Increased Cardiac Expression of Tissue Inhibitor of Metalloproteinase-1 and Tissue Inhibitor of Metalloproteinase-2 Is Related to Cardiac Fibrosis and Dysfunction in the Chronic Pressure-Overloaded Human Heart

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Background—Alterations in the balance of matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs) are involved in left ventricular (LV) remodeling. Whether their expression is related to interstitial fibrosis or LV dysfunction in patients with chronic pressure overload–induced LV hypertrophy, however, is unknown.

Methods and Results—Therefore, cardiac biopsies were taken in 36 patients with isolated aortic stenosis (AS) and in 29 control patients without LV hypertrophy. Microarray analysis revealed significantly increased mRNA expression of collagen types I, III, and IV and transcripts involved in collagen synthesis, including procollagen endopeptidase and lysine and proline hydroxylases, in AS compared with control patients. Collagen deposition was greater in AS than in control patients and was most pronounced in AS patients with severe diastolic dysfunction. Cardiac mRNA expression of TIMP-1 and TIMP-2 was significantly increased in AS compared with control patients (mRNA transcript levels normalized to GAPDH: TIMP-1, \(0.67 \pm 0.1\) in AS versus \(0.37 \pm 0.08\) in control patients; TIMP-2, \(9.5 \pm 2.6\) in AS versus \(1.6 \pm 0.4\) in control patients; \(P<0.05\) for both) but did not differ significantly for MMP-1, -2, or -9. Cardiac TIMP-1 and -2 transcripts were significantly related to the degree of interstitial fibrosis and proportional to diastolic dysfunction in AS patients.

Conclusions—Cardiac expression of TIMP-1 and TIMP-2 is significantly increased in chronic pressure-overloaded human hearts compared with controls and is related to the degree of interstitial fibrosis. (Circulation. 2005;112:1136-1144.)

Key Words: metalloproteinases ■ remodeling ■ hypertrophy ■ hypertension ■ collagen

Left ventricular (LV) hypertrophy is an adaptive process that compensates for pressure overload caused by aortic stenosis (AS). This remodeling process consists of hypertrophic changes of cardiomyocytes and abnormalities of the extracellular network, which are both responsible for changes in systolic and diastolic function.

Altered levels of matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs) have been involved in remodeling of the extracellular matrix during LV hypertrophy and in the failing heart (reviewed previously). Recently, Hein and colleagues showed that the extent of myocyte degeneration in patients with severe AS correlated with the severity of systolic dysfunction, whereas Polyakova et al showed enhanced protein expression of TIMPs and MMPs in these patients. These studies included a significant number of patients with moderate to severe systolic dysfunction, and cardiac samples were taken at the subvalvular septum, where myocyte strain and fibrosis are most pronounced. Whether cardiac TIMP or MMP transcript levels in patients with cardiac hypertrophy because of chronic pressure overload correlate with the degree of interstitial fibrosis or with the severity of cardiac dysfunction, however, has not been investigated.

Therefore, in the present study, we first performed a microarray analysis to investigate changes in expression levels of genes involved in collagen synthesis and degrada-
TABLE 1. Clinical Preoperative Data

