: CC1042, Chemicon; EMMPRIN: Human Breast Cancer Preparation).

Myocardial Immunoblotting

Past studies identified a cell-surface protein in specific tumor cell lines that caused the induction of MMP expression termed extracellular matrix metalloproteinase inducer (EMMPRIN).9–12 Membrane-type MMP (MT1-MMP) is a recently discovered MMP that has the capacity to activate other MMPs, including collagenase-3 (MMP-13).1,9,11 Accordingly, relative EMMPRIN, MT1-MMP, and MMP-13 levels were measured in normal and DCM myocardial samples by immunoblotting.2,3,6 A positive control was included in all immunoblots (MMP-13: CC068, MT1-MMP: CC1042, Chemicon; EMMPRIN: Human Breast Cancer Preparation).

Myocyte Isolation and Immunohistochemistry

The LV myocardial biopsy was placed in a collagenase solution within a microtitration system developed by this laboratory.5,8,14 A yield of >60% of viable myocytes was obtained from each isolation. LV myocytes were washed, incubated with primary antisera for MT1-MMP (1:300) or EMMPRIN (1:20) overnight at 4°C, washed, and incubated with conjugated goat-FITC antisera (1 hour; 25°C). LV myocytes were double-stained with α-actinin antibodies conjugated to rhodamine red-X and nuclei identified by propidium iodide with confocal microscopy as described previously.14 Colocalization of the cytoskeletal–sarcomeric–associated protein α-actinin, MT1-MMP, and EMMPRIN were performed by means of confocal microscopy (Olympus FluoView BX50WI).

Data Analysis

For comparisons of MMP zymography and ELISA values between normal, ischemic DCM, and nonischemic DCM, an ANOVA was first performed. If ANOVA revealed significant differences, pairwise tests of individual group means were compared by means of Bonferroni probabilities. For relative comparisons between groups for immunoblotting results, the Kruskal-Wallis ANOVA was used. Pharmacological treatment was considered a categoric variable, and MMP activity was examined by ANOVA. Results are presented as mean±SEM. Values of P<0.05 were considered statistically significant.

Results

All quantitative data with respect to MMP activity, MMP/TIMP abundance, and EMMPRIN abundance are shown in the Table.

MMP Zymographic Activity in Normal and DCM Human Myocardium

LV myocardial MMP zymographic activity was detected in all human LV myocardial samples at 50 to 90 kDa, consistent with the molecular weights of several MMP species including MMP-2 and MMP-9 (Figure 2).4,7 Moreover, MMP zymographic activity was increased in both forms of DCM. LV myocardial MMP zymographic activity after trypsin activation increased by >3-fold from basal values (Figure 2). Total

### Table

<table>
<thead>
<tr>
<th>Sample</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-9</th>
<th>COMP</th>
<th>TIMP-1</th>
<th>EMMPRIN abundance, %</th>
<th>MT-MMP-1 abundance, %</th>
<th>Sample size, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>780±148</td>
<td>380±45*</td>
<td>294±35*</td>
<td>1920±172</td>
<td>1369±154*</td>
<td>754±36</td>
<td>474±39</td>
<td>1099±139*</td>
<td>100</td>
</tr>
<tr>
<td>DCM</td>
<td>394±91</td>
<td>675±61*</td>
<td>365±67†</td>
<td>394±91</td>
<td>675±61*</td>
<td>365±67†</td>
<td>365±67†</td>
<td>1099±139*</td>
<td>100</td>
</tr>
<tr>
<td>Ischemic DCM</td>
<td>535±93</td>
<td>549±36</td>
<td>447±39</td>
<td>535±93</td>
<td>549±36</td>
<td>447±39</td>
<td>447±39</td>
<td>1099±139*</td>
<td>100</td>
</tr>
</tbody>
</table>

*P<0.05 vs normal. †P<0.05 vs DCM. ‡Computed as percentage of normal.
recruitable MMP zymographic activity was higher in both nonischemic and ischemic DCM. There was no relation between increased MMP activity in DCM patients when stratified with respect to pharmacological treatment ($P > 0.9634$).

**MMP Abundance in Normal and DCM Human Myocardium**

MMP-9 was increased in both ischemic and nonischemic DCM. MMP-2 was increased 2-fold in nonischemic DCM but was unchanged in ischemic DCM. MMP-1 was reduced in both nonischemic and ischemic DCM. A robust immunoreactive signal was observed for collagenase 3 (MMP-13) in normal human myocardium (Figure 3) and was increased with both forms of DCM. Stromelysin (MMP-3) was increased in nonischemic DCM but was unchanged with ischemic DCM.

