Matrix Metalloproteinases and Their Tissue Inhibitors in Pressure-Overloaded Human Myocardium During Heart Failure Progression

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OBJECTIVES
We studied the role of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in fibrosis formation in the transition from hypertrophy to heart failure (HF) as well as the cellular source of MMPs and TIMPs.

BACKGROUND
Human pressure-overloaded hearts are characterized by a significant increase in cardiac fibrosis. However, the contribution of the proteolytic/antiproteolytic system in aortic stenosis (AS) during hypertrophy progression has not yet been elucidated.

METHODS
Three groups of AS patients (I: EF >50%, n = 12; II: EF 50% to 30%, n = 10; III: EF <30%, n = 12) undergoing aortic valve replacement and seven controls were studied. Tissue samples were investigated by immunoconfocal microscopy, Western blotting, and zymography.

RESULTS
Quantitative analysis by immunoconfocal microscopy and Western blotting showed an upregulation of MMP-1, -2, -3, -9, -13, and -14 in group I and further increases in later stages. Tissue inhibitors of metalloproteinase-1 and -2 were enhanced and TIMP-4 was decreased in comparison to control. Gelatinolytic activity of MMP-2 significantly (p < 0.05) increased 1.2-fold (group I), 1.5-fold (group II), and 1.6-fold (group III) over control. The level of collagen I was significantly upregulated in all AS groups. Immunoconfocal microscopy showed that MMPs and TIMPs are produced predominantly by fibroblasts. The number of proliferating fibroblasts was significantly elevated during the transition to HF (0.67 n/mm²-control, 5.03-group III, p < 0.05).

CONCLUSIONS
In human hearts a continuous turnover of the extracellular matrix occurs during the progression from compensated hypertrophy to HF that is characterized by the upregulation of MMPs and inadequate inhibition by TIMPs. The altered balance between proteolysis/antiproteolysis with accompanying proliferation of fibroblasts results in fibrosis progression. (J Am Coll Cardiol 2004;44:1609–18) © 2004 by the American College of Cardiology Foundation

Recently, our group showed that in patients with hypertrophy due to aortic valve stenosis (AS) a continuous remodeling process of the left ventricular (LV) myocardium takes place and appears to be the major factor determining the development of heart failure (HF) (1). It was documented that a close correlation exists between cardiac function and myocardial morphology and that the deterioration of clinical LV parameters occurred in parallel with progressive fibrosis, myocyte degeneration, and cell death. However, the possibility of an involvement of the enzymatic systems of proteolysis and antiproteolysis in extracellular matrix (ECM) remodeling development has not been studied. Matrix metalloproteinases (MMPs) represent the major proteolytic system for the ECM and, together with their tissue inhibitors (TIMPs), are of significant importance in cardiac remodeling. The activity and expression of MMPs and TIMPs essentially vary in different cardiac diseases. In experimental hypertrophy the MMP portfolio has been reported to be affected by diverse factors including the type of cardiac overload (2). In human myocardium an MMP/TIMP imbalance was observed in end-stage HF (3,4), whereas in experimental models a variable MMP expression depending on the severity of hypertrophy was demonstrated (5–8). However, data about specific profiles of MMPs and TIMPs in AS during the transition from compensated to decompensated hypertrophy are missing.

Despite the fact that numerous reports demonstrate the ability of different cell types to synthesize MMP in vitro (9,10), the cellular source of proteinases and their local inhibitors in vivo is still an issue.

We hypothesized that fibrotic changes in the interstitium of hypertrophied myocardium in AS patients are accompanied by the consecutive development of an MMP/TIMP imbalance and that mainly interstitial cells synthesize these enzymes. The aim of the present research was two-fold: 1) to evaluate the levels of MMPs and TIMPs in AS during the transition from compensated to decompensated hypertrophy are missing.

We hypothesized that fibrotic changes in the interstitium of hypertrophied myocardium in AS patients are accompanied by the consecutive development of an MMP/TIMP imbalance and that mainly interstitial cells synthesize these enzymes. The aim of the present research was two-fold: 1) to evaluate the levels of MMPs and TIMPs in different stages of hypertrophy and their involvement in ECM remodeling; and 2) to determine the tissue source of these proteolytic and antiproteolytic systems.