<table>
<thead>
<tr>
<th></th>
<th>Control Patients</th>
<th>Aortic Stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=29)</td>
<td>(n=36)</td>
</tr>
<tr>
<td>Age, y</td>
<td>64±2</td>
<td>69±2</td>
</tr>
<tr>
<td>Men/women, %</td>
<td>80</td>
<td>58*</td>
</tr>
<tr>
<td>EF, %</td>
<td>64±1</td>
<td>64±2</td>
</tr>
<tr>
<td>Valve orifice, cm²</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mean gradient, echo, mm Hg</td>
<td>5±1</td>
<td>58±3*</td>
</tr>
<tr>
<td>Gradient, invasive, mm Hg</td>
<td>2±0.5</td>
<td>72±5*</td>
</tr>
<tr>
<td>LV ED, mm</td>
<td>47±1</td>
<td>48±1</td>
</tr>
<tr>
<td>LV septum WT, mm</td>
<td>11±0.4</td>
<td>15±0.4*</td>
</tr>
<tr>
<td>LV posterior WT, mm</td>
<td>10±0.3</td>
<td>13±0.4*</td>
</tr>
<tr>
<td>LV relative WT</td>
<td>0.43±0.02</td>
<td>0.61±0.02*</td>
</tr>
<tr>
<td>LV mass/m², g/m²</td>
<td>95±4</td>
<td>147±6*</td>
</tr>
<tr>
<td>Sokolow-Lyon, mm</td>
<td>20±2</td>
<td>34±2*</td>
</tr>
<tr>
<td>Cornell voltage, mm/ms</td>
<td>1740±145</td>
<td>3495±221*</td>
</tr>
<tr>
<td>Presence of CAD, %</td>
<td>100% (29/29)</td>
<td>31% (11/36)*</td>
</tr>
<tr>
<td>No. of affected vessels</td>
<td>2.6±0.1</td>
<td>0.6±0.2*</td>
</tr>
<tr>
<td>Internal mammary artery, %</td>
<td>100% (29/29)</td>
<td>82% (9/11)</td>
</tr>
<tr>
<td>Venous bypass grafts, %</td>
<td>62% (16/29)</td>
<td>27% (3/11)</td>
</tr>
<tr>
<td>ACE inhibitors, %</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>AT1 receptor antagonists, %</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Diuretics, %</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>β-Blockers, %</td>
<td>72</td>
<td>45†</td>
</tr>
<tr>
<td>Ca antagonists, %</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>28</td>
<td>26</td>
</tr>
</tbody>
</table>

*P<0.0001, †P<0.05 in aortic stenosis vs control.

EF indicates ejection fraction; ND, not determined; EDD, end-diastolic diameter; WT, wall thickness; and CAD, coronary artery disease.

M-mode LV end-diastolic diameter was measured and normalized to body surface area. Diastolic patterns of LV filling were evaluated by Doppler echocardiography at the level of the mitral valve and pulmonary vein and were considered normal, prolonged relaxation, pseudonormal, or restrictive as previously described in detail.9

**Tissue Sampling**

During open-heart surgery, before patients were placed on the extracorporeal circulation, 2 or 3 transmural true-cut needle biopsy specimens, each weighing 1 to 2 mg, were taken from the anterior LV at close proximity between the left descending coronary artery and the circumflex coronary artery. One biopsy was immediately frozen in liquid nitrogen and stored at −80°C, whereas the second one was mildly fixed in 1% paraformaldehyde and embedded in paraffin. For electron microscopy, samples were embedded in Epon, sectioned, and stained according to a standard protocol.10

**RNA Isolation, Real-Time Polymerase Chain Reaction, and Microarray Analysis**

RNA was isolated from biopsies of 24 AS patients and 20 control patients with the RNasey Mini kit (Qiagen).11 RNA quality was measured with a Bioanalyzer Nanochip (Agilent Technologies), and RNA quantity with the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies).

Cardiac mRNA levels of MMP-1, MMP-9, TIMP-1 and TIMP-2, collagen αI type I (COLIa1), and collagen αI type III (COLIIIa1) were determined by use of real-time fluorescence detection. The genes of interest and a housekeeping gene (GAPDH) were amplified with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Transcript levels were determined in duplicate, and the results were expressed relative to GAPDH. The primer and probe sequences are listed in the Data Supplement Part II.

For microarray analysis, 50 ng total RNA from 19 AS patients and 7 control patients were amplified in 2 rounds with the 2-cycle cDNA synthesis kit from Affymetrix. First-round cDNA (600 ng) was used in a second round of amplification with biotin labeling. Biotin-labeled cDNA (11 μg) was then hybridized to Affymetrix human U133A GeneChips containing 18 400 genes. Gene transcript levels were determined with Microarray Analysis Suite Software, version 5.0 (MAS 5.0) (Affymetrix).