**MMP Inhibition in Normal and DCM Human Myocardium**

A mechanism for controlling MMP activity in normal tissue is through a family of closely related inhibitors known as tissue inhibitors of MMPs, or TIMPs.$^{4,7}$ One of the more ubiquitous TIMPs, TIMP-1, has been demonstrated in LV myocardium previously.$^6$ With both nonischemic and ischemic DCM, TIMP-1 abundance was similar to normal values. However, actual MMP-1/TIMP-1 complex abundance was reduced with nonischemic DCM and was further reduced with ischemic DCM.

**Local MMP Induction and Activation in Normal and DCM Myocardium**

EMMPRIN levels, as measured by immunoblotting, were increased in both nonischemic and ischemic DCM (Figure 3). Relative EMMPRIN levels were increased in ischemic DCM when compared with nonischemic DCM. MT1-MMP, as measured by immunoblotting (Figure 3), was localized at $\approx 50$ kDa. MT1-MMP was substantially increased in ischemic and nonischemic DCM.

In the first series of studies, purified LV myocyte sarcolemmal preparations were prepared and immunoblotting was performed (Figure 4). A robust signal was obtained in LV sarcolemmal preparations for both MT1-MMP and EMMPRIN (Figure 3). Both MT1-MMP and EMMPRIN were localized by immunofluorescence to human LV myocytes by confocal microscopy (Figure 5). Colocalization of both MT1-MMP and EMMPRIN to the LV myocyte sarcolemma was observed. A punctate and well-defined colocalization with $\alpha$-actinin was also observed with MT1-MMP.

**Discussion**

Although the activation of MMPs has been identified to occur in a number of disease states, whether and to what degree of activation of this local proteolytic system occurs in failing human myocardium as the result of DCM remain unknown. There were 3 significant findings of the present study regarding myocardial MMP activity and expression in severe end-stage human DCM. First, MMP zymographic activity was increased in DCM and was accompanied by increased levels of certain MMP species, including the emergence of unique MMPs without a compensatory increase in the levels of the endogenous inhibitors of MMPs (TIMPs). Second, a local induction/activation system was localized to the LV myocyte sarcolemma that consisted of EMMPRIN and MT1-MMP. Third, this local inducer/activation system was significantly upregulated in DCM. These findings provide new mechanistic insight into the cellular and molecular basis for...
fibrillar collagens such as collagen type I and III. Thus, the report to demonstrate the emergence of this MMP species in microscopy was performed to localize MMP-9 is synthesized by myocytes, fibroblasts, and smooth laminin and fibronectin. Past studies have demonstrated that MMPs possess the capacity to degrade a number of interstitial because of a high affinity for this substrate. However, these network to degradation.

MMP levels within the DCM myocardium may contribute to the increased LV myocardial MMP activity that occurs in end-stage human heart failure.

**MMP and TIMP Species Expression**

With both ischemic and nonischemic DCM, a common pattern of myocardial content for the interstitial collagens MMP-1 and MMP-13 was observed. Specifically, MMP-1 was reduced with DCM and was accompanied by increased levels of collagenase 3, or MMP-13. MMP-13 has been identified to be expressed in human breast carcinoma and in osteoarthritic chondrocytes. In both forms of DCM, MMP-9 levels were increased without a concomitant increase in TIMP-1 levels. TIMP-1 binds to the active site of the MMPs by blocking access to their substrate.

MMP-2 was increased only in nonischemic DCM. The MMPs contain certain regulatory DNA sequences within the promoter region that bind transcription factors that are influenced by a number of intracellular signaling pathways. The development of DCM is accompanied by increased neurohumoral system activity such as increased levels of norepinephrine and tumor necrosis factor-α. These bioactive molecules can induce formation of transcription factors, which will bind to the MMP promoter region. MMP-9 and MMP-2 contain dissimilar promoter sequences and regulatory elements. Thus, the different levels of MMP-2 with nonischemic and ischemic forms of DCM probably are due to the differences in the underlying cause of the disease process, which in turn may result in the stimulation of different intracellular signaling pathways and selective activation of MMP-2. Although further studies regarding the regulatory mechanisms of MMP expression in the myocardium are warranted, results from the present study demonstrated that MMPs are differentially regulated in end-stage DCM.

MMP-1 induction may depend on cooperative binding of the PEA-3 element and the formation of the transcription factor AP-1, whereas stromelysin, or MMP-3 transcription, can occur independent of the AP-1 response. Thus, increased extracellular stimuli such as neurohormones and cytokines, which occur with DCM, may induce differential levels of MMP-1 and MMP-3 expression. In the present study, MMP-1 levels were reduced in both forms of DCM, whereas MMP-3 levels were increased in nonischemic DCM. MMP activation can be achieved through a final common enzymatic pathway requiring MMP-3. Moreover, MMP-3 can participate in the activation cascade required to achieve activation of MMP species relevant to the LV remodeling process. Thus, the increased MMP-3 expression that occurred in nonischemic DCM may be an important contributory mechanism for the LV remodeling in this form of heart failure.

