Atrial Extracellular Matrix Remodeling and the Maintenance of Atrial Fibrillation

Jun Xu, MD, PhD; Guanggen Cui, MD; Fardad Esmailian, MD; Mark Plunkett, MD; Daniel Marelli, MD; Abbas Ardehali, MD; Jonah Odim, MD; Hillel Laks, MD; Luyi Sen, MD

Background—Remodeling occurs in both ventricle and atrium in dilated cardiomyopathy and heart failure. However, the alteration of atrial extracellular matrix components during remodeling and its effect on the electrical remodeling and atrial arrhythmia have never been explored.

Methods and Results—Atrial tissue samples of 53 explanted hearts from patients with dilated cardiomyopathy and end-stage heart failure who underwent heart transplantation were examined. Nineteen patients had permanent atrial fibrillation (PmAF), 18 had persistent AF (PsAF), and 16 had no documented AF (NAF). Sixteen donor left atria (LA) were used as controls (CNs). Western Blot analysis revealed a selective downregulation of tissue inhibitor of metalloproteinase (TIMP)-2 in PmAF and PsAF groups compared with the NAF and CN groups and an upregulation of atrial metalloproteinase (MMP)-2 that was most pronounced in the PmAF group followed by the PsAF and NAF groups. Immunofluorescent staining revealed that in the LA, type I collagen volume fraction (CVF-I) increased significantly in the PmAF group followed by the PsAF and NAF groups compared with that in CN. LA CVF-I significantly correlated with LA dimension and TIMP-2 to MMP-2 ratio. In the PsAF group, CVF-I/CVF-III ratio was significantly correlated with AF duration and the frequency of AF recurrence.

Conclusions—Atrial extracellular matrix remodeling manifested by the selective downregulation of TIMP-2 along with upregulation of MMP-2 and CVF-I in the atrium is associated with the development of sustained atrial fibrillation in patients with cardiomyopathy and heart failure. (Circulation. 2004;109:363-368.)

Key Words: fibrillation ■ metalloproteinases ■ heart failure ■ atrium ■ collagen

Atrial fibrillation (AF), one of the most common arrhythmias in clinical practice, is related to increasing disability and mortality. The detailed mechanism behind this arrhythmia is still unclear. During the development of AF in dilated cardiomyopathy and congestive heart failure, obvious structural changes in atrial myocytes may occur, including increase in cell size, perinuclear accumulation of glycogen, myolysis, alterations in connexin expression, changes in mitochondrial shape, and homogeneous distribution of nuclear chromatin, which may facilitate AF recurrence and maintenance.1-3 Because extracellular matrix (ECM) not only provides supportive scaffolding for myocytes and maintains the structural integrity and geometry of the heart4 but also interplays with myocytes in activation conduction, we hypothesize that changes of ECM components in atrium may be associated with the development of sustained AF.

Collagens are the major ECM proteins in the heart. Of the 5 different collagen isoforms found in the heart, fibrillar collagen type I and III comprise approximately 85% of the cardiac interstitium.5 Therefore, in the present study, we examined atrial collagen type I, type III, and related metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), in the atrium of explanted hearts from patients with end-stage heart failure with and without AF.

