Differential Expression of Tissue Inhibitors of Metalloproteinases in the Failing Human Heart

Yun You Li, MD, PhD; Arthur M. Feldman, MD, PhD; Yi Sun, MD, PhD; Charles F. McTiernan, PhD

Background—Extracellular matrix turnover is regulated by matrix metalloproteinases (MMPs) and a family of tissue inhibitors of metalloproteinases (TIMPs). Together, these proteins may contribute to myocardial remodeling in congestive heart failure. We hypothesized that the expression of MMPs and TIMPs might be differentially regulated in the failing human heart.

Methods and Results—Northern blot analyses were performed with probes to TIMP-1 to -4 and GAPDH with poly A+ mRNA from ventricular tissues of patients with ischemic cardiomyopathy (ICM, n=16) or idiopathic dilated cardiomyopathy (DCM, n=15) and nonfailing control hearts (n=15). TIMP-1 to -4 and MMP-9 proteins were quantified by ELISA and/or Western blot, and the total gelatinolytic activity was studied by gelatin zymography. The results showed that cardiac expression of TIMP-1 and -3 transcripts and proteins was significantly reduced in ICM and DCM. No significant difference was observed in TIMP-2 and -4 transcripts. However, TIMP-4 protein was significantly reduced in ICM myocardium. MMP-9 protein content and total gelatinolytic activity were upregulated in the same samples.

Conclusions—These studies demonstrated a selective downregulation of TIMPs along with upregulation of MMP-9 and gelatinolytic activity in the failing hearts, alterations that favor matrix degradation and turnover. These findings might be of pathophysiological significance and might suggest new therapeutic targets for limiting the ventricular remodeling and dilatation process characteristic of the failing human heart. (Circulation. 1998;98:1728-1734.)

Key Words: heart failure ■ metalloproteinases ■ remodeling

The pathology of the end-stage failing human heart is characterized by myocyte loss, myocardial collagen accumulation and collagen fibril disruption, remodeling of the extracellular matrix, and disorganization of the cardiac myofibrils. It has been proposed that this maladaptive remodeling contributes to the diminished systolic function as well as the decreased compliance of the failing human heart.1 Furthermore, investigators have suggested that the increased fibrosis and ventricular dilatation are secondary to either myocardial damage due to ischemic cardiomyopathy (ICM)2 or inflammation-mediated damage in patients with idiopathic dilated cardiomyopathy (DCM). However, fibrosis is often identified in the absence of an inflammatory infiltrate, and factors that regulate fibrosis and remodeling are not well understood but probably include local tissue-generated substances.3 Therefore, the mechanisms responsible for the alterations in the extracellular matrix in the failing human heart remain undefined.

Recently, 2 families of proteins have been identified that regulate the extracellular matrix in a variety of tissues, including the myocardium: the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).4 An increase in the expression or activity of MMPs results in increased proteolytic activity in the extracellular spaces, leading to increased extracellular remodeling. Alternatively, decreased expression of TIMPs can effect a similar tissue response. Increased activity of MMPs or decreased expression of TIMPs can result in enhanced proteolytic activity, fibrillar collagen degradation, progressive myocyte loss, and ventricular dilatation and sphericalization. Indeed, increased MMP activities have been observed in the failing human heart.5 However, the expression and the role of TIMPs in the failing human heart are not well defined.

Four TIMPs have been cloned and purified, the most recent being TIMP-4.6 Each is encoded by a unique gene, yet all share both structural and functional similarities, with conservation of 12 cysteine residues important for tertiary structure. Although each TIMP has tissue-specific expression, all are expressed in the heart, with TIMP-4 being the most cardiac-specific.6 Furthermore, the various TIMP proteins respond differently to pharmacological and physiological stimuli.7 Recent studies using differential display suggested that car-

Received February 18, 1998; revision received April 17, 1998; accepted May 27, 1998.
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dilomyocyte TIMP-3 is differentially regulated by proinflammatory cytokines.\textsuperscript{8} Although studies have evaluated the expression of TIMP-1 in failing human heart, there was no concordance between steady-state levels of TIMP-1 mRNA and the quantity of TIMP-1 protein.\textsuperscript{9,10} Furthermore, expression of remaining 3 TIMPs in failing myocardium remains unexplored. In the present study, we quantify TIMP-1 to TIMP-4 at both the mRNA and protein levels, in addition to MMP-9 and gelatinolytic activity, in failing and nonfailing human hearts. These studies suggest a downregulation of TIMP-1, -3, and -4 and an upregulation of MMP-9 and gelatinolytic activity, which might play an important role in the pathogenesis of congestive heart failure (CHF).

**Methods**

This study was performed according to the guidelines outlined in the Declaration of Helsinki. Before tissue harvest, consent was obtained from transplant patients for the use of tissue specimens for research. All chemicals were purchased from Sigma Chemical Co unless otherwise indicated.

**Cardiac Tissue Sample Collection**

Patients diagnosed with ICM or DCM in NYHA functional classes III to IV were included in the present study. Diagnosis of ICM or DCM was based on patient charts, findings of 2-dimensional echocardiography, hemodynamics, and coronary angiography. The end-stage heart failure patients had a relatively standard therapeutic regimen, including diuretics, digoxin or intravenous inotropes, and ACE inhibitors. The explanted samples were immediately immersed in ice-cold St Thomas cardioplegic solution (mmol/L: NaCl 147.2, MgCl\textsubscript{2} 16, KCl 20, NaHCO\textsubscript{3} 10, and CaCl\textsubscript{2} 2.25) for transport to the laboratory. The samples were snap-frozen in liquid nitrogen, and stored at −80°C until use. The explanted samples were immediately immersed in liquid nitrogen, and stored at −80°C until use (Table 1). All but 3 of the samples were transmural sections taken from the free wall of the left ventricle in areas that were free of infarction or scarring. The 3 samples from the right ventricular free wall (the left ventricular free wall was not available for research purposes) were removed from patients with bised heart failure. Nonfailing ventricular samples were obtained from cardiac donors whose hearts were unsuitable for transplantation.

**Total RNA and poly A\textsuperscript{+} mRNA Isolation**

Tissue samples were pulverized under liquid nitrogen, and total RNA was isolated via acid phenol extraction.\textsuperscript{11} RNA samples were enriched for polyadenylated species by oligo(dT)/magnetic bead capture (Promega).

**Northern Blot Analysis**

The TIMP-1, -2, and -4 and GAPDH cDNA probes were prepared by reverse transcription–polymerase chain reaction amplification of heart RNA with primers shown in Table 2. The TIMP-3 cDNA probe (177 bp, corresponding to GenBank L27424 nucleotides 920 to 1096) was isolated from differential display of rat cardiomyocyte RNA.\textsuperscript{8} The nucleotide sequences of all cDNA probes were determined to confirm their identities. Poly A\textsuperscript{+} mRNA samples enriched from 110 μg of total RNA were subjected to Northern blot analysis, as previously reported.\textsuperscript{8} The membranes were stripped after each hybridization and rehybridized sequentially with radiolabeled TIMP-1 to -4 and GAPDH probes. The hybridization results of

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<th>Table 1. Clinical Characteristics of the Patient Population</th>
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LVED indicates left ventricular end-diastolic diameter; LVES, left ventricular end-systolic diameter; PCWP, pulmonary capillary wedge pressure; and n, number of patients available for those data.

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<th>Table 2. Primers Used for Reverse Transcriptase–Polymerase Chain Reaction Amplification of cDNA Probes</th>
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<td>GenBank No.</td>
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<tr>
<td>Timp-1 L31883</td>
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TIMPs were quantified with ImageQuaNT software (Molecular Dynamics) and were normalized to that of the GAPDH probe and in turn normalized to the mean of the nonfailing controls, which was arbitrarily set as 100%.

ELISA
Frozen cardiac samples were homogenized in 50 mmol/L Tris-HCl, 75 mmol/L NaCl, and 1 mmol/L PMSF, pH 7.5, with 1 mL buffer per 50 mg tissue. The homogenates were centrifuged at 10 000g at 4°C for 20 minutes. The protein concentration of the supernatants was measured with a modified Bradford reaction (Bio-Rad Laboratories) with bovine globulin as a standard. The supernatants were assayed for TIMP-1 and -2 contents with commercially available ELISA kits and human TIMP-1 and -2 standards, respectively (Amersham Life Science). The absorbance at 630 nm was read spectrophotometrically with a microtiter plate reader. The kits detect total TIMP-1 or -2, both free and complexed with metalloproteinases. All assays were performed in duplicate.

Western Blot Analysis
The same samples as used for ELISA of TIMP-1 and -2 proteins were also used for Western blot analyses of TIMP-1, -3, and -4 and MMP-9 proteins. The Western blot analysis for TIMP-1 served to validate the ELISA. Equal amounts (60 µg) of proteins were separated on 12% SDS-PAGE and electroblotted onto nitrocellulose membrane (Micron Separations). TIMP-1 and -3–positive controls were included and electroblotted in parallel with the myocardial samples. TIMP-1 protein was detected with a monoclonal antibody (1 µg/mL, Oncogene Research Products). TIMP-3 protein was probed with polyclonal antiserum (1:600) or with a monoclonal anti–human TIMP-3 antibody (5 µg/mL, Oncogene Research Products). TIMP-4 protein was probed with rabbit anti-TIMP-4 polyclonal antibody (1:3000, Chemicon International). The MMP-9 protein was detected with a rabbit polyclonal antibody against activated human MMP-9 (1:2000, Chemicon International). Horse-radish peroxidase–conjugated goat anti-rabbit IgG (1:7500, Schleicher & Schuell) and anti-mouse IgG (1:20 000, Pierce) were used as secondary antibodies for the polyclonal or monoclonal primary antibodies, respectively. The reactions were developed with enhanced chemiluminescence reagents (NEN Life Science or Pierce), and the images were obtained by exposure to x-ray films. The films were digitized and quantified with the ImageQuaNT software. The results were presented as percent change compared with nonfailing controls, the means of which were arbitrarily set as 100%.

Gelatin Zymography
Gelatin zymography of myocardial protein extracts was performed as described.13 MMPs in 30 µg myocardial protein extracts were activated with 7 µg/mL trypsin for 15 minutes. The trypsin was then inhibited by addition of PMSF to 50 mmol/L; 10 mmol/L freshly containing 2.5% SDS without -mercaptoethanol or boiling and sample loading buffer containing 2.5% SDS without β-mercaptoethanol or boiling and elecrophoresed onto nitrocellulose membrane (Micron Separations). TIMP-1 and -3–positive controls were included and electroblotted in parallel with the myocardial samples. TIMP-1 protein was detected with a monoclonal antibody (1 µg/mL, Oncogene Research Products). TIMP-3 protein was probed with polyclonal antiserum (1:600) or with a monoclonal anti–human TIMP-3 antibody (5 µg/mL, Oncogene Research Products). TIMP-4 protein was probed with rabbit anti-TIMP-4 polyclonal antibody (1:3000, Chemicon International). The MMP-9 protein was detected with a rabbit polyclonal antibody against activated human MMP-9 (1:2000, Chemicon International). Horse-radish peroxidase–conjugated goat anti-rabbit IgG (1:7500, Schleicher & Schuell) and anti-mouse IgG (1:20 000, Pierce) were used as secondary antibodies for the polyclonal or monoclonal primary antibodies, respectively. The reactions were developed with enhanced chemiluminescence reagents (NEN Life Science or Pierce), and the images were obtained by exposure to x-ray films. The films were digitized and quantified with the ImageQuaNT software. The results were presented as percent change compared with nonfailing controls, the means of which were arbitrarily set as 100%.

Statistical Analysis
The data are presented as mean±SEM. One-way ANOVA was applied to compare changes in expression levels of MMP-9 and TIMPs in different groups. When a significant F value was obtained, comparison among the means was performed with the post hoc Student-Newman-Keuls analysis test with SPSS statistical analysis software.14 Statistical significance was considered at P<0.05.

Results
Table 1 presents clinical characteristics of patients included in the studies. On the basis of NYHA classification and findings from physical examination, 2-dimensional echocardiography, and cardiac catheterization, all heart failure patients displayed markedly diminished cardiac function. The nonfailing donors included a slightly younger population with more equivalent representation of male and female patients. Complete cardiac functional studies were not available on any of the nonfailing heart donors, although none presented a history of heart failure.

As seen in Figure 1, one TIMP-1 transcript was detected in the poly A+ mRNA; minor bands at 1.8 and 4.2 kb are believed to correspond to residual 18S rRNA and 28S rRNA. The TIMP-1 mRNA levels were significantly downregulated in the failing human heart (P<0.01). Consistent with mRNA levels, there was significantly less TIMP-1 protein in the failing heart, as quantified by ELISA (P<0.01). Furthermore, in contrast to mRNA levels, TIMP-1 protein was significantly
lower in patients with ICM than in patients with DCM (P < 0.05, Figure 1B and 1C). The measurement of TIMP-1 protein via ELISA was confirmed by Western blot analysis with a monoclonal antibody, which showed close correlation between the results obtained by the 2 different methods (Figure 1D).

The TIMP-2 cDNA probe detected 2 transcripts in the same membrane as used for TIMP-1 Northern blot. However, neither of these 2 transcripts changed in the failing heart compared with nonfailing ones (Figure 2A). Consistent with the mRNA levels, the protein level of TIMP-2 was similar in both failing and nonfailing hearts (Figure 2B).

The TIMP-3 cDNA probe detected 3 transcripts of 4.5, 2.3, and 0.9 kb. Downregulation of the 4.5- and 2.3-kb TIMP-3 transcripts was observed in the failing heart compared with nonfailing controls (P < 0.04, Figure 3A). This change was demonstrated in patients with either ICM or DCM. Interestingly, the 0.9-kb transcript showed no change. Consistent with the Northern blot analysis, expression of the 23-kDa TIMP-3 protein was downregulated in the myocardium of both ICM and DCM patients (P < 0.01). The 29-kDa band, which showed coregulation with the 23-kDa TIMP-3, is believed to correspond to the glycosylated form of TIMP-3.

One additional unidentified protein of 64 kDa was also detected by both the polyclonal and monoclonal TIMP-3 antibodies (Figure 3B).

The human TIMP-4 cDNA probe detected 1 major (1.4-kb) and 1 minor (2.1-kb) transcript on the same membrane as used for Northern blot analysis of the other 3 TIMPs. Neither transcript showed altered levels of expression between nonfailing and failing hearts (Figure 4A). Western blot analysis detected a robust 24-kDa band in the myocardial homogenates (Figure 4B). In contrast to the mRNA expression, TIMP-4 protein levels were significantly decreased (P < 0.01) in ICM hearts but not in DCM hearts (n = 8 each).

The levels of TIMP-1 to -4 in the 3 right ventricles of bisided heart failure patients did not differ from those in the left ventricles. Although reduced TIMP levels in general (including nonfailing controls as a whole) were associated with reduced cardiac function, the individual TIMP protein level in a given patient correlated poorly with pulmonary
capillary wedge pressure, cardiac index, left ventricular shortening fraction, or end-diastolic and end-systolic diameters (linear regression data not shown).

Because the TIMP-1 Western blot yielded higher-molecular-weight species that could conceivably arise from MMP/TIMP-1 complexes and prior studies suggested specific interactions between MMP-9 and TIMP-1,15,16 we also assessed the levels of MMP-9 protein by reprobing the same filters as used for TIMP-1 Western blot. The active forms of MMP-9 (67 and 64 kDa) showed significant upregulation in the failing hearts (Figure 5A). Because other MMPs may also contribute to the proteolysis in the myocardium, we assessed the total gelatinolytic activities in the failing hearts with gelatin zymography. As seen in Figure 5B, the multiple banding pattern reflects MMP gelatinolytic activity. The bands at 64 and 62 kDa, similar to those reported previously,13 are likely to reflect activated MMP-2 or MMP-9. The overall gelatinolytic activities were markedly increased in both DCM and ICM hearts. Furthermore, the levels of TIMP-1 and MMP-9 in the same group of patients were antithetically regulated (Figure 5C).

Discussion
Congestive heart failure has been associated with maladaptive extracellular matrix remodeling resulting from an imbalance in the synthesis and degradation of extracellular matrix components.17 Increased degradation of extracellular matrix proteins due to augmented activity of MMPs observed in the heart of patients with cardiomyopathies18 may facilitate ventricular dilatation and remodeling. Conversely, an important modulation of extracellular matrix turnover is the local expression of physiological inhibitors of MMPs: the TIMPs. Thus, both TIMPs and MMPs are involved in the turnover of extracellular matrix and appear to play an important role in...
the remodeling of myocardium and blood vessels during the transition from compensation to decompensation.1,19

We have systematically studied both DNA and protein expression of all known TIMPs, the naturally occurring inhibitors of MMPs in human heart, and demonstrated that TIMPs are differentially regulated in failing hearts compared with nonfailing controls. In the case of TIMP-1 and -3, expression was downregulated at both the mRNA and protein levels, regardless of disease origin. However, TIMP-4 mRNA was not altered in the failing hearts, yet its protein was downregulated selectively in the hearts of ICM patients.

It was not surprising that there was differential regulation among the 4 TIMPs, because each is encoded by a different and unique gene. In addition, analysis of the promoter regions shows that 3 of the TIMP genes contain a diverse array of regulatory sequences.12,20,21 Because of this apparent complexity of regulation, it is unclear why differential expression of TIMPs occurs in patients with heart failure of different causes, such as idiopathic and ischemic. However, the alteration of TIMP-4 at the protein but not at the mRNA level suggests a role for posttranscriptional regulation of TIMP expression in the failing myocardium. Finally, it should be noted that recent reports suggest that the expression of TIMPs may be regulated by proinflammatory cytokines,22–24 and therefore, variations in cytokine levels25 might contribute to the differential regulation of TIMPs seen in our patients. Because TIMP-1, -2, and -4 form complexes with pro–MMP-2 or pro–MMP-9,16,26 it is also possible that the downregulation of free TIMPs results from upregulation of pro-MMPs. In the case of TIMP-1 and -2, levels in the study reflect the total, because the assay detects both the free and the complexed forms of TIMPs. In patients with ICM, we cannot rule out possible conversion of free TIMP-4 to species not recognized by anti–TIMP-4 antibody.

MMP-9 is one of the family of MMP proteins expressed in the heart,9,27 and one that may specifically interact with TIMP-1.15,16,28 We observed an antithetical expression of TIMP-1 and MMP-9 in the failing hearts (Figure 5C). Recent studies of the 92-kDa MMP-9 in a human fibrosarcoma cell line show that activation of the proenzyme results in an intermediate form of 83 kDa and 2 fully active forms of 67 and 64 kDa.29,30 All these forms would be recognized in the Western blotting, because the antibody was raised against active forms of MMP-9. Indeed, Western blot identified the activated forms of MMP-9 (67 and 64 kDa) in addition to the pro–MMP-9 form and the intermediate form of 83 kDa (Figure 5A).

The overall downregulation of TIMPs in the presence of increased content and activities of MMPs (Figure 5A and 5B)10,31 may affect extracellular matrix remodeling in the failing heart. Indeed, disregulated collagen degradation and synthesis, fibrosis, myofibril disarray, and progressive cardiomyocyte loss are characteristic findings in the failing human heart.17 Because each of the failing hearts came from a patient with end-stage disease at the time of cardiac transplantation, the cardiac functional data were not sufficiently scattered to allow for meaningful correlations of TIMP expression with severity of cardiac dysfunction. Furthermore, because cardiac function is determined by various factors, it is not surprising to find poor correlation between the levels of TIMPs with the pulmonary capillary wedge pressure, cardiac index, left ventricular diameters, or shortening fraction. The various permutations of pharmacotherapies made it difficult to tease out the relative contributions of any one pharmacotherapy to alterations of gene expression. Therefore, we would note that animal studies that allow for controlled use of specific therapies will be required to better understand the effects of various medications on TIMP and MMP expression in the heart.

In summary, TIMPs are differentially downregulated in the failing human heart. TIMP-1 and -3 are significantly downregulated at both the mRNA and protein levels in both DCM and ICM, whereas TIMP-4 protein is downregulated only in ICM. The downregulation of TIMPs along with the upregulation of MMP and gelatinolytic activity favor matrix degradation and turnover during cardiac remodeling. This finding might be of pathophysiological significance and might suggest new therapeutic targets for limiting the ventricular remodeling and dilatation process characteristic of the failing human heart.

Acknowledgments
We thank Drs Robert Kormos, Si Pham, and Brack Hattler (Division of Cardiothoracic Surgery, University of Pittsburgh Medical Center) for their assistance in the collection of cardiac tissue specimens.

References


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Circulation. 1998;98:1728-1734
doi: 10.1161/01.CIR.98.17.1728

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