Differential activation of matrix metalloproteinases in heart failure with and without ventricular dilatation

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Abstract

Objective: Remodeling of extracellular matrix (ECM) is considered to contribute to progression of left ventricular (LV) remodeling and matrix metalloproteinases (MMPs) play crucial roles in regulation of ECM. Activation of MMPs is observed in systolic heart failure (SHF) and is suggested to be responsible for LV dilatation in SHF. Diastolic heart failure (DHF) that is not associated with LV dilatation is also accompanied with collagen accumulation; however, differences in ECM regulatory system, especially activation of MMPs, between SHF and DHF remain to be clarified. This study was conducted to clarify whether MMPs are activated even in DHF, and if so, to characterize the difference in activation of MMPs between SHF and DHF for identification of a target for the prevention of LV remodeling in SHF. Methods: To exclude effects of differences in underlying cardiovascular diseases and genetic background on the comparison between DHF and SHF, we used Dahl salt-sensitive rats fed on high salt diet starting at 7 weeks of age as DHF model and at 8 weeks as SHF model, both of which our laboratory recently developed. Results: LV fibrosis progressed in the DHF and SHF model rats. MMP-2 was activated to the same degree in both rats. Activation of MMP-9 was enhanced in the DHF model rats, but the activity was more enhanced in the SHF rats. Film in situ zymography showed that enhanced gelatinolytic activity appeared only in the mid layer of the LV wall in the DHF rats and throughout the wall in the SHF rats. The distribution of gelatinolytic activity was consistent with that of expression of MMP-9 as assessed in immunohistochemical study. Conclusions: MMP-9 rather than MMP-2 may be involved in LV dilatation in SHF and be a specific target for the prevention of LV remodeling.

Keywords: Extracellular matrix; Fibrosis; Heart failure; Hypertension; Remodeling

1. Introduction

The death rate for cardiovascular diseases has decreased in the past decades [1,2], however, prevalence of heart failure is still increasing [3]. Most common heart failure is associated with left ventricular (LV) systolic dysfunction and dilatation, which is called systolic heart failure (SHF). Progressive LV dilatation is associated with an increased incidence of morbidity and mortality in SHF [4,5]. Quantitative and qualitative changes in extracellular matrix (ECM) accompany the development of SHF [6–9], and are likely to contribute to LV remodeling [10–12]. Matrix metalloproteinases (MMPs), an endogenous family of zinc-dependent enzymes, are responsible for regulation of ECM and several clinical studies reported activation of MMPs in association with LV remodeling [13–15]. Thus, there are growing interests in MMPs as novel therapeutic targets for the prevention of LV remodeling.

A number of recent community-based epidemiological studies have revealed that 30–40% of cases of heart failure...
2. Methods

This study conforms to the guiding principles of Osaka University Graduate School of Medicine with regard to animal care and to the position of the American Heart Association on Research Animal Use.

2.1. Study subjects

Laboratory chow containing 0.3% sodium chloride was fed to weaning male Dahl-S rats (DIS/Eis, Eisai, Tokyo, Japan) and switched to laboratory chow containing 8% sodium chloride at 7 weeks (n=6) (DHF model) or at 8 weeks (n=6) (SHF model). Overt heart failure is developed around 19 weeks in the DHF model rats and around 26 weeks in the SHF model rats [20]. Male Dahl-S rats continuously fed on laboratory chow containing 0.3% sodium chloride were used as age-matched control at 19 weeks (n=6) and 26 weeks (n=6), respectively. Systolic blood pressure was measured during the study protocol with a tail cuff system (BP-98A, Softron, Tokyo, Japan).

2.2. Echo and hemodynamic study

The rats were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and transthoracic echo recordings were obtained around 19 weeks for the DHF model rats and their age-matched control rats and around 26 weeks for the SHF model rats and their age-matched control to determine LV cavity size and wall thickness as previously described [20]. These schedules were decided according to our previous study [20]. LV endocardial and mid-wall fractional shortenings were calculated as described previously [20]. LV end-systolic stress was calculated as previously described [21].