Methods

Study Subjects

Atrial myocardium samples of explanted hearts were taken from 53 patients who consecutively underwent heart transplantation at the University of California Los Angeles Medical Center from 1993 to 2002 because of dilated cardiomyopathy and end-stage heart failure. Thirty-seven explanted hearts were from patients who had electrophysiologically documented AF, and 16 were from patients with no documented AF (NAF). Electrocardiographic diagnosis of AF was made according to Bellet’s definition.6 Patients with AF were subdivided into 2 groups: permanent AF group (PmAF), including 19 explanted hearts from patients with permanent AF, and persistent AF group (PsAF), including 18 explanted hearts from patients with persistent AF. AF with duration of 7 days or longer is classified as persistent AF.7 Cessation of persistent AF normally requires pharmacological or nonpharmacological intervention. Persistent AF may be a single episode (which, by definition, would also be new-onset AF) or recurrent. PsAF was defined when the persistent AF had resisted all attempts to restore sinus rhythm or when the physician and patient decided that no such attempt (or additional attempts)
Clinical Characteristics of Study Population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PmAF</th>
<th>PsAF</th>
<th>NAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male/female</td>
<td>15/4</td>
<td>18/0</td>
<td>12/4</td>
</tr>
<tr>
<td>Age, y</td>
<td>52.26±12.15</td>
<td>57.22±8.20</td>
<td>52.19±11.38</td>
</tr>
<tr>
<td>Clinical diagnosis</td>
<td>Ischemic</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Idiopathic</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Valvarular</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Congenital</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Course of disease, mo</td>
<td>138±93.83*</td>
<td>72.89±56.67</td>
</tr>
<tr>
<td>Accompanied disease</td>
<td>Hypertension</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hyperlipidemia</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>AF duration, mo</td>
<td>94.38±70.80</td>
<td>40.91±36.80</td>
</tr>
<tr>
<td>Echocardiography</td>
<td>LVEF, %</td>
<td>21.42±7.59</td>
<td>21.44±6.33</td>
</tr>
<tr>
<td></td>
<td>LVEDD, mm</td>
<td>67.2±11.09</td>
<td>69.24±14.02</td>
</tr>
<tr>
<td></td>
<td>LAD, mm</td>
<td>57.85±16.92</td>
<td>53.50±5.24†</td>
</tr>
<tr>
<td>Hemodynamic</td>
<td>RAP, mm Hg</td>
<td>13.47±10.05†</td>
<td>9.5±4.32</td>
</tr>
<tr>
<td></td>
<td>PAP, mm Hg</td>
<td>35.47±7.41</td>
<td>33.16±9.21</td>
</tr>
<tr>
<td></td>
<td>PCWP, mm Hg</td>
<td>24.27±9.67</td>
<td>23.81±8.28</td>
</tr>
<tr>
<td></td>
<td>CO, L/min</td>
<td>4.25±1.11</td>
<td>4.40±1.11</td>
</tr>
<tr>
<td>Drugs</td>
<td>ACEI/ATA</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Spironolactone</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>AICD</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

The clinical characteristics are listed in the Table. Trimming-off tissues of left atrium (LA) (1 cm away from pulmonary vein orifices) were obtained from patients’ explanted hearts and 16 donor hearts (used as control [CN]). Because the bicaval anastomosis technique was used in most of the cases, right atrium (RA) trimming-off tissue samples from donor hearts were not available. These donor hearts were trauma victims and were free of cardiovascular pathology. None of the patients included in this study had any kind of ablations or were taking dobutamine or dopamine at the time of harvest in donors or explantation in recipients. This study was approved by the Committee for the Protection of Human Subjects at David Geffen School of Medicine at University of California, Los Angeles.

Immunohistochemistry Staining for Collagen Type I and Type III

Cryosections of both atria were obtained by embedding a small cube of atrial tissues in optimal cutting temperature compound and cutting into 5-μm sections by cryostat and were then stored at −80°C before staining. Frozen slides were blocked in 10% normal horse serum (Southern Biotechnology), incubated with goat anti-collagen I and collagen III antibodies (Southern Biotechnology, 1:1000) for 30 minutes, and then incubated with rabbit anti-goat IgG–FITC–conjugated secondary antibody (Southern Biotechnology, 1:1000) diluted with 0.01% Evan’s blue for 30 minutes at room temperature. Slides were observed under fluorescent microscopy. Ten vessel-free fields (×40) were captured, and the images were analyzed using NIH image software. Collagen volume fraction (CVF) was expressed as the percentage of pixels of positive collagen staining divided by total pixels of the image.

Western Blotting

Tissue samples (~100 mg) were homogenized in lysis buffer and centrifuged at 10 000g for 20 minutes. Supernatants were collected, and the protein concentrations were measured with a modified Lowry essay (Pierce Endogen Biotechnology). Equal amounts of proteins were separated on SDS gels and electrophoresed onto nitrocellulose membrane. Membranes were incubated overnight with monoclonal mouse anti-human MMP-1, MMP-2, and TIMP-1 antibodies (1:1000) and monoclonal anti-human TIMP-2 antibody (1:200, Oncogene Research Products). Horseradish peroxidase–conjugated goat anti-mouse IgG (1:5000, Kirkegaard & Perry Laboratories) and rabbit anti-mouse IgG (1:1000, Kirkegaard & Perry Laboratories) were used as secondary antibodies for MMPs and TIMP proteins, respectively. Purified proteins of TIMP-1 and TIMP-2 (Oncogene) were used as positive control. Negative controls were obtained by using mouse IgG (Southern Biotechnology) instead of monoclonal antibodies as primary antibody. Monoclonal antibody against α-actin (Santa Cruz Biotech) was used in every experiment for the internal control. The reactions were developed with enhanced chemiluminescence reagents, and images were obtained by exposure to films. The images were scanned, and densities of each band were analyzed by TotalLab software (Nonlinear Dynamics). The results are presented as percent change compared with that in donor controls after normalization to the actin bands of each sample.