TIMPs bind to the active site of the MMPs by blocking access to extracellular matrix substrates. The MMP/TIMP complex is formed in a stoichiometric 1:1 molar ratio and forms an important endogenous system for regulating MMP activity in vivo. Although up to 4 TIMPs have been identified to date, the most well characterized of these endogenous inhibitors of MMPs are TIMP-1 and TIMP-2. MMP-1, TIMP-1, and TIMP-2 form a complex with several MMPs, which include MMP-1 and MMP-9. In the present study, there was no change in absolute TIMP-1 levels with DCM, but a reduction in MMP-1/TIMP-1 complex formation occurred. One contributory factor for the reduction in the levels of this specific MMP/TIMP complex was the absolute reduction in MMP-1 levels with DCM. TIMP-1 binds to other MMPs such as proMMP-9, which slows the activation process of this MMP species. In both forms of DCM, MMP-9 levels were increased without a concomitant increase in TIMP-1 levels. Thus, the ratio of MMP-9 to TIMP-1 was reduced, which in turn may have contributed to the increased MMP zymographic activity and the reduction in MMP-1/TIMP-1 complex formation observed with DCM. Although the role of other myocardial TIMPs remains to be defined, the focus of this study as well as past reports was MMP induction and activation.

MMPs are secreted in a proenzyme form and require proteolytic cleavage for activation, most notably by serine...
proteases. The present study demonstrated heightened MMP activity in DCM by zymography that increased after preactivation with the serine protease trypsin. Endogenous myocardial MMP activation by serine proteases could involve chymase, which has been demonstrated to be increased during both pressure- and volume-overload states. There are problematic issues that surround in vitro zymographic measurements that prevent direct extrapolation to in vivo LV myocardial MMP activity. First, this assay does not provide a measure of total MMP content and species identification. Second, the zymographic assays were performed under optimal enzymatic conditions and substrate availability and in the absence of the influence of TIMPs. These issues were addressed in part by the present study through the direct measurement of MMP and TIMP abundance and MMP/TIMP complex formation. Another limitation of the present study is that MMP zymographic activity was measured in end-stage DCM. Whether increased MMP activity exists in milder forms of heart failure remains to be established. Finally, although the entire complement of LV myocardial cells including fibroblasts, smooth muscle cells, endothelial cells, and the LV myocyte itself have been demonstrated to produce MMPs, the focus of the present study was first, to examine global MMP expression and activity in whole myocardial tissue, and second, to determine the existence of a local cellular MMP induction and activation system in LV myocardium. Given that the present study has identified both EMMPRIN and MT1-MMP in LV myocytes, and this laboratory routinely performs isolated LV myocyte studies, future directions will entail a more careful examination of this local induction and activation system in an isolated LV myocyte system.

MMP Induction and Activation

Although serine proteases such as plasmin and trypsin contribute to MMP activation through proteolytic cleavage of the C-terminal propeptide domain, recent studies have demonstrated that the membrane-bound MMPs, MT-MMPs, can induce local MMP activation. MT1-MMP has been clearly shown to participate in the MMP activation process in a number of cell systems and in extracellular matrix degradation. For example, pro–MMP-2 will bind to a specific extracellular domain of MT1-MMP, resulting in full activation of MMP-2. Past studies have demonstrated that in human fibroblast cultures, MMP-13 activation occurs by MT1-MMP. Although the complete mechanism of MT-MMP mediated MMP activation is still not completely understood, MT-MMPs provide a more precise means for localizing MMP activation and extracellular matrix degradation. Furthermore, MT1-MMP is not inhibited by TIMP-1, which provides further support that increased levels of MT1-MMP would result in increased local extracellular proteolytic activity and degradation. In the present study, a significant increase in the levels of myocardial MT1-MMP occurred with DCM and could be localized to the LV myocyte sarcolemma. With the use of confocal microscopy, sarcomeric α-actinin and MT1-MMP coalesced at the sarcolemma, consistent with the location of costameres. This would suggest that MT1-MMP is in proximity to extracellular binding domains, which would implicate MT1-MMP in modulating local myocyte adhesion to the extracellular matrix. It has been demonstrated that cytokines such as tumor necrosis factor-α upregulate MT1-MMP in several cell systems. Moreover, it has been demonstrated that MT1-MMP can yield the mature form of tumor necrosis factor-α, which suggests that an important autoinduction system may exist for MT1-MMP in DCM. Because DCM is accompanied by increased neurohormonal and cytokine synthesis, it is likely that these extracellular signals contribute to the induction of myocardial MT1-MMP.