MATERIALS AND METHODS

Patients. Thirty-four patients with isolated aortic valve stenosis were subdivided into three different groups on the basis of EF determined echographically at the time of
admission: I group, EF > 50% (n = 12); II group, EF 50% to 30% (n = 10); III group, EF < 30% (n = 12). All patients had no evidence of any other cardiovascular disease and underwent surgical aortic valve replacement. Controls consisted of three donor hearts that were not used for transplantation and four biopsies from patients undergoing closure of an atrial septum defect with normal left ventricles. Clinical data are presented in Table 1. The institutional Ethical Committee approved the study. All patients gave informed consent.

**Tissue sampling.** During open-heart surgery myectomy samples of the LV subvalvular septum weighing about 30 to 80 mg were removed, immediately frozen in liquid nitrogen, and stored at −80°C. The tissue samples were mounted in tissue Tec and cryosections 5 μm thick were prepared.

**Immunolabeling and confocal microscopy.** Cryosections were air dried and fixed for 10 min in acetone (−20°C), 4% formalin, or Carnoy’s solution (room temperature). After rinsing in phosphate-buffered saline (PBS), sections were incubated with 1% bovine serum albumin for 30 min to ensure that the image collected demonstrated a full range of fluorescent intensity from 0 to 255 pixel intensity level and were air dried and stored at 80°C. The tissue samples were mounted in Mowiol and coverslipped. The samples were examined by confocal laser microscopy (Leica TCS-NT). Digital images were further processed for three-dimensional reconstruction on a Silicon Graphics Octane Workstation using software “Imaris” and “Selima” (Bitplane, Zürich).

**Morphometry.** Measurements of immunofluorescence were done with a Leica TCS-NT confocal microscope. For quantitative analysis all sections were immunolabeled simultaneously using identical dilutions of primary and secondary antibodies and other reagents. Sections were scanned under identical parameters of imaging, zoom, pinholes, objectives, and laser power. Sections exposed to PBS instead of primary antibodies served as negative controls. One channel with the identical parameters of imaging, zoom, pinholes, objectives, and laser power. Sections exposed to PBS instead of primary antibodies served as negative controls. One channel with the identical parameters of imaging, zoom, pinholes, objectives, and laser power.

**Table 1. Clinical Data**

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, yrs</th>
<th>Men/women</th>
<th>EF, %</th>
<th>LVEDP, mm Hg</th>
<th>ΔP mean, mm Hg</th>
<th>LV mass/m²</th>
<th>Aortic valve area, cm²</th>
<th>Diuretics, n</th>
<th>Digitals, n</th>
<th>ACE inhibitors, n</th>
<th>Beta-blockers, n</th>
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<td>3/4</td>
<td>61 ± 8</td>
<td>7 ± 2</td>
<td>11 ± 2</td>
<td>102 ± 12</td>
<td>0.9 ± 0.3</td>
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<tr>
<td>I</td>
<td>70 ± 6</td>
<td>5/7</td>
<td>59 ± 8</td>
<td>15 ± 2</td>
<td>62 ± 17</td>
<td>137 ± 26</td>
<td>0.6 ± 0.3</td>
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<td>1</td>
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<td>3</td>
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<tr>
<td>II</td>
<td>67 ± 12</td>
<td>5/5</td>
<td>41 ± 5</td>
<td>18 ± 6</td>
<td>58 ± 12</td>
<td>131 ± 57</td>
<td>0.6 ± 0.2</td>
<td>7</td>
<td>1</td>
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<tr>
<td>III</td>
<td>71 ± 7</td>
<td>7/5</td>
<td>24 ± 5</td>
<td>32 ± 8</td>
<td>48 ± 15</td>
<td>156 ± 34</td>
<td>0.5 ± 0.2</td>
<td>9</td>
<td>3</td>
<td>6</td>
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</tbody>
</table>

*p < 0.05 vs. control. Data are expressed as mean ± SEM or number of patients. EF = ejection fraction; LVEDP = left ventricular end-diastolic pressure; LV = left ventricular.