**Histological Analysis**

Primary antibodies against desmin, ubiquitin, MMP-1, MMP-2, MMP-9, TIMP-1 (all R&D Systems) and TIMP-2 (Calbiochem) were used for immunostaining. Relative surface area of MMP/TIMP immunoreactivity was expressed as percentage immunoreactive area divided by total area. The collagen fraction in the endocardial to epicardial regions was determined after collagen-specific picrosirius red staining with a Zeiss Axioplan2 microscope, a 3CCD video camera (DXC-930P, Sony), and KS300 software as previously described.10

**MMP Zymography and Immunoblotting Analysis**

For in situ zymography, cryosections of AS and control biopsies were covered with fluorescently labeled pigskin gelatin (Oregon Green 488; Molecular Probes) and incubated for 72 hours at 37°C.12 The gelatinase activity, indicated by the gray discoloration caused by gelatin degradation, was scored on a scale of 0 (no activity); 1, focal activity; 2, moderate; and 3, diffuse activity by a blinded observer. To confirm specificity of gelatinase activity in the degradation assay, consecutive sections were coincubated with EDTA, an inhibitor of MMP activity.

To semi-quantitatively investigate protein expression of MMP-2 and MMP-9 in cardiac extracts, immunoblotting of MMP-2 and MMP-9 (antibodies by R&D Systems) was performed on additional pooled biopsies of AS and control patients.11

**Statistics**

Data are expressed as mean±SEM. Normal distribution of all continuous variables was tested by use of the method of Kolmogorov...
Patients with AS had marked systolic dysfunction (ejection fraction [EF] was similar in AS and control patients. Only 2 patients with overt LV dilatation (end-diastolic diameter >5.8 cm) was present in only 3 AS patients, including the 2 patients with an EF of >50%. Overt LV dilatation (end-diastolic diameter >5.8 mm) was present in only 3 AS patients, including the 2 patients with an EF of <50%. All patients with AS except one presented with diastolic dysfunction at echocardiography. Twenty-one patients with AS presented with an abnormal relaxation without signs of increased LV filling pressures, whereas the 16 remaining patients showed a pseudonormal or restrictive filling, indicating increased LV filling pressures. Systolic or diastolic dysfunction was absent in the control group (exclusion criteria). No significant difference in drug intake, except for β-blockers, was observed between the 2 groups.

Collagen Synthesis, Fibrosis, and Systolic Function

Microarray analysis showed that collagens and transcripts involved in collagen synthesis were significantly upregulated in AS compared with control patients (Data Supplement Table I and Figure 1). Expression of COLIa1 and COLIIIa1 and Smirnov. The characteristics data all passed the normality test, and therefore, unpaired t tests were used for comparing patient characteristics in AS with those of control patients. Histological data and cardiac mRNA expression levels were compared by the unpaired t test or the Mann-Whitney test when the standard deviations of 2 groups were significantly different. A standard Pearson correlation test was used when 2 groups followed Gaussian distribution, whereas the nonparametric (Spearman) correlation was selected when groups did not follow Gaussian distribution.

Results

Myocyte Hypertrophy

Patients with AS showed typical characteristics of concentric cardiac hypertrophy, including significantly increased LV mass index (LV mass/m2), septal and posterior wall thickness, relative wall thickness, ECG criteria (Table 1), and cross-sectional area of cardiomyocytes (Table 2). Systolic function was similar in AS and control patients. Only 2 patients with AS had marked systolic dysfunction (ejection fraction [EF] <50%). Overt LV dilatation (end-diastolic diameter >5.8 mm) was present in only 3 AS patients, including the 2 patients with an EF of <50%. All patients with AS except one presented with diastolic dysfunction at echocardiography. Twenty-one patients with AS presented with an abnormal relaxation without signs of increased LV filling pressures, whereas the 16 remaining patients showed a pseudonormal or restrictive filling, indicating increased LV filling pressures. Systolic or diastolic dysfunction was absent in the control group (exclusion criteria). No significant difference in drug intake, except for β-blockers, was observed between the 2 groups.

Collagen Synthesis, Fibrosis, and Systolic Function

Microarray analysis showed that collagens and transcripts involved in collagen synthesis were significantly upregulated in AS compared with control patients (Data Supplement Table I and Figure 1). Expression of COLIa1 and COLIIIa1 was 1.3- and 2.3-fold upregulated, respectively. Upregulation of COLIa1 and COLIIIa1 mRNA was confirmed by Taqman real-time polymerase chain reaction (PCR) and expressed as fold increase in AS compared with control patients (COLIa1, 2.6-fold, P=0.05; COLIIIa1, 2.8-fold, P=0.02).