2.3. Tissue sampling

Following the echo study and adequate anesthesia, the lung and the heart were harvested and the lung and the left ventricle were weighed. The LV myocardium was harvested, immediately placed in liquid nitrogen and stored at −80°C for the measurement of mRNA levels, hydroxyproline content and in vitro zymography. LV hydroxyproline content was measured and the results were calculated as the hydroxyproline content per wet weight of tissue as previously described [22]. Samples for in situ zymography and immunohistochemistry were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechical, Tokyo, Japan) and frozen on dry ice. The LV weight corrected for body weight was determined as LV mass index as previously described [20].

2.4. Immunohistochemistry

Serial cryostat transverse sections with 4-μm thickness were fixed in acetone for 10 min, air-dried, and stained by the indirect immunohistological method as previously described [22]. Tissue sections were treated with 0.3% H2O2 solution for 10 min to inhibit endogenous peroxidase activity and then incubated with blocking solution (10% donkey serum, Rockland) followed by 2-h incubation with purified rabbit anti-rat MMP-9 polyclonal antibody (1:500 dilution, TP-221P Torrey Pines Biolabs., San Diego, CA, USA) or purified rabbit anti-mouse MMP-2 monoclonal antibody (1:40 dilution, F-73 Fuji, Toyama, Japan). After washing in phosphate buffered saline for 10 min, the sections were incubated with secondary donkey anti-rabbit antibody and visualized with 3- amino-9-ethyl carbazole as substrate (AEC; Merck). The nuclei were stained with hematoxylin and the sections were viewed with a light microscope both at low (×20) and high (×100) magnifications.

2.5. Quantification of gene expression

Quantification of mRNA levels of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), TIMP-2, type I collagen and type III collagen in the left ventricle was determined by real-time quantitative PCR with Prism 7700 Sequence Detector (Perkin-Elmer, Foster, CA, USA) as previously described [22,23]. Sequences of all oligonucleotides used as forward primers, reverse primers and detection probes are summarized in Table 1. To correct the efficiency of cDNA synthesis, the amounts of each measured mRNA were divided by the amounts of GAPDH mRNA used as internal standards.
### Table 1
Sequences of all oligonucleotides used as forward primers, reverse primers and detection probes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Detection probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TATCGGACGCTGGTTACCA</td>
<td>TTAGACTGTCCGGTGAACTTG</td>
<td>CCATCAACGACCCCTTCATATTGACCTC</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>CATAATCTGAGGCCCTCGTCAGC</td>
<td>GAGGATCTGATCCTGCCAACAGC</td>
<td>CTGTTCAGGACCTTGTGGAATTGAC</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>CCCAGAAGAAGAGGCTTTGCAAACTGG</td>
<td>AGTCCATCCAGAGGCACTCATC</td>
<td>TGGCCCTATGATCCCCATGTACATCTC</td>
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<tr>
<td>Type I</td>
<td>TGCTGTCTGCAGTAAAGTCG</td>
<td>TCAAACCATCCTCTGCGCTCG</td>
<td>ACTGAGACGAGGACGGCGTGGAC</td>
</tr>
<tr>
<td>collagen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>TGCCCAAGCCCTTCTACACCT</td>
<td>CAGCCACCTCCTCCACTCCAG</td>
<td>CTGTGTCTCCTGATGCGCATGCCATGGAC</td>
</tr>
</tbody>
</table>

Abbreviations: TIMP, tissue inhibitor of metalloproteinase.

### 2.6. In vitro gelatin zymography studies

LV myocardial samples were homogenized (20-s bursts) in 1.5 ml of an ice-cold extraction buffer containing cacodylic acid (10 mmol/l), NaCl (0.15 mol/l), ZnCl (20 mmol/l), NaN (1.5 mmol/l), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4 °C, 3000 rpm for 10 min), and the supernatant decanted and saved on ice. The protein concentration of the myocardial extracts was determined with a standardized colorimetric assay (Bio-Rad protein assay). The extracted samples were then aliquoted and stored at −80 °C. Zymography was performed using Yagai gel electrophoresis apparatus, zymogram gels and reagents (Yagai, Yamagata, Japan). The thawed extracts samples on ice were mixed with an equal volume of sample buffer at room temperature for 15 min. A 40-μg amount was loaded onto electrophoretic gels (SDS–PAGE) containing gelatin. The gels were run at 15 mA/gel through the stacking phase and at 30 mA/gel for the separating phase, maintaining a running buffer temperature of 4 °C for about 80 min, and then incubated 40 h at 37 °C in zymogram developing buffer. Areas of MMP digestion were visualized by negative staining with Coomassie blue. Zymogram gels were digitized with scanner (Studio Scan II si, Agfa, Belgium) set at high-resolution. The lysis bands for each sample were determined by quantitative image analysis, and the final results were expressed as pixel density per lane [24]. Each densitometric result was normalized to mean value of control rats at 19 weeks.