**Table 2. Primary Antibodies**

<table>
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<th>Antibody</th>
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<td>Biotrend</td>
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<td>Biotrend</td>
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<tr>
<td>MMP-3</td>
<td>Mono-</td>
<td>Stromelysin 1</td>
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<tr>
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<tr>
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<td>Biotrend</td>
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<td>Mono-</td>
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<td>Oncogene</td>
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<td>MMP 14</td>
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<td>—</td>
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<td>Mono-</td>
<td>—</td>
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<tr>
<td>TIMP-4</td>
<td>Mono-</td>
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MMP = matrix metalloproteinase; TIMP = tissue inhibitors of metalloproteinase.
Zymography. Zymography was done according to a well-described protocol (6). In brief, LV samples were homogenized in ice-cold extraction buffer and centrifuged at 8,000 g for 30 min at 4°C. Proteins were determined by Bradford assay. The myocardial extracts were loaded onto electrophoretic gels (SDS-PAGE) containing a gelatin (0.1%) or casein (0.1%) substrate under non-reducing conditions. After electrophoresis the gel was incubated twice in renaturing buffer for 30 min each (25°C), rinsed in water, and incubated for 20 h in developing buffer (25°C). Samples were incubated in the presence of 1 mM of phenylmethylsulphonylfluoride (inhibitor of serine proteinase) and 5 mM iodoacetamide (inhibitor of cysteine protease). To study inhibition of the enzymes, an identical gel was incubated in reaction buffer containing 20 mM ethylene-diaminetetraacetic acid, an inhibitor of MMPs. After the reaction, the gel was stained with 0.25% Commissie brilliant blue R 250. Zones of lysis were visualized as clear bands against the blue background. To test the linearity of zymographic activity different concentrations of proteins were used. The zymograms were digitized using constant light intensity. The size of bands indicating MMP activity was determined by quantitative image analysis.

Western blot. Frozen tissue was homogenized in lysis buffer and centrifuged for 10 min. Left ventricular myocardial extracts were loaded onto 4% to 12% polyacrylamide gel and separated under reducing conditions. Proteins were electrotransferred onto nitrocellulose membrane (Invitrogen) and blocked with 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) at 4°C. After washing with TBST, proteins were exposed overnight at 4°C to monoclonal antibodies (Table 2) against MMP-1, -2, -3, and -9,
TIMP-4 (1 mg/ml), MMP-13, MT1-MMP, TIMP-1 and -2 (2 mg/ml) diluted in TBS with 5% powdered milk. Bound antibodies were detected by peroxidase-conjugated human anti-mouse or anti-rabbit immunoglobulin G horse-radish peroxidase-conjugated and SuperSignal WestFemto (Pierce) detection system and exposed to X-ray film. In order to equalize data, immunoblotting for sarcomeric alpha-actin was performed and all specific values of proteins were standardized to sarcomeric actin.

Quantification of immunoblots and zymograms was done by scanning on a STORM 860 (Amersham Pharmacia Biotech) using ImageQuant software.

**Statistical analysis.** All data are presented as means ± SEM. Differences by parametric analysis of variance followed by Bonferroni’s multiple comparison test or nonparametric Kruskal-Wallis with Dunn’s post-test were considered significant at p < 0.05.

## RESULTS

**MMPs (TIMPs) staining patterns.** Matrix metalloproteinases and TIMPs showed similar staining patterns within the ventricular myocardium with heterogeneous degrees of expression. We observed specific immunofluorescence labeling in some interstitial cells (Fig. 1A), multilocal or diffuse immunoreactivity in the perivascular areas (Fig. 1B), and multilocal or diffuse staining in fibrotic regions (Fig. 1C).

**MMPs’ zymographic activity.** Proteolytic activity of MMPs was detected in all tissues by zymography. Clear bands represent gelatinolytic activity of MMP-9, pro-MMP-2, and MMP-2 (Fig. 2). The zymography revealed that the majority of the detected activity could be attributed to MMP-2, which is significantly increased in patients with AS. We found that zymographic activity was linear with respect to protein content in both control and AS samples. The levels of MMP-3 and -13 were assessed by casein zymography, but no EDTA-sensitive bands were found.

