Matrix Metalloproteinase-9 Contributes to Human Atrial Remodeling During Atrial Fibrillation

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OBJECTIVES The purpose of this study was to determine the relationship between matrix metalloproteinases (MMPs)-1, -2, and -9, and tissue inhibitors of metalloproteinases (TIMP)-1 and the atrial structural remodeling during atrial fibrillation (AF).

BACKGROUND Matrix metalloproteinases, a family of proteolytic enzymes and TIMPs, regulate the extracellular matrix turnover in cardiac tissue.

METHODS Tissue samples were obtained from 25 patients without a history of AF (regular sinus rhythm [RSR]) and 13 patients with AF (paroxysmal AF: 6, chronic AF: 7) undergoing cardiac operations. We performed a western blotting analysis of the MMP-1, -2, and -9, and quantitatively analyzed the expression of the MMP-9 and TIMP-1 by real time polymerase chain reaction and ELISA. The localization of the MMP-9 was investigated by in situ zymography and immunohistochemistry.

RESULTS The active form of the MMP-9 was significantly increased in the AF group in comparison to that in the RSR group (p < 0.05), but there were no differences between the groups in the protein level of the latent form of the MMP-9 and active and latent forms of the MMP-1 and MMP-2. We also demonstrated that the expression of the MMP-9 was significantly more increased in the atria of the AF group than in that of the RSR group for both the messenger ribonucleic acid (mRNA) (AF: RSR; 1: 1.5) and protein levels (AF: RSR; 3.9 ± 1.3: 1.5 ± 0.4 ng/mg atrium). The expression level of the MMP-9 was also higher in the PAF group than in the RSR group, however, the diameter of the left atrium was similar in both groups. The gelatinase activity and left atrium diameter were positively correlated (p < 0.05, R = 0.766). The relative expression of the mRNA for the monocyte chemoattractant protein-1 was higher in the AF group than in the RSR group. Immunohistochemical analysis revealed that the MMP-9 was distributed within the perivascular area and under the epicardium of the atria.

CONCLUSIONS We clearly showed that the expression of the MMP-9 increased in fibrillating atrial tissue, which may have contributed to the atrial structural remodeling and atrial dilatation during AF. (J Am Coll Cardiol 2004;43:818–25) © 2004 by the American College of Cardiology Foundation

Atrial fibrillation (AF) is the most common arrhythmia (1) and is well known as a source of thromboembolic events (2). The longer the duration of AF, the more persistent it becomes because of atrial remodeling. A shortening of the atrial refractory period, termed “electrical remodeling” (3–7), occurs first. Then, contractile remodeling, meaning a decrease in the atrial contractility, occurs, followed by structural remodeling, which guarantees the persistence of AF (8,9). Several reports have demonstrated that electrical remodeling was caused by a reduction in the L-type Ca²⁺ channels and various kinds of potassium channels in atria with AF (10–13). However, both the mechanism and the role of the structural remodeling of AF still remain unclear. Remodeling of cellular ultrastructures, such as myolysis occurring in the atrial myocardium, is known to develop progressively during AF (14). An increase in the expression of the gap junctions (connexin 40) has been reported to induce changes in the biophysical properties of the atrial tissue during AF (15,16). Enhanced disintegrin and metalloproteinase (ADAM) activity was also reported to be a molecular mechanism contributing to the dilation of fibrillating human atria (17).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, and they regulate the extracellular matrix turnover in a balance with the tissue inhibitors of metalloproteinases (TIMPs) (18,19). The extracellular matrix degradation by MMPs has been known to be associated with the pathogenesis of cardiovascular diseases, including atherosclerosis, restenosis, dilated cardiomyopathy, and myocardial infarction (MI) (20,21). In particular, many reports have demonstrated that MMP-9...
plays an important role in ischemia–reperfusion–induced myocardial matrix remodeling and could be a target for the prevention or treatment of acute ischemic myocardial injury (19,21–25).

The purpose of this study was to investigate the role of MMP-1, -2, and -9 and TIMP-1 in AF-induced human atrial remodeling.