Gelatin Zymography

Protein samples (6 μg) were loaded on standard 10% polyacrylamide gel containing 1 mg/mL gelatin (Sigma) under nonreducing condition. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for 15 minutes each and then incubated in development buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, and 0.02% NaCN) overnight. After incubation, gels were stained with 0.1% Coomassie brilliant blue R-250 overnight and destained with 45% (vol/vol) methanol and 45% (vol/vol) glacial acetic acid in water.

Statistical Analysis

All group data were expressed as mean±SD. Group differences were analyzed by 1-way ANOVA. In some experiments, a Student’s t test was used to determine differences between 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 (SPSS). Statistical significance was considered at P<0.05.

Results

Patients Characteristics

Time course of diseases was longer in the PmAF group compared with the PsAF and NAF groups (P<0.001, Table). In the PmAF and PsAF groups, LA dimensions estimated by echocardiography were significantly greater than in the NAF group (P<0.01, Table). The difference between the PmAF and PsAF groups was not statistically significant. There were no differences in age, gender, clinical diagnosis of etiology, and accompanied disease among the patient groups.
Collagen Subtypes Distribution
Representative sections of immunofluorescent staining of LA collagen I (green). Myocytes were counterstained by Evan’s blue (red) (magnification ×40). In the control group, almost no collagen I could be seen in the image; in the NAF group, little collagen I was visible among muscle fascicles. In the PsAF group, more collagen I can be seen. In the PmAF group, interstitial space increased with much more collagen I around muscle fascicles. White bar in lower right corner of control represents 20 μm. B, Bar graph shows a comparison of collagen I volume fraction of both LA and RA among groups. C, Histogram shows collagen III volume fraction of both LA and RA among groups. *P<0.05 compare with CN; **P<0.01 between groups.

Expression of Matrix Metalloproteinases
Quantitative Western blot analysis showed that MMP-1 and MMP-3 protein expression in both LA and RA was the same among all patient groups and in the CN group (data not shown). As shown in Figure 2, LA MMP-2 protein expression was significantly higher in patient groups than in CN group. LA MMP-2 expression was prominent in the PmAF group compared with the PsAF and NAF groups (P<0.05). In the PsAF group, LA MMP-2 expression was higher than that in NAF groups, but the difference was not statistically significant (P<0.05). MMP-2 protein level in RA was significantly higher than LA in every patient group (P<0.05, data not shown). However, there was no difference of RA MMP-2 protein expression among 3 patient groups. LA MMP-9 protein level was significantly increased in the PmAF group (P<0.05); however, the trends of increase did not reach statistical significance in the PsAF group (P=0.051). As in RA, MMP-9 was significantly increased in the PmAF group (P<0.05), but it was increased to a lesser extent in the PsAF group that in the NAF group (data not shown). RA MMP-9 was slightly higher than LA MMP-9 in both AF groups, but the differences were not statistically significant (P=0.57, data not shown).
sis. As shown in Figure 3A, clear proteolytic bands showed on gelatin zymography gels corresponding to both gelatinase MMP-9 (92 KD) and MMP-2 (72 KD). Quantitative analysis demonstrated that the increase of MMP-2 activity was more pronounced in the PmAF than in the PsAF group (Figure 3B). The change in the NAF group was not significant. MMP-9 activity was extensively increased in the PmAF group (P<0.01), and there was a moderate increase in both PsAF and NAF groups compared with the CN group (P<0.05) (Figure 3C).

Tissue Inhibitor of Metalloproteinases
As shown in Figure 4, TIMP-1 protein level was compatible among all groups, whereas TIMP-2 was downregulated significantly in both PmAF and PsAF groups compared with the CN group (P<0.01). In the NAF group, the abundance of TIMP-2 was not significantly changed compared with that in the CN group (P>0.05). Both TIMP-1 and TIMP-2 contents were the same between LA and RA in all groups.