**MMP expression.** All MMPs demonstrated a tendency to be upregulated already in group I and the upregulation further increased with the progression of hypertrophic changes (Figs. 3A to 3F). Data obtained from quantitative immunohistochemical (IHC) analysis were in agreement with Western blot (WB) findings but not identical, most probably because of differences in methodical approaches. MMP-1 IHC expression significantly increased in all AS groups in comparison with control (Fig. 1D), whereas by WB, MMP-1 was significantly enhanced only in group III (Fig. 3A). The other collagenase, MMP-13, which normally is totally absent or present in scanty amounts in normal heart (10), was conspicuously elevated in diseased myocardium (Figs. 1D and 3C). Matrix metalloproteinase-3 abundance was significantly upregulated in severe hypertrophy in both IHC and WB analysis (Figs. 1D and 3B). The MT1-MMP protein content was enhanced in all AS groups, whereas IHC showed an insignificant increase only in group I as compared to control. MMP-13 was enhanced with the transition to HF (Figs. 1D and 3E) that correlated with its gelatinolytic activity (Fig. 2). In contrast, the elevation of MMP-9 protein (Fig. 3F) was not matched by an increased activity (Fig. 2).
TIMP expression. Increased expression of TIMP-1 and -2 in decompensated hypertrophy was demonstrated by both IHC and WB (Figs. 1D, 3G, and 3H). Nevertheless, the IHC amount of TIMP-1 was rather poor in comparison with myocardial ECM collagen I and MMP-1, even in group III with the highest tissue content of this inhibitor (Figs. 4A to 4C). At the same time the abundance of TIMP-4 observed in controls tended to be decreased in the hypertrophy groups I and III and was significantly depressed in group II (Fig. 3I).

Fibrosis. The level of collagen I was upregulated in all groups of AS patients as compared with control. Myocardial fibrosis significantly increased with the transition to HF (Fig. 4D).

Tissue source of MMP (TIMP) production. To determine the tissue source of MMP (TIMP), we used both double sequential immunolabeling and serial sections. Immunohistochemical analysis showed that the contribution of endothelial cells (Figs. 5A and 5B) as well as smooth muscle cells (Figs. 5C and 5D) in the secretion of MMPs is rather negligible. In contrast, double staining for MMPs and vimentin demonstrated that the majority of cells in the MMP-containing area were fibroblasts (Figs. 6A to 6D). Between fibroblasts we found a few macrophages (Fig. 6D) that were often in close contact with fibroblasts (data not shown) and most likely take part in fibroblast activation.

Surprisingly, we found that fibroblasts are producing not only matrix proteinases but also their tissue inhibitors (TIMP-1, -2, and -4), of which TIMP-1 is shown as an example (Figs. 6E and 6F).

As previously reported, in hypertrophied myocardium only a few inflammatory cells were present. Therefore, their participation in MMP (TIMP) synthesis is least of all probable or insignificant. In cardiomyocytes occurrence of MMP (TIMP) was not found.

Ki-67 associated antigen and myofibroblasts. Proliferation of myocardial fibroblasts was a rare event in the control group, whereas in patients of group III the number of Ki-67-positive fibroblasts was increased 7.5-fold (Figs. 7A and 7B). In addition, in patients with decompensated hypertrophy we observed myofibroblasts (Figs. 7C and 7D).

DISCUSSION

Several studies have reported a selective increase of MMP and TIMP levels and MMP activity in myocardium of patients with end-stage HF in both non-ischemic and ischemic cardiomyopathies (11).  

The present study. The novel observation of the present study is a characterization of different MMP and TIMP profiles in patients with AS depending on the degree of...
hypertrophy. We demonstrate that in AS the amount and distribution of both MMPs and their inhibitors are involved in the remodeling of myocardium in different stages of hypertrophy. Matrix metalloproteinases and TIMPs were situated in the ECM and mainly colocalized with fibrosis.

In normal human myocardium, the protein content of MMPs was comparatively low. In failing myocardium, all MMPs studied here were significantly or insignificantly upregulated and correlated with the degree of fibrosis and hypertrophy. Interestingly, almost all MMPs had sharply increased already in group I, where fibrosis is already elevated but cardiac EF is still normal. Similarly, TIMP-1 and -2 were low in control tissue, increased (TIMP-2) or tended to be upregulated (TIMP-1) in compensated hypertrophy (group I), and were highest in failing hearts (group II, III), whereas TIMP-4 demonstrated a tendency to be lower in all AS groups than in controls. It is evident that the myocardial ECM in AS is in a highly dynamic state and varies depending on the degree of hypertrophy progression. Changes in MMP and TIMP expression can serve as early markers of remodeling in pressure-overloaded myocardium.

In our previous study the level of fibronectin (one of the markers of fibrosis) was shown to be increased in the ECM of myocardium in patients with AS (1). We documented a negative correlation between EF and fibrosis and showed that patients with the highest level of fibrosis (group III) were characterized by incomplete postoperative recovery. In our present work we measured the level of collagen I, the marker of mature fibrosis and the main substrate of MMP-1 and other MMPs. The degree of fibrotic changes correlated with upregulation of MMPs, TIMP-1 and -2, and the decrease of TIMP-4. That indicates the contribution of these enzymes to the remodeling of ECM and the clinical development of HF.

Study limitations. Because for ethical reasons the removal of repetitive biopsies was not possible, we subdivided the patients in representative groups. Repeated clinical studies of these AS patients confirmed the strong relationship of functional/structural characteristics that led us to interpret changes in groups with different EF as certain stages of hypertrophy progression. Taking into account the possible interference of turbulence on tissue structure and composition of myoectomies, the innermost endocardial part of the samples was not analyzed. Several samples of LV free wall were studied for the purpose of MMP and TIMP expression. The findings were similar to the current data, but myoectomies were further analyzed because they are easier for surgical and ethical reasons to obtain.
Tissue source of MMPs and TIMPs. Myocardial MMPs are produced by a variety of cells such as fibroblast-like cells, vascular smooth muscle cells, endothelial cells, infiltrating inflammatory cells and by cardiomyocytes (9,10,12–14). Moreover, according to numerous recent reports, practically all parenchymal and mesenchymal cells are able to synthesize extracellular proteinases. At the same time, there are only few data concerning the cellular origin of TIMPs (15). However, the overwhelming majority of investigations were done in vitro and the data cannot be completely extrapolated to the situation in vivo (16). In this study we have shown that the main cellular sources of MMPs and TIMPs in myocardium of AS patients are fibroblasts and that the contribution of other cells is negligible.

The role of fibroblasts in ECM remodeling. About 90% of the myocardial ECM cells are fibroblasts. Nevertheless, we found an increased number of proliferating fibroblasts in decompensated hypertrophy, likely because of intensive remodeling of the ECM. It has been illustrated in several reports that fibroblasts are a rather heterogeneous population that preserves its heterogeneity in vitro, demonstrating different responses to some cytokines (17,18). In the myocardial ECM of patients with progressing hypertrophy, we observed by electron microscopy the presence of fibroblasts characterized by diverse size and shape with different numbers of subcellular organelles and varying nuclear configuration (data not shown) and myofibroblasts. It remains unknown whether different matrix proteins including MMPs and TIMPs are synthesized by the same fibroblasts or whether there are different subpopulations of fibroblasts with limited functional specialization. Until now it has been established only that myofibroblasts have the capacity to produce both structural ECM proteins and proteinases (19). It is probable that a number of neurohormonal and cytokine signaling cascades determine the myocardial MMP and TIMP expression and activity in different cardiovascular disease states. Future studies are necessary to investigate the “switching over” phenomenon of cardiac fibroblasts and the factors involved in determining their synthetic specificity. This may represent a promising therapeutic target.

The paradox of the study. Collagen accumulation increased with the progression of hypertrophic changes in spite of enhanced MMP levels. Augmentation of MMP expression and activity in failing human myocardium was accompanied by an increase of TIMP-1 and -2 that would imply reduced MMP activity. This is at first sight a paradox, but it may be a natural phenomenon as long as MMPs not only degrade matrix components but also modulate collagen synthesis. It is not clear which event is initial, reactive fibrosis or MMP activation, as they both were present already in patients with compensated hypertrophy and can be provoked by the same factors. In a previous study we revealed a significant upregula-
tion of transforming growth factor-beta and angiotensin-converting enzyme inhibitors in AS patients (1). Their localization and concentration-dependent release may favor the imbalance between degradation and synthesis of ECM proteins with a prevalence of fibrotic changes. On the other hand, this discordance can be caused by insufficient inhibitory control of hypertrophy progression. In normal myocardium the cleavage of ECM proteins by MMPs is strictly limited and ECM is characterized by the presence of native “undenatured” interstitial proteins including collagen. Upregulation of MMPs at different levels of hypertrophy suggests an insufficiency of the inhibitory mechanism. It is known that TIMP-2, on one hand, is able to specifically inhibit MMP-2 but, on the other hand, to promote its activation (20). Tissue inhibitor of metalloproteinase-1 inhibits most of the known MMPs by forming complexes in a stoichiometric ratio of 1:1 although it poorly inhibits membrane-type MMPs and MMP-19 (20,21). Data from IHC analysis revealed a very low tissue content of TIMP-1 relative to the elevated levels of fibrotic changes (Figs. 6A to 6C). Therefore, the observed elevation of TIMP-1 and -2 protein levels may not necessarily translate into reduced MMP activity because in failing human LV the amount of MMP/TIMP complexes can be decreased while the ratio of

Figure 6. Cellular sources of MMP and TIMP (A to F). Double labeling for MMP-1 (green) (A) and vimentin (red) (B) showing that fibroblasts are the majority of cells in the MMP occupied area. Serial sections stained for MMP-2 (green) (C) and vimentin (red), CD68 (green) (D). Note: most of the cells in the MMP containing regions are fibroblasts and only a few macrophages are present (arrows). Sequential staining for TIMP (TIMP-1 shown as an example) (green) (E) and vimentin (red) (F) demonstrating fibroblasts in TIMP-positive areas. Nuclei are blue. Abbreviations as in Figure 1.
free to bound TIMP will increase (11). In addition, gelatinsases can be activated by stromelysin MMP-3 even in the presence of TIMPs (22). Tissue inhibitor of metalloproteinase-4 is the most cardiospecific among the known inhibitors of MMPs and possesses a higher inhibitory potential than the other TIMPs (23). In the present study, TIMP-4 was found to be repressed, which is in agreement with other reports (3,24,25). A decreased level of this inhibitor testifies to weakening of its cardioprotective abilities against an elevated MMP background and is presumably a decisive factor in HF progression. In hypertrophied myocardium, an insufficient increase or lack of stimulation of TIMPs fails to supply the necessary control of upregulated MMPs that is accompanied by accumulation of “abnormal” collagen and other extracellular components. Li et al. (26) consider that matrikines, side products of MMP activity, stimulate fibrosis progression.

It is also very probable that fibroblasts are the main affected link in the chain of stoichiometric MMP/TIMP imbalance development. Cardiac fibroblasts represent a heterogeneous population, which is able to produce ECM proteins as well as proteolytic and antiproteolytic agents. The stimulation of fibroblasts by profibrotic cytokines such as TGF-beta and angiotensin-converting enzyme inhibitors might override any protein degradation effects of MMPs. The net result of the activity of profibrotic and antifibrotic influences is a significant and continuously increasing degree of fibrosis.

**Conclusions.** In human hearts the progression from compensated hypertrophy to HF is accompanied by altered MMP and TIMP levels. Cardiac fibroblasts proliferate and take an active part in ECM remodeling during hypertrophy progression. In AS the upregulation of MMPs and inadequate inhibition by TIMPs occur in an early stage and continue in further stages of hypertrophy that finally contribute to a high degree of fibrosis and reduction of cardiac function.

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**REFERENCES**