METHODS

Tissue collection. Right atrial appendages were obtained from 38 patients undergoing cardiac operations, which consisted of 25 patients with regular sinus rhythm (RSR) without a history of AF, and 13 patients with AF (paroxysmal AF [PAF]: 6 patients; chronic AF [CAF]: 7 patients). The institutional ethics committee of the Graduate School of Biomedical Science, Hiroshima University, approved all procedures involving human tissue usage. Informed consent was obtained from patients before tissue harvesting. A transverse section (5 to 8 mm) of the atrial appendage was divided into three sections. One was embedded in an OCT compound (Sakura, Torrance, California) and frozen with liquid nitrogen. The tissue blocks were stored at −80°C until sectioning. Another section was quickly frozen with liquid nitrogen and stored at −80°C until the protein would be extracted. The other section was preserved in RNAlater (Ambion, Austin, Texas) at 4°C and used to extract the RNA.

ELISA and Western blotting analysis. Frozen cardiac samples were homogenized in a lysis buffer (20 mmol/l Tris; 1% Triton X; 10% glycerol; 1% DOC; 0.1% SDS; 50 mmol/l NaF; 10 mmol/l NaP₂O₄; 1 mmol/l DTT; 1 mmol/l Banadate; 10 μg/ml leupeptin, pH 7.4) with a Zilconia bead at 30 frequency/s and 4°C for 8 min using an MM 300 (Quagen, Hilden, Germany) and centrifuged at 14,000 rpm at 4°C for 10 min. The protein concentrations of the supernatants were determined by DC protein assay (Bio-Rad, Richmond, California). Equal amounts of the proteins were assayed for human MMP-9 and TIMP-1 production by ELISA kits purchased from R&D Systems (Minneapolis, Minnesota) according to the manufacturer’s instructions. All assays were performed in duplicate.

The same samples used for the ELISA assay of the MMP-9 were also used for the Western blot analysis of the MMP-1, -2, and -9. Equal amounts of protein (2 μg) were separated by SDS–PAGE and immunoblotted to a nitrocellulose membrane with anti-MMP antibodies (Oncogene Research Products, San Diego, California), which recognized their latent or active forms. The MMP protein bands were visualized using HRP-conjugated anti-IgG secondary antibodies and ECL plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed, dried, and exposed to X-ray film (Kodak). The images were analyzed with National Institutes of Health (NIH) images and quantified by normalizing them with that from beta-actin.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Tissue samples taken from the RNA-later (Ambion, Austin, Texas) were pulverized in Trizol reagents (1 ml/100 mg tissue), and the total RNA was extracted by the Trizol method supplied by Invitrogen. Precisely 1 μg of the total RNA from each sample was reverse-transcribed to generate complementary deoxyribonucleic acid (cDNA) using Ready-To-Go RT-PCR beads (Amersham Pharmacia, Piscataway, New Jersey) and the random primer pd (IV/6). This mixture was placed in a thermal cycler (model 9600) at 42°C for 20 min and 95°C for 5 min. Quantitative PCR was performed using a Light Cycler (Roche, Mannheim, Germany). The cDNA (10 μl each) was diluted to a volume of 20 ml with a PCR mixture (Light Cycler Fast Start DNA Master SYBR Green I kit [Roche]) containing 0.5 μmol of specific primers (Light Cycle Primer Set) for the MMP-9, TIMP-1, monocyte chemoattractant proteins (MCP-1), and tumor necrosis factor-alpha (TNF-α). Initial denaturation at 95°C for 10 s was followed by amplification, involving 35 cycles with denaturation at 95°C for 10 s, annealing at 68°C for 10 s and elongation at 72°C for 16 s. All samples were analyzed in triplicate. The fluorescence intensity of the SYBR Green I reflected the amount of PCR products actually formed.

Film in situ zymography. In situ zymography using FIZ films was used to determine the localization of the MMP-2, -3, -7, and -9 activity. The FIZ films were coated with gelatin in either the presence (FIZ-GI film) or absence (FIZ-GN film) of an MMP inhibitor (1,10-phenanthroline) (FUJIFILM, Japan). Unfixed frozen sections (6 mm) were placed on the films and incubated for 24 h at 37°C with 100% humidity and stained with Biebrich scarlet. The MMP-2, -3, -7, and -9 activity was visualized by both the loss of staining in the FIZ-GN films and staining in the FIZ-GI films. Images of the FIZ-GN films were scanned, and the gelatinase activity was analyzed using NIH images.

Immunohistochemical staining of MMP-9 and CD11b. We conducted double immunofluorescent staining for MMP-9 (Santa Cruz, California) and CD11b (macrophage marker, Dako Japan, Kyoto). The nuclei were stained with Hoechst 33342. Frozen sections of atrium were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.5% Triton X-100/PBS for 5 min. After blocking with 1% BSA/PBS
and incubating with primary antibodies in 1% BSA/PBS, the sections were incubated with secondary antibodies (Alexa 488/568). Specimens were observed using confocal laser scanning microscopy (Leica DM IRBE).

Statistical analysis. Clinical characteristics and quantitative data were compared between the AF and RSR groups by the Mann-Whitney $U$ test. The Kruskal-Wallis test was used to compare the three groups (RSR, PAF, and CAF). Data are presented as mean ± SD. A $p$ value $<0.05$ was considered statistically significant.

RESULTS

No differences existed in the gender, age, or percentage of oral administration with angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, diuretics, and beta-blockers between the AF and RSR groups. From the cardiac echocardiography, the left ventricular (LV) function and LV mass were similar among the AF and RSR groups (Table 1). The left atrial diameter was similar in the RSR and PAF groups, but larger in the CAF group than in the RSR group (35.7 ± 6.1 mm in the RSR group, 40.2 ± 4.8 mm in the PAF group, and 51.8 ± 10.5 mm in the CAF group, $p < 0.05$).

As a result of Western blotting analysis, the active form of the MMP-9 (88 kDa) was significantly increased in the AF group in comparison to that in the RSR group ($p < 0.05$), but no difference existed in the protein level of the latent form (92 kDa) of the MMP-9 and active and latent forms of the MMP-1 (43 and 55 kDa, respectively) and MMP-2 (66 and 72 kDa, respectively) between the two groups (Fig. 1). Also, the ELISA analysis showed that the protein level of the MMP-9 was significantly increased in the AF group in comparison to that in the RSR group (Fig. 2A, $p < 0.05$).

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$p < 0.05$, atrial fibrillation vs. regular sinus rhythm.
A = aortic valve; AF = atrial fibrillation; ARB = angiotensin II receptor blocker; EF = ejection fraction; IVSTd = interventricular septum thickness; LAD = left atrial diameter; LV = left ventricular; LVdD = left ventricle diastolic diameter; M = mitral valve; RSR = regular sinus rhythm; T = tricuspid valve; Thrombectomy = left atrial or left atrial appendages thrombectomy; Valve (A/M/T) = valve replacement or plasty.

![Figure 1. Results of the measurement of the matrix metalloproteinase (MMP)-1, -2, and -9 using the Western blotting analysis. The active form of MMP-9 (88 kDa) significantly increased in the atrial fibrillation (AF) group in comparison to that in the regular sinus rhythm (RSR) group ($p < 0.05$), but there was no difference in the protein level of the latent form of MMP-9 (92 kDa) (top panel). No difference existed in the protein level of the active and latent forms of the MMP-1 (middle panel) and MMP-2 (bottom panel) between the two groups. Closed bars = AF group; open bars = RSR group. $p < 0.05$, AF vs. RSR.](image-url)
The TIMP-1 protein showed a tendency to rise in the AF patients as compared to that in the RSR patients, but it was not significant (p > 0.1, Fig. 2A). Interestingly, the expression level of the MMP-9 was also higher in the AF group (closed bar) than in the RSR group (open bar) (p < 0.05), and the TIMP-1 protein showed a tendency to rise in the AF patients as compared to that in the RSR patients (p < 0.1). Comparison of the expression of the MMP-9 protein among the three groups. The expression level of MMP-9 was higher not only in the CAF group, but also in the paroxysmal atrial fibrillation (Paf) group, than that in the RSR group. Closed bars = AF group; open bars = RSR group. *p < 0.05, AF vs. RSR. Abbreviations as in Figure 1.

The TIMP-1 protein showed a tendency to rise in the AF patients as compared to that in the RSR patients, but it was not significant (p < 0.1, Fig. 2A). Interestingly, the expression level of the MMP-9 was also higher in the PAF group than in the RSR group (RSR: PAF, p < 0.05, Fig. 2B). The level of the TIMP-1 protein was positively correlated to the left atrial diameter (p < 0.05, R = 0.453).

The relative expression of the MMP-9 mRNA was higher in the AF group than in the RSR group (Fig. 3A, p < 0.05). The difference in the expression of the TIMP-1 mRNA between the two groups was not significant. The ratio of the MMP-9 to TIMP-1 was higher in the AF group than that in the RSR group (p < 0.05). Expression of the MCP-1 mRNA was higher in the AF group than in the RSR group, whereas that of the TNF-α mRNA was similar between the groups (Fig. 3B).

Hematoxylin-eosin staining showed that, in the atrium, the cell layer under the epicardium was thicker in the AF group than in the RSR group (Figs. 4A and 4B). In situ zymography revealed that the activity of the gelatinase, represented as unstained spots with Biebrich scarlet in the FIZ-GN films, was higher in patients with AF than in those with RSR (Figs. 4C and 4D). The unstained spots in the FIZ-GN films completely disappeared in the MMP inhibitor-coated FIZ-GI films, meaning that all the spots reflected the activity of the MMP (gelatinase) (Figs. 4E and 4D). The activity of the gelatinase was distributed under the epicardium, in the lumens of blood vessels, and in the interstitium of cardiac myocytes. Gelatinase activity was higher in the AF group than in the RSR group (13.1 ± 2.3% vs. 7.3 ± 2.3%). The left atrial diameter and gelatinase activity were positively correlated (Fig. 5, p < 0.05, R = 0.766).

Immunohistochemical analysis showed that, in the atria with AF, the MMP-9 was found mainly in the perivascular area, under the epicardium, and sporadically in the interstitium of the cardiomyocytes (Figs. 6A and 6B). Conversely, in the atria with RSR, we could not detect any MMP-9 in the perivascular area, and there were thin layers of the MMP-9 under the epicardium. The staining of the MMP-9 appeared as fibers in the enlarged view (Fig. 6C). In the same place, we observed collagen fibers stained blue with Masson trichrome (Fig. 6D). The double staining of the MMP-9 and CD11b revealed that the distribution of the MMP-9 was not always coincident with that of the CD11b (Fig. 7).

**DISCUSSION**

Atrial fibrillation is a common arrhythmia (1) and, unfortunately, a potential source of thromboembolic events (2). As AF has a tendency to become persistent due to atrial remodeling, new strategies for early termination of AF should be established by clarifying the mechanisms of the
atrial remodeling induced by AF. Many investigators have demonstrated that changes in the electrophysiological properties occur during AF, and ionic channel remodeling factors, such as a reduction in the L-type Ca\(^{2+}\) channels and downregulation of several kinds of potassium channels, have been represented as probable explanations for these changes (10–13). Some researchers have shown that the expression of gap junctions (connexin 40) increased in the atria during AF(15,16). Recently, it was reported that ADAM activity increased in fibrillating human atria (17). These changes in the expression of gap junctions and ADAMs cause structural changes in the atrium that are considered to be part of the pathogenesis of sustained AF. However, there still has been little information about the mechanisms of structural remodeling in the atria with AF.

Matrix metalloproteinases are a family of proteolytic enzymes that regulate the extracellular matrix turnover together with the inhibitory mediators of the MMPs called TIMPs (18,19). The MMPs have been known to be involved in the pathogenesis of many kinds of cardiovascular diseases, including atherosclerosis, restenosis, dilated cardiomyopathy, and MI (20,21). The MMP-1, -2, -3, -9, -13, and -14 exist in the mammalian myocardium. It has also been reported that MMP-9 especially plays a pivotal role in myocardial remodeling in ischemic cardiomyopathy and chronic heart failure (19, 21–25). We selected MMP-1, -2, and -9 to investigate the mechanism of the atrial remodeling by AF in the present study. In addition, compared to the vast information on ventricular myocardial remodeling, there has been little information as to whether or not the MMPs exist in human atrial tissue, and certainly there has been no data on the relationship between the MMPs and the atrial remodeling in AF.

In this study, we first investigated whether or not the
expression of MMP-1, -2, and -9 differed between human atria with AF and with RSR. As a result of Western blotting analysis, the active form of MMP-9 was significantly increased in the AF subjects in comparison to those in the RSR group (p < 0.05), but no difference was observed in the protein level of the latent form of the MMP-9 and active and latent forms of the MMP-1 and MMP-2 between the two groups of subjects. Also, the ELISA analysis showed that expression of the MMP-9 in the atria increased step by step, as the rhythm changed from RSR to PAF and from PAF to CAF. As for the TIMP-1, the level of the TIMP-1 protein was positively correlated to the left atrial diameter. The positive correlation between the left atrial diameter and the expression of the TIMP-1 protein suggested that the TIMP-1 protein increased in compensation for the MMP-9 increase. We also demonstrated that the expression of the MMP-9 was more increased in the atria of the AF group than in that of the RSR group, not only for the protein level, but also the mRNA level. Based on these data, the MMP-9 was judged to be a strong candidate for being associated with the atrial structural remodeling during AF. As the MMP-9 overexpressed, the TIMP-1 compensatingly increased, but it did not catch up to the MMP-9 increase, and the balance between them broke down, thus possibly resulting in the atrial structural remodeling of AF.

We first demonstrated the existence and distribution of the MMP-9 in the atrial tissue in the present study. In the atria with AF, the MMP-9 exists mainly in the perivascular area, under the epicardium, and sporadically in the interstitium of cardiomyocytes, whereas in the atria with RSR, it was found only under the epicardium as a thin layer and rarely in the interstitium. The staining of the MMP-9 appeared as fibers, and the image reflected the secreted MMP-9 ridden of collagen fibers.

The MMP-9 is usually found in human and ventricular...
myocardial remodeling. It has been known to be associated with macrophages, infiltrating neutrophils, fibroblasts, and vascular smooth muscle cells (26–28). Inflammatory changes have been known to contribute to the atrial structural remodeling and to increase the propensity for AF to persist (29). Recently, investigators reported that inflammatory cells indicative of chronic inflammation had infiltrated the dilated atrium (30). Monocyte chemoattractant protein-1 is a type of chemokine, caused by changes in the renin-angiotensin system and shear stress; it is an important mediator in many pathologies recruited by multiple leukocytes, and it is known to be closely related to MMPs (31). In this study, the relative expression of the mRNA of the MCP-1, indicative of chronic inflammation, was higher in the AF group than in the RSR group, but the TNF-α, indicative of acute inflammation, was similar for both groups. However, double staining of the MMP-9 and Mac-1 revealed that the distribution of the MMP-9 did not always coincide with that of the Mac-1, suggesting that the MMP-9 in atria with AF partially originates from blood cells.

Expression of the MMP mRNA can be modulated by various chemical agents, such as neurohormones, corticosteroids, and cytokines influencing the MMP gene expression through the formation of transcription factors binding to specific response elements on the MMP gene promoters (21). The common response elements contained in the promoter region of the MMP genes are known to be activator protein-1 (AP-1) and NF-κB sites (32). The TNF-α, a cytokine, is known to be a contributor to LV myocardial remodeling. Moreover, TNF-α was reported as one of the molecular triggers for the induction of myocardial MMPs in LV remodeling (33). In our study, the relative expression of the TNF-α was similar for both the atria with AF and those with RSR.

In the present study, we clarified that the expression of the MMP-9 had increased in the fibrillating atria, but the mechanism of the MMP-9 increase is the subject of a separate study. The molecular mechanisms of AF were not completely clarified in previous studies, but there was a report that an ACE-dependent increase in the amount of activated extracellular signal-regulated kinases Erk1/Erk2 in atrial interstitial cells might spur on atrial fibrosis in atria with AF (34). Angiotensin II (Ang II) was reported to mediate the upregulation of the matrix gelatinase (MMP-9) and to be related to the cardiac hypertrophy process and cardiac remodeling (35). It was also reported that Ang II blockade prevented myocardial fibrosis, and the long-term combined endothelin-A receptor and ACE inhibition improved cardiac failure (36). Angiotensin II was reported to be involved in the mechanism of the atrial electrical remodeling, and blockade of Ang II may lead to a better therapeutic management of human AF (37). Hence, alterations in the renin-angiotensin system in hearts with AF may be a possible trigger of the MMP secretion.

In conclusion, we clarified that the expression of the MMP-9 increased in the fibrillating atrial tissue, and the alteration in the MMP-9/TIMP-1 may contribute to the atrial structural remodeling and atrial dilation during AF. Additionally, we clarified that the distribution of the MMP-9 was in the perivascular area and under the epicardium in the atria.

Acknowledgments
We especially thank Takafumi Ishida, MD, and Syuichi Nomura, MD, for their excellent advice on this study. We also thank Yoshiko Kondo, Terumasa Hibi (NILS), and Yuka Umeda for their technical assistance, and Yuko Ohmura for her secretarial assistance.

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