### 2.7. Film in situ zymography studies

Serial cryostat transverse sections with 4-μm thickness were placed on a polyethylene terephthalate base-film coated with crosslinked gelatin of 7 μm thickness. The films with sections were incubated in a moist chamber at 37 °C for 48 h. After incubation, the specimens were stained in a Coplin jar over 15 min with Amido Black 10B in 1.5 ml of an ice-cold extraction buffer containing (Wako, Osaka, Japan), which diluted with 1.0% solution with 70% methanol and 10% acetic acid. These films were then decolored for 20 min with a destaining solution3 (pH 5.0). The homogenate was then centrifuged (4 °C, containing 1.0% solution with 70% methanol and 10% acetic acid). The degraded gelatin was not stained with Amido Black, and areas of gelatinolytic activity were visualized as white to pale blue area on the blue black background and detected as brightness [25].

### 2.8. Statistics

Results are expressed as mean values±S.E.M. The statistical analysis was conducted with a commercially available statistical software (STATVIEW 5.0, SAS Institute, Cary, NC, USA). Differences at specific stages among groups were assessed using one-way ANOVA and Fisher’s protected least significant difference test. A probability value of less than 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Characteristics of the DHF and SHF models

Blood pressure steeply elevate from 7 to 13 weeks in the DHF model; thereafter, kept constant at higher level than in the control rats (Fig. 1). Blood pressure gradually increased from 8 to 15 weeks in the SHF model and then remained constant at a higher level than in the control rats.

Results of echocardiographic study are shown in Table 2. LV end-diastolic dimension, LV end-systolic stress and endocardial and mid-wall fractional shortenings were not
advanced fibrosis in the presence of similar LV hypertrophy.

3.2. Gene expression of ECM regulatory systems

The mRNA levels of type I collagen, type III collagen, TIMP-1 and TIMP-2 were significantly elevated in the DHF model rats as compared with the control rats, respectively (Fig. 2). Gene expression of type I collagen, TIMP-1 and TIMP-2 was significantly enhanced in the SHF model rats as compared with the control rats, and mRNA level of type III collagen also tended to be elevated. As compared with the DHF model rats, mRNA levels of TIMP-1 and TIMP-2 were significantly elevated in the SHF model rats.

3.3. Gelatinolytic activities by in vitro zymography

Representative in vitro gelatin zymography is shown in Fig. 3. Activity of 72-kDa gelatinase (MMP-2) was similarly increased in the SHF and DHF model rats. Gelatin lysis in the region of 92-kDa gelatinase (MMP-9) was enhanced in both rats; however, it was more prominent in the SHF model rats.

3.4. Gelatinolytic activities by film in situ zymography

This technique allowed to localize the portions of gelatinolytic activity in fresh tissue sample (Fig. 4). Control tissue displayed minimal gelatin digestion. The tissue from the DHF rats showed apparent digestion limited to the mid layer in the LV wall. In the SHF model rats, enhanced gelatinolytic activity was observed throughout the wall.

### Table 2

Hemodynamic and structural characteristics of each group

<table>
<thead>
<tr>
<th></th>
<th>Control at 19 weeks</th>
<th>DHF model</th>
<th>Control at 26 weeks</th>
<th>SHF model</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>LVDD (mm)</td>
<td>8.9±0.2</td>
<td>9.2±0.4</td>
<td>8.9±0.3</td>
<td>10.6±0.7*†</td>
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<tr>
<td>PWd (mm)</td>
<td>1.3±0.1</td>
<td>2.1±0.2*</td>
<td>1.4±0.1</td>
<td>1.9±0.1*</td>
</tr>
<tr>
<td>Endocardial FS (%)</td>
<td>33±2</td>
<td>34±2</td>
<td>33±1</td>
<td>27±1*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV ESS (10^7 dynes/cm²)</td>
<td>80±8</td>
<td>102±9</td>
<td>92±10</td>
<td>141±15*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mid-wall FS (%)</td>
<td>18±2</td>
<td>18±1</td>
<td>19±1</td>
<td>13±1*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV mass index (mg/g)</td>
<td>2.0±0.1</td>
<td>4.2±0.3*</td>
<td>1.9±0.1</td>
<td>4.1±0.4*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV pro-OH (µmol/g)</td>
<td>2.7±0.4</td>
<td>3.7±0.1*</td>
<td>3.1±0.1</td>
<td>4.8±0.4*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung weight (mg/g)</td>
<td>3.3±0.1</td>
<td>8.2±1.9*</td>
<td>3.3±0.1</td>
<td>11.0±2.7*&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>/body weight (mg/g)</td>
<td></td>
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</table>

Data are shown as mean±S.E.M.