A large portfolio of extracellular stimuli can alter the expression of MMPs in various cell systems. In severe end-stage congestive heart failure such as DCM, it is likely that a number of neurohormonal and cytokine signaling cascades contribute to alterations in myocardial MMP expression and activity. However, it remained unclear whether a local MMP induction system existed at the level of the LV myocyte and whether this system may be upregulated in DCM. EMMPRIN is a 58-kDa, membrane-bound protein that has been identified in both normal (keratinocytes) and diseased human tissue (breast and lung carcinoma). Exposure of human fibroblasts to recombinant EMMPRIN caused an induction of MMP-1, MMP-2, and MMP-3. Whether and to what degree other MMPs are induced by EMMPRIN remain to be defined.

Basal expression of EMMPRIN has been reported in a number of tissue types, suggesting multiple roles for this transmembrane protein. The present study clearly defined increased abundance of EMMPRIN in failing human myocardium. This provides circumstantial evidence that EMMPRIN may facilitate MMP expression in DCM myocardium. Identification of extracellular binding domains that interrupt EMMPRIN function would be an important avenue of future study that may provide a definitive approach for detecting the role of EMMPRIN in myocardial remodeling. In the present study, EMMPRIN was identified in normal human LV myocardium. With confocal microscopy, a definitive staining pattern for EMMPRIN was localized to the LV myocyte sarcolemma. Using multicellular culture systems, it has been postulated that EMMPRIN induces MMP expression by a cell-cell interaction or a paracrine-mediated effect. Increased myocardial EMMPRIN levels were observed in both forms of DCM and could be localized to the LV myocyte sarcolemma. Thus, increased sarcomemal levels of EMMPRIN in DCM may enhance MMP induction through this cell-cell interaction.

Through immunoprecipitation experiments, Berditchevski and colleagues demonstrated that EMMPRIN forms a complex with αβ1 integrin. Functions of integrins include cell-cell adhesion, extracellular matrix-cell adhesion, and transduction of cellular signaling cascades. The coexistence of EMMPRIN and αβ1 integrin suggests that EMMPRIN-mediated MMP induction may be influenced by both the composition of and stress placed on the extracellular matrix. The intracellular signaling pathways by which EMMPRIN facilitates MMP expression remain to be fully elucidated but probably involve tyrosine kinase pathways. The EMMPRIN protein sequence contains a PKC phosphorylation site, which may also be an important intracellular regulatory mechanism.
Digoxin, diuretics, and were treated with conventional medical therapy (ie, ACE inhibition, of disease progression. In the present study, patients with DCM changes in chamber geometry are an important prognostic indicator to MMP induction and activation (Figure 6). The local myocardial MMP induction/activation system identified in the present study may represent an important system responsible for LV remodeling in the heart failure process.

Although the causes of CHF are diverse, a common event in the progression of this disease process is LV remodeling, resulting in increased wall stress and subsequent pump dysfunction. An important cause of CHF is DCM, in which significant LV remodeling and changes in chamber geometry are an important prognostic indicator of disease progression. In the present study, patients with DCM were treated with conventional medical therapy (ie, ACE inhibition, digoxin, diuretics, and β-adrenergic antagonists). Such therapeutic approaches for CHF have been focused on modifying LV load or by interrupting the effects of specific neurotransmitters. Despite the high treatment penetrance, MMP activity and expression remained increased in patients with DCM. This observation would suggest that current conventional therapy for CHF fails to significantly influence MMP expression and activation processes. Past studies as well as present observations would support the concept that direct modulation of the LV myocardial remodeling process through the control of MMP expression and activity would be an important therapeutic strategy in the setting of developing CHF. The present study identified that a local MMP induction and activation pathway exists in the normal human LV myocardium that is upregulated with DCM. Moreover, localization of this system to the LV myocyte suggests that a paracrine loop exists with respect to MMP induction and activation (Figure 6). The local myocardial MMP induction/activation system identified in the present study may represent an important system responsible for LV remodeling in the heart failure process.

This study was supported by National Institutes of Health grants HL-45024 (F.G.S.), HL-97012 (F.G.S.), and PO1-HL-48788 (F.G.S.).

References
A Matrix Metalloproteinase Induction/Activation System Exists in the Human Left Ventricular Myocardium and Is Upregulated in Heart Failure

doi: 10.1161/01.CIR.102.16.1944
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
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:
http://circ.ahajournals.org/content/102/16/1944

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/