Prolyl 4-hydroxylase and lysyl hydroxylase, critical for procollagen synthesis in the endoplasmic reticulum, were upregulated 1.6- and 1.4-fold. Procollagen type III N-endopeptidase and procollagen type I and type II C-protease enhancer, which catalyze the cleavage of procollagens to ready-to-secrete collagens, were upregulated 1.3-, 1.2-, and 2.0-fold, respectively, in AS patients. Furthermore, lysyl oxidase like-2, which cross-links collagens to form triple helices in the extracellular space, was upregulated 1.4-fold. In addition, TIMPs were slightly but significantly upregulated by 1.4-fold (TIMP-1) and 1.2-fold (TIMP-2), whereas there was no differential expression of MMP-1 and -9 and a 1.2-fold increase in MMP-2 expression.

Transmural interstitial fibrosis as visualized by Sirius red staining was pronounced in all biopsies of AS compared with control patients (Table 2; Figure 2, A and B). Collagen deposition in AS patients was most prominent in the suben-
Cardiac MMP/TIMP Expression and Fibrosis

To quantitatively assess the differential expression of MMP/TIMPs in AS (n=21) compared with control patients (n=19), transcript levels of MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 were investigated by real-time PCR. Transcript levels of TIMP-1 and TIMP-2 and TIMP-1/MMP-2, TIMP-2/MMP-2, and TIMP-2/MMP-9 ratios were significantly increased in AS compared with control patients (Figure 3A–C). Importantly, transcript levels of both TIMP-1 and TIMP-2 were significantly related to the degree of fibrosis in AS patients (Figure 4A and B). In contrast, mRNA expression of MMP-1, -2, or -9 did not differ significantly between AS and control patients (Figure 3A). Nor was it related to collagen deposition (MMP-1, r=-0.11, P=NS; MMP-2, r=-0.18, P=NS; MMP-9, r=-0.14, P=NS) in AS patients. Significantly higher transcript levels of TIMP-1 and TIMP-2 and TIMP-2/MMP-2 ratio were observed in AS patients with pseudonormal or restrictive patterns compared with normal or abnormal relaxation (mRNA expression relative to GAPDH:...
TIMP-1, 1.89 ± 0.4 versus 0.41 ± 0.04, respectively, P < 0.001; TIMP-2, 18.7 ± 5.3 versus 3.2 ± 1.2 respectively, P < 0.01; TIMP-2/MMP-2, 538 ± 213 versus 139 ± 71, respectively, P = 0.02).

Semiquantitative analysis revealed significantly increased TIMP-1 and -2 immunoreactivity in the left ventricle of AS (Table 2; Figure 2, D and F) compared with control patients (Table 2; Figure 2, C–E), whereas MMP-1, -2, or -9 immunoreactivity did not differ significantly between the 2 groups (Table 2; Figure 5, A–F). In AS hearts, immunoreactivity of TIMP-1 and -2 was superimposable and was most prominent in areas of pronounced hypertrophy and in the extracellular matrix (Figure 2, D and F) but was weak in normal myocardium (Figure 2, C–E). Cardiomyocytes surrounding fibrotic areas also strongly expressed TIMP-1 and -2, suggesting that not only fibroblasts but also cardiomyocytes produce TIMPs.

MMP Zymography and Immunoblot Analysis

Immunoblotting of extracts from additional pooled biopsies (n = 4 per group) showed similar levels of protein expression of MMP-2 and MMP-9 in AS compared with control patients (% expression in AS patients relative to control patients: MMP-9, 89 ± 8% in AS versus 100 ± 15% in control patients; for MMP-2, 122 ± 13% in AS versus 100 ± 24% in control patients) (Figure 6G), concordant with similar MMP-2 and MMP-9 immunoreactivity.

In situ gelatin (MMP-2 and MMP-9) zymography of cryosections (n = 6 per group) revealed consistently decreased MMP gelatinolytic activity in AS compared with control patients (Figure 6, A–F). All of the 6 randomly studied AS patients showed grade 0 (minimal) gelatinolytic activity, whereas 4 of 6 control patients showed grade 2 (moderate), 1 grade 3 (diffuse), and 1 grade 1 (focal) gelatinolytic activity (gray coloration), which was inhibