Excessive Activation of Matrix Metalloproteinases Coincides With Left Ventricular Remodeling During Transition From Hypertrophy to Heart Failure in Hypertensive Rats

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OBJECTIVES

We sought to elucidate how the local activation of matrix metalloproteinases (MMPs) is balanced by that of the endogenous tissue inhibitors of MMP (TIMPs) during left ventricular (LV) remodeling.

BACKGROUND

Although it is known that the extracellular matrix (ECM) must be altered during LV remodeling, its local regulation has not been fully elucidated.

METHODS

In Dahl salt-sensitive rats with hypertension, in which a stage of concentric, compensated left ventricular hypertrophy (LVH) at 11 weeks is followed by a distinct stage of congestive heart failure (CHF) with LV enlargement and dysfunction at 17 weeks, we determined protein and messenger ribonucleic acid (mRNA) levels of LV myocardial TIMP-2 and -4 and MMP-2, as well as their concomitant activities.

RESULTS

No changes were found at the LVH stage. However, during the transition to CHF, TIMP-2 and -4 activities, protein and mRNA levels were all sharply increased. At the same time, the MMP-2 mRNA and protein levels and activities, as determined by gelatin zymography, as well as by an antibody capture assay, showed a substantial increase during the transition to CHF. The net MMP activities were closely related to increases in LV diameter (r = 0.763) and to systolic wall stress (r = 0.858) in vivo.

CONCLUSIONS

Both TIMPs and MMP-2 remained inactive during hypertrophy, per se; they were activated during the transition to CHF. At this time, the activation of MMP-2 surpassed that of TIMPs, possibly resulting in ECM breakdown and progression of LV enlargement. (J Am Coll Cardiol 2002;39:1384–91) © 2002 by the American College of Cardiology Foundation

Left ventricular hypertrophy (LVH) is an adaptive response to various mechanical and hormonal stimuli, but eventually it causes congestive heart failure (CHF) (1). Among several pathologic factors that might be activated during the transition from adaptive LVH to CHF, qualitative and quantitative changes of the myocardial extracellular matrix (ECM) may be the critical ones inducing changes in myocyte alignment and, thus, remodeling of the left ventricular (LV) geometry (2–5). However, the cellular and molecular mechanisms regulating myocardial ECM remain poorly understood.

Matrix metalloproteinases (MMPs) belong to a family of proteolytic enzymes that has been implicated as playing a key role in ECM degradation in several disorders (6). Experimental animal hearts (7,8) and human hearts (9) with advanced heart failure have also shown increases in MMP enzyme activity or messenger ribonucleic acid (mRNA) levels. However, the actual proteolysis that occurs might depend on a balance between MMPs and the tissue inhibitors of MMP (TIMPs) (10). Also, the net activity of MMPs and its correlation with the process of chamber remodeling have not yet been clarified. Thus, in the present study, using an animal model in which the process from mechanically compensated LV hypertrophy to heart failure can be clearly distinguished, we examined whether an imbalance between MMPs and TIMPs may occur concurrently with the progression of hypertrophy or with the transition to heart failure (11).

METHODS

Experimental animals and in vivo assessment of LV geometry and function. Male inbred Dahl salt-sensitive (DS) rats were fed an 8% sodium chloride (high-salt) diet after the age of six weeks (11). The animals were sacrificed at the stages of LVH (11 weeks, n = 16) and CHF (17 weeks, n = 15). Normotensive Dahl salt-resistant (DR) rats were used as the age-matched control animals (sacrificed at 11 weeks [LVH stage, n = 14] and CHF stage, n = 14)]. Systolic blood pressure and the echocardiographically guided LV dimension were measured (11) on the day before sacrifice. The animals were treated in accordance with the “Position of the American Heart Association on Research Animal Use,” adopted by the
Abbreviations and Acronyms
bp = base pair
cDNA = complementary deoxyribonucleic acid
CHF = congestive heart failure
DR = Dahl salt-resistant
DS = Dahl salt-sensitive
ECM = extracellular matrix
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
LV = left ventricular
LVH = left ventricular hypertrophy
MMP = matrix metalloproteinase
mRNA = messenger ribonucleic acid
RT-PCR = reverse transcriptase-polymerase chain reaction
TIMP = tissue inhibitor of matrix metalloproteinase

Association in November 1984, and with the institutional guidelines of Kyoto University Graduate School of Medicine.