Correlation Among MMP Regulation, Atria ECM Remodeling, and the AF Maintenance
The LA MMP-2 to TIMP-2 ratio significantly correlated with LA CVF-I (r=0.373, P<0.01, Figure 5A). The correlation between RA MMP-2/TIMP-2 ratio and RA CVF-I was also significant (r=0.43, P<0.01, data not shown); however, the data from RA in the CN group were not included in this analysis, because the RA tissue of CN was not available. LA CVF-I was closely correlated with LA dimension (r=0.509, P<0.01, Figure 5A). RA CVF-I was also significantly correlated with RA pressure (r=0.387, P<0.05, data not shown). In the PsAF group, the CVF-I/CVF-III ratio was significantly correlated with AF duration (r=0.595, P<0.01) and frequency of AF recurrence (r=0.537, P<0.01) (Figure 5B).

Discussion
Main Findings
This study explored for the first time the finding that alterations of atrial ECM components, especially collagen subtype distribution and their related MMPs/TIMPs, in patients with end-stage heart failure is distinct from those reported previously in ventricular myocardium.8 The increased level of collagen I associated with selective downregulation of TIMP-2, along with upregulation of MMP-2 expression and activity in atrium, correlates with left atrial
dimension and the maintenance and recurrence of AF in end-stage heart failure.

**Atrial Extracellular Matrix Remodeling and the Maintenance of Atrial Fibrillation**

Numerous studies have suggested that electrical remodeling manifested by altering transmembrane ionic currents and shortening atrial effective refractory period is the potential substrate for increased stability of AF in a few hours to a few days. An underlying structural remodeling might occur before, during, and after electrical remodeling and plays an important role in progressing sustained AF. Besides an ultrastructural degeneration found in patients with sustained AF, in an experimentally induced AF animal model, a variety of structural alterations were suspected of being the cellular and molecular basis of electrical remodeling. Most of these are alterations in the atrial myocytes, which include a loss of contractile elements, accumulation of glycogen, the expression and distribution of gap junction proteins, and myocytes dedifferentiation. On the other hand, an increase in the amount of connective tissue was found in dogs and cats with mitral valve disease and cardiomyopathy, both of which developed spontaneous AF without significant transmembrane action potential changes. In a canine pacing-induced heart failure model, Li et al found that AF could be easily induced without an altered atrial refractory period, refractoriness heterogeneity, or conduction velocity but with extended interstitial fibrosis in the atrium. Homogeneous conduction of atrial activity not only relies on myocyte integration but also can be affected by ECM among the atrial myocytes. Augmented interstitial fibrosis may facilitate local inatraial conduction block and increase atrial susceptibility to AF. The present results demonstrate that during AF, collagen type I, not type III, increased gradually from that in NAF to PsAF and then to PmAF, regardless of the etiology of the cardiomyopathies, ischemic or idiopathic. More collagen I, which means more thick fibers associated with a remarkable heterogeneity of fiber thickening and disarray, was found in patients in the PmAF group and PsAF group with longer AF duration and higher frequency of recurrence. Moreover, selectively increased collagen type I/III ratio was significantly correlated with the duration and the frequency of recurrence of AF in the PsAF group. In contrast, in the ventricular tissue of failing hearts, a dominant upregulation of collagen type III and decrease of collagen type I/III ratio were observed, along with the decrease in the abundance of collagen types I, IV, and VI. This distinct pattern of collagen subtype deposition in the atrium might just be the part of the substrate of electrical remodeling by increasing the heterogeneity of atrial conduction and playing an important role in the maintenance of AF. Conversely, prolonged AF could cause atrial dilatation. In a goat model, prolonged AF induced by electrical stimulation resulted in a significant increase in the ECM surface area per myocyte, and it was reversed after AF was terminated by cardioversion. The correlation between the increase of collagen-I volume fraction and LA dimension and RA pressure suggests the increase of ECM might just be the consequence of the mechanical or volume overloading of LA or prolonged AF.