*<sup>p<0.05 versus control rats at 19 weeks.
†<sup>p<0.05 versus control rats at 26 weeks.
‡<sup>p<0.05 versus DHF rats.

Abbreviations: LVDD, left ventricular end-diastolic dimension; PWd, posterior wall thickness; FS, fractional shortening; ESS, end-systolic wall stress; pro-OH, hydroxyproline content.
3.5. Immunohistochemical studies

Immunohistochemical staining for MMP-2 was detected throughout the LV wall both in the DHF and SHF model rats (Fig. 5). In contrast, the expression of MMP-9 was different between the models: the staining for MMP-9 appeared only at the mid layer of the LV wall in the DHF model rats and was detected throughout the LV wall in the SHF model rats (Fig. 6). The staining for MMP-2 or MMP-9 was not apparent in the control rats.

4. Discussion

The DHF and SHF model rats with pressure overload showed similar degree of pulmonary congestion as indicated by the data of lung weight. LV hypertrophy and fibrosis occurred in both models. LV enlargement and systolic dysfunction were observed in the SHF model rats, but not in the DHF model rats. Progression of whichever phenotype of heart failure was associated with enhanced gene expression of TIMP-1, TIMP-2, and type I and III collagens; however, gene expression of TIMP-1 and TIMP-2 was more upregulated in the SHF model rats. The expression and activity of MMP-2 were enhanced to the same degree in both phenotypes of heart failure; however, those of MMP-9 were significantly promoted in the SHF model rats as compared with the DHF model rats.

Previous studies demonstrated expression and activity of MMPs were increased in animal models with SHF and in patients with dilated cardiomyopathy [15,26,27]. Because MMPs are responsible for ECM degradation and remodeling, the previous studies concluded that the activation of MMPs plays crucial roles in LV dilatation and is therapeutic targets for the prevention of LV remodeling. However, it remains unknown whether all of the activated MMPs equally contribute to LV remodeling. Alterations in ECM including collagen accumulation occur in DHF without LV dilatation as well as in SHF [19]. Therefore, regulatory system of ECM may be altered even in DHF, and if so, changes in MMP activity observed in SHF may not necessarily account for LV dilatation. The current study demonstrated that activation of MMP-2 occurred in association with progressive myocardial fibrosis and was independent of LV dilatation. In contrast, although MMP-9 was somewhat activated in DHF, its activity was more enhanced in SHF with LV dilatation. In addition, the immunohistochemical study and the in situ zymography
Fig. 3. (A) Representative gelatinolytic bands at 72 kDa (MMP-2). (a, b) Control rats at 19 weeks; (c, d) DHF rats; (e, f) control rats at 26 weeks; (g, h) SHF rats; (i) positive control for MMP-2. (B) Summary data of activity in 72 kDa gelatinase. (C) Representative gelatinolytic bands at 92 kDa (MMP-9). (a, b) Control rats at 19 weeks; (c, d) DHF rats; (e, f) control rats at 26 weeks; (g, h) SHF rats; (i) positive control for MMP-9. (D) Summary data of activity in 92-kDa gelatinase. *, P<0.05 versus control rats at 19 weeks; †, P<0.05 versus the DHF rats.

Fig. 4. Visualization of net gelatinolytic activity by film in situ zymography. The brightness under the light microscope shows the digestion with gelatinases. The tissue from a DHF rat (right upper) showed apparent digestion limited to the mid layer in the LV wall. In a SHF model rat, enhanced gelatinolytic activity was observed throughout the wall. Age-matched control rats for DHF (left upper) and SHF (left lower) showed minimal gelatin lysis.
Fig. 5. Localization of MMP-2 expression in the left ventricular tissue by immunohistochemical staining in a control rat for DHF, a DHF model rat, a control rat for SHF and a SHF model rat. Representative examples of the staining are shown at a low magnification (left panels) and at a high magnification (right panels). The expression of MMP-2 was observed throughout the wall in both the DHF and the SHF rats.

The current results suggest that MMP-9 rather than MMP-2 plays an important role in LV dilatation in hypertensive heart disease. Ducharme et al. [28] reported that LV dilatation was attenuated even with enhanced expression of MMP-2 after myocardial infarction in MMP-9 knockout mice. SHF is induced by different causes, and MMP-9 is likely to contribute to LV dilatation irrespective of underlying cardiovascular diseases and may be one of specific therapeutic targets in the prevention of LV remodeling.

The reasons why MMP-9 activity was different between these phenotypes of heart failure in spite of the same underlying cardiovascular disease and the same genetic background remain to be clarified. Li et al. [29] reported downregulation of MMP-9 in patients with dilated cardiomyopathy following support with LV assist devices. Their study suggested that an increased LV filling pressure and hence an elevated LV wall stress are responsible for enhanced expression of MMP-9. In this study, although the data of LV filling pressure were lacking, LV end-systolic stress was significantly higher in the SHF model than in the DHF model. Thus, the difference in the wall stress may be partly responsible for the difference in MMP-9 activity, although the higher LV end-systolic stress in the SHF model might be a result rather than a cause of LV dilatation.

MMP-9 is inactivated through forming a specific complex with TIMP-1 [30,31], and the predominant enhancement of gene expression of TIMP-1 in SHF may well lead to the suppression of MMP-9 activity. However, roles of pro-MMP-9–TIMP-1 complex are complicated. In addition, TIMP-1 is inactivated by proteinases such as neutrophil elastase [30] and MMP-3 affects the formation of pro-MMP-9–TIMP-1 complex and the activity of MMP-9 [32]. The activity of the proteinases and MMP-3 or the amount of the complex was not assessed in the current study. Thus, the increased mRNA level of TIMP-1 does not necessarily correspond to the decrease in MMP-9 activity. Recently, TIMP-1 has been shown to exert other effects than inhibition of MMPs [33], and further studies are necessary to elucidate roles of TIMP-1 and its effects on MMP-9 activity in heart failure.

MMP-2 was similarly activated in the SHF and DHF models, and its contribution to pathophysiology of these phenotypes of heart failure remains unclear. A recent animal study reported that enhanced activity of MMP-2 and MMP-1 was associated with LV diastolic stiffening...
Fig. 6. Localization of MMP-9 expression in the left ventricular tissue by immunohistochemical staining in a control rat for DHF, a DHF model rat, a control rat for SHF and a SHF model rat. Representative examples of the staining are shown at a low magnification (middle panels) and at a high magnification (left and right panels). The left panel represents the staining of the mid layer of the LV wall and the right panel represents that of the epicardial layer. The expression of MMP-9 was observed only in the mid layer in the DHF rat and throughout the wall in the SHF rat.

[34], and MMP-induced stiffening was explained by facilitated interstitial edema [35]. Chamber stiffness is increased irrespective of phenotype of heart failure when LV filling pressure is elevated enough to induce pulmonary congestion. Thus, activated MMP-2 may be partly responsible for chamber stiffening in both DHF and SHF, resulting in elevation of LV filling pressure, pulmonary congestion and overt heart failure.

Collagen accumulation progressed even though MMP activity was enhanced in both SHF and DHF models. Gene expression of collagen synthesis was also enhanced, and thus, collagen production may well overcome activation of MMPs and induce imbalance between ECM production and degradation. In both phenotypes of heart failure gene expression of TIMPs was enhanced, and their suppressive effects on MMP activity might be responsible for insufficient activation of MMPs. However, activation and production of other species of MMPs or collagenase activity was not investigated in this study. Thus, we can raise the possibility that collagenase activity was attenuated in contrast to enhanced gelatinase activity. At present there is no established method to assess collagenase activity, which makes it difficult to clarify mechanisms of progressive LV fibrosis in heart failure.

5. Conclusions

MMP-2 was activated to the same degree in DHF and SHF. Activity of MMP-9 was more enhanced in SHF as compared with DHF. Thus, MMP-9 rather than MMP-2 may be responsible for LV dilatation in SHF and a specific target for the prevention of LV remodeling.

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References