Northern and Western blot analyses. Total ribonucleic acid was isolated from the LV by using the acid guani- dinium thiocyanate-phenol-chloroform method. The com- plementary deoxyribonucleic acid (cDNA) probes used in this study were the following: 1) TIMP-2, a 378-base pair (bp) fragment isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, with primers complementary to positions 513 to 890 of the rat cDNA sequence (12); 2) TIMP-4, a 375-bp fragment isolated by RT-PCR amplification, with primers complementary to positions 111 to 485 of the rat cDNA sequence (13); 3) MMP-2, a 301-bp fragment isolated by RT-PCR, with primers complementary to positions 1666 to 1966 of the rat cDNA sequence (14); and 4) human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), purchased from the American Type Culture Collection (Manassas, Virginia). The polymerase chain reaction products were purified and subcloned into pBluescript (Stratagene, La Jolla, California). The results of Northern blotting of each sample were normalized by the amount of GAPDH mRNA.

Forty micrograms of homogenized LV protein was electrophoresed under reducing conditions on 8% polyacrylamide gels and transferred to nitrocellulose filters (Hybond ECL, Amersham, Buckinghamshire, United Kingdom). Membranes were incubated with specific rabbit anti-TIMP-2, TIMP-4 and MMP-2 antibodies (Chemicon International, Temecula, California) as the primary antibod- ies, at a dilution of 1:3,000, and with horseradish peroxidase-conjugated goat anti-rabbit antibody as the sec- ondary antibody, at a dilution of 1:2,000. The immune complexes were detected with a chemiluminescence kit (ECL, Amersham).

Quantitative zymography and reverse zymography. The zymographic method for the measurement of MMPs in the rat myocardium has been described previously (15). Gelatin (Sigma Chemical Co., St. Louis, Missouri) was used as a substrate and was added to a standard 8% polyacrylamide gel mix to achieve a final concentration of 1 mg/ml. Samples (40 μg) and 0.2 to 1.0 μg of purified human MMP-2 (Chemicon) were directly loaded onto gels under nonreducing conditions. After electrophoresis, the gels were twice soaked in 2.5% Triton X-100 (Sigma) for 30 min at room temperature, rinsed in water and incubated overnight (12 h) at 37°C in substrate buffer (50 mmol/l Tris–HCl [pH 8], 5 mmol/l CaCl2 and 0.02% NaN3). After incubation, the gels were stained for 30 min in 0.125% Coomassie blue R-250 and then destained in 30% ethanol and 10% acetic acid in water.

Quantitative reverse zymography was performed as recently described (16). Protein extracts were subjected to 12% polyacrylamide gels containing 2.25% gelatin and latent 72-kDa gelatinase (Biogenesis, Poole, England). After incubation overnight at 37°C, the gels were stained with Coomassie blue R-250. Activity of TIMP appeared as a dark band where MMP-related gelatinase activity was blocked.

Measurement of active MMP-2 levels by antibody capture assay. The endogenous levels of active MMP-2 were measured without p-aminophenylmercuric acetate by using an antibody capture method, according to the manufacturer’s instructions (RPN2631, Amersham Pharmacia Biotech) (17).

Statistical analysis. All data are expressed as the mean value ± SEM. The significance of differences among the mean values of two factors—age (LVH at 11 weeks and CHF at 17 weeks) and type (DS and DR)—was analyzed by two-way factorial analysis of variance. We have also described three p values: p_a for the age factor; p_t for the type factor; and p_i for the interaction. When there was an interaction between two factors, various groups were ana- lyzed with post-hoc comparisons by using the Tukey- Kramer test. The other groups were analyzed with the t test for each factor. In all tests, p < 0.05 was considered statistically significant.

RESULTS

Heart weight, systolic blood pressure and echocardiogra- phy at the LVH and CHF stages. The systolic blood pressure, LV weight and echocardiographic measurements are presented in Table 1. At 11 weeks, the DS rats showed concentric LVH, as reported previously (11). Systolic LV wall stress was equal for these two groups, implying that mechanical compensation had been established for the increased afterload. At 17 weeks, the LV diameter increased by 18% in the DS rats, and this was associated with a 56% decrease in fractional shortening and a 3.4-fold increase in LV wall stress. Thus, along with the process of mechanical decompensation against excessive afterload, the LV geometry showed an alteration from the concentric to the eccentric pattern of hypertrophy (i.e., LV remodeling).
Expression of TIMP during the transition from LVH to CHF. The TIMP activity was quantified as the density of a 21-kDa single band on the reverse zymogram, which corresponded to TIMP-2 and TIMP-4 (Fig. 1). The activity was the same for the DS and DR rats at 11 weeks. However, it showed an increase of 103% at 17 weeks (1.81 ± 0.2 in 17-week-old DS rats vs. 0.89 ± 0.1 in 17-week-old DR rats; p < 0.01). The levels of TIMP protein and mRNA were also quantified using the same method (Fig. 2). In the 17-week-old DS rats, TIMP-2 and -4 protein levels were increased by 21.7% and 56.8%, respectively. The TIMP-2 mRNA increased by 112% in the 11-week-old DS rats and by 142% in the 17-week-old DS rats. The TIMP-4 mRNA levels remained unchanged at 11 weeks; however, it increased by 77% at 17 weeks. Taken together, the TIMPs were regulated at their transcriptional level and were not activated by hypertrophy, per se. However, they were activated during the transition to heart failure.

Expression of MMP during the transition from LVH to CHF. The activity of MMP-2 in cardiac extracts was quantified as a band of gelatinolytic activity, based on its specific molecular weight (54, 68 and 72 kDa), which was confirmed by comparison with commercially available, pure MMP-2 (Fig. 3A) (18), whose proteolytic activity was inhibited by ethylenediamine-tetracetic acid but not by phenylmethyl sulfonyl fluoride (data not shown). Only MMP-2 activity was detected on the gelatin zymogram. The endogenous MMP activity was unchanged for both the DS and DR rats at 11 weeks. In contrast, at 17 weeks, the net MMP activity in the DS rats showed an 89.2% increase, as compared with that in the age-matched DR rats (Fig. 3B). We also calculated the ratio of 68- to 72-kDa bands on the zymogram (Fig. 3C). The ratio was significantly increased in rats with CHF as compared with the age-matched DR rats and DS rats at 11 weeks. The protein level of MMP-2 showed changes that parallelled its gelatinolytic activity (1.7 ± 0.2 arbitrary unit in the DS rats with CHF vs. 1.0 ± 0.1 in 17-week-old DR rats; p < 0.01) (Fig. 4A). The MMP-2 mRNA level was also significantly increased at 17 weeks in the DS rats (2.26 ± 0.05, p < 0.01), as compared with that of the DS rats at 11 weeks (1.48 ± 0.15) and the age-matched DR rats (1.21 ± 0.22, p < 0.01) (Fig. 4B). When the net MMP activity of each animal was plotted against the in vivo LV variables, the MMP activity showed a linear correlation with the LV end-diastolic diameter (r = 0.763) and with LV systolic wall stress (r = 0.858). Thus, MMP activation occurred in a manner parallel to that of LV remodeling.

Endogenous levels of active MMP-2. The endogenous levels of active MMP-2 were further determined by an antibody capture assay. The active MMP-2 level of the DS rats with CHF (2.14 ± 0.04 ng/ml) was significantly higher than that of the age-matched DR rats (1.81 ± 0.06 ng/ml, p < 0.01) and the DS rats at 11 weeks (1.95 ± 0.04 ng/ml, p < 0.01), by using the t test. The results were consistent
with the MMP-2 activity determined by gelatin zymography. In this case, the three p values are $p_a = 0.0390$, $p_t < 0.0001$ and $p_i = 0.0675$.

**DISCUSSION**

**Cardiac remodeling and MMPs.** The activation of MMPs has been reported in the failing myocardium in several experimental animal models. Dynamic expression and activation of MMPs have been implicated in the morphologic changes that occur after myocardial infarction in either infarct-related or peri-infarct regions (8). In swine with pacing-induced heart failure, the activities of MMP-1, -2 and -3 increased, along with an increase in the LV end-diastolic diameter (7). Thus, the increased MMPs might be closely associated with the geometric remodeling in diseased hearts. This hypothesis was further supported by long-term pharmacologic interventions in rats (17,19). In the present study, although the MMP activation occurred during the transition to CHF around the age of 17 weeks in DS rats, the collagen level did not show a decrease; rather, it was increased by 13% (data not shown). That is, the increased fibrosis could mask a small but significant decrease in the structural collagen matrix that surrounds myocytes, myofibrils and muscle bundles. Increased MMP activity may induce this decrease in the structural collagen matrix during the process of LV remodeling in the transition to heart failure. This issue can be addressed by an evaluation of the ultrastructural collagen matrix, but this is beyond the scope of our current research.

Our data indicate that the net MMP-2 activation may be the determining factor for LV enlargement and dysfunction. Two structural mechanisms have been identified in the pathogenesis of ventricular dilation in the failing heart. One is the side-to-side slippage of myocytes, and the other is the elongation of myocytes (20). Spinale et al. (4) reported that MMP activity was increased and the fibrillar collagen weave appeared reduced and disrupted in pigs with CHF caused by pacing-induced supraventricular tachycardia. Tyagi et al. (18) measured soluble collagen-derived peptide of type I collagen in cardiac extracts obtained from patients with
Figure 2. Results of measurement of tissue inhibitor of matrix metalloproteinase (TIMP) protein (A) and messenger ribonucleic acid (mRNA) (B) in left ventricular extracts of Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) rats at the left ventricular hypertrophy (LVH) and congestive heart failure (CHF) stages. The TIMP-2 and -4 proteins and mRNA were measured by Western and Northern blot analyses, respectively. The TIMP mRNA level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The data are expressed as arbitrary units (values for 11-week-old DR rats were set at 1.0, and remaining values were adjusted accordingly) and mean values ± SEM. *p < 0.01, †p < 0.05. (A) p, = 0.0207, p, = 0.7292 and p, = 0.0127 for TIMP-2 by Western blot; p, = 0.0002, p, = 0.0002 and p, = 0.0021 for TIMP-4 by Western blot. (B) p, = 0.0746, p, < 0.0001 and p, = 0.1669 for TIMP-2 mRNA by Northern blot; p, = 0.0007, p, = 0.0050 and p, = 0.1804 for TIMP-4 mRNA by Northern blot. W = weeks.
dilated cardiomyopathy. They demonstrated that the collagen-derived peptide level, which indicates collagen degradation, was increased in the myocardium with dilated cardiomyopathy, as compared with normal hearts. Because MMP activation directly leads to degradation or damage of the fibrillar collagen network that connects myocytes, this mechanism might lead to a reduction in the myocardial contractile force, muscle fiber slippage and realignment, with thinning of the myocardium, and eventual chamber dilation.

Relationship between MMPs and TIMPs. The TIMPs participate in the endogenous system, which provides post-translational regulation of MMP activity in various tissues, including the myocardium (21). In this study, TIMP-2 and -4 were detected by reverse zymography and immunoblotting of the LV myocardium. Both TIMP proteins were upregulated in a manner parallel with MMPs. Active forms of MMPs are inhibited by an interaction with the amino-terminal domain of TIMPs, which form tight-binding, 1:1 complexes with active enzyme sites. In addition, TIMP-2 and -4 form specific complexes with latent MMP-2, presumably by an interaction between the carboxyl-terminal domains of both the enzyme and inhibitor (22). In contrast, recent studies have demonstrated that the MMP-2 activation process required the formation of the ternary activating complex of MMP-2/TIMP-2/membrane type 1-MMP. Thus, TIMP-2 promotes the activation of MMP-2 by forming complexes with MMP-2 and membrane type 1-MMP. Mazzieri et al. (23) reported that surface-bound pro-MMP-2 also can be activated by the cell surface-associated urokinase-type plasminogen activator/plasmin system. These lines of evidence suggest that the simple ratio of TIMP-2 to MMP-2 can be misleading in terms of measuring the absolute amount of proteolytic activity. Therefore, we determined the absolute MMP-2 activity by an antibody capture method.

Characteristics of cardiac TIMPs in heart failure. Although each of the four known TIMPs is encoded by a unique gene and has tissue-specific expression, it is known that all four isoforms are expressed in the human heart. Li et al. (24) reported that TIMP-1 and -3 were significantly reduced in patients with end-stage heart failure, whereas the TIMP-2 and -4 transcripts appeared unchanged in the failing myocardium. In the present study, we could not detect the activities of other TIMPs (TIMP-1 and -3) by reverse zymography in the LV myocardium of experimental rats. This difference may result from several causes, including the following explanation: in patients with end-stage heart failure, the disease process is usually over a period of years, during which a number of pharmacologic interventions, including angiotensin-converting enzyme inhibitors, beta-adrenergic blockers or catecholamines, might be continuously prescribed. In our animal model showing the transition to heart failure, the controlled experimental condition revealed that TIMP-1 and -3 activation does not necessarily occur in this setting, and that TIMP activation was not persistent, but rather timed with the stress event.

Actually, Chua et al. (25) reported that angiotensin II induces upregulation of TIMP-1 in endothelial cells of the rat heart. Thus, it is conceivable that factors such as...
interleukin-1-beta, tumor necrosis factor-alpha and transforming growth factor-beta1, which have been strongly associated with the transition to heart failure in this animal model (26), might regulate TIMP expression (10).

Conclusions. Both TIMPs and MMPs remained inactive during the process of hypertrophy, per se, but were activated during the transition to CHF. At this time, the activation of MMPs surpassed that of TIMPs, which might induce the structural collagen matrix breakdown, resulting in the progression to LV enlargement.

Acknowledgment
The authors wish to thank Dr. Shunzo Maetani for his critical review of the statistical analyses.

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