**Regulation of MMP Expression and Activation in the Development of Sustained AF**

Keeping a suitable concentration and proportion of different types of collagen lies on the balance between collagen synthesis and degradation. Little is known so far about the detailed pathway leading to collagen oversynthesis in atrium related to AF. Goette et al reported downregulation of angiotensin-1 receptors, upregulation of angiotensin-2 receptors, and increased expression of ACE in patients with AF. Shi et al also reported that ACE inhibitor attenuated atrial fibrosis in a canine model of congestive heart failure and associated AF promotion. In the present study, we found an alteration of MMP/TIMP associated with the collagen I upregulation in atrium from patients with end-stage heart failure and AF. In most previous studies, an increase in the abundance and activity of MMP-3 and MMP-9 and decrease in MMP-1 was observed, accompanied by either increased or decreased MMP-2 expression level in the left ventricular tissue of failing hearts. Our results show that the abundance of MMP-1 and MMP-3 was not changed in the atrium of end-stage heart failure. Instead, the expression level of MMP-2 and the activity of MMP-9 were significantly increased. The differential regulation of MMPs again laid the foundation for the distinct ECM remodeling between atrium and ventricle in heart failure. On the other hand, a greater stepwise increase of MMP-2 expression and MMP-9 activity was found in tissue of the failing heart without AF to PsAF than PmAF. Additionally, a striking increase in both abundance and activity of atrium MMP-2 and MMP-9 in patients with PMAF suggests the importance of these 2 gelatinases not only in the ECM remodeling of the failing heart but also in...
the development of sustained AF. Unlike MMP-9, MMP-2 not only degrades gelatin and collagens IV, V, and VII, but also degrades collagen I. Recently, Ritty and Herzog\textsuperscript{24} reported the influence of collagen I on both the production and the state of activation of these 2 gelatinases, MMP-2 and MMP-9, in cultured endotenon and sheath cells. This positive-feedback regulation cycle might also exist in the atrium of failing heart. Collagen degradation or synthesis is modulated by MMPs, in this case at least MMP-2, which in turn could be influenced by the alignment/attachment of collagen I. The final result is an increased amount of MMPs, accompanied by increased fibrosis and vice versa.\textsuperscript{25} A new homeostasis of collagen/MMPs is thus established during the process of atrial structural remodeling in the heart failure, which becomes the substrates for promoting sustained AF. MMP-9 has not been shown to affect collagen I through a similar feedback mechanism so far. However, although it is activated by collagen I, MMP-9 could certainly influence the cascade of other collagen types.

In the present study, upregulated atrial MMP-2 protein was found to be associated with a selectively downregulated TIMP-2 protein in AF patients with cardiomyopathy and end-stage heart failure. TIMP-1 was not changed. These changes were similar to those in the ventricle during heart failure.\textsuperscript{21,22} The MMP-2/TIMP-2 ratio was significantly correlated with the CVF-I, suggesting a potential target for prevention and possible reversal atrial fibrosis, thereby reducing AF occurrence.

Potential Limitations
First, all patients in our study had cardiomyopathy and end-stage heart failure. Therefore, the conclusions drawn from this study cannot be applied to a population with AF that is free of end-stage heart failure. Second, we were not able to compare the data from the RA of patient groups with the CN group.

Conclusion
Atrial extracellular matrix remodeling manifested by the selective downregulation of TIMP-2 along with upregulation of MMP-2 and CVF-I in the atrium is associated with the development of sustained atrial fibrillation in patients with cardiomyopathy and heart failure. These alterations might result from the atrial mechanical or volume overloading or prolonged AF. Additional study needs to be done to confirm whether extracellular matrix remodeling might in turn make certain contributions to the increase in the atrial heterogeneity, thereby enhancing the propensity to AF recurrence and self-perpetuation.

Acknowledgments
This study was supported in part by a Grant-in-Aid from the American Heart Association, Western States Affiliate, the Zee Foundation, and the Foundation of Cardiovascular Disease and Transplantation.

References
4. Sackner-Bernstein JD. The myocardial matrix and the development and progression of ventricular remodeling. 
5. Weber KT. Cardiac interstitium in health and disease: the fibrillar collagen network. 
\textit{Am J Cardiol}. 1982;82:18N–28N.
8. Spinale FG. Matrix metalloproteinases: regulation and dysregulation in the failing heart. 
10. Allessie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. 
Atrial Extracellular Matrix Remodeling and the Maintenance of Atrial Fibrillation
Jun Xu, Guanggen Cui, Fardad Esmailian, Mark Plunkett, Daniel Marelli, Abbas Ardehali, Jonah Odim, Hillel Laks and Luyi Sen

Circulation. 2004;109:363-368; originally published online January 19, 2004; doi: 10.1161/01.CIR.0000109495.02213.52

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/109/3/363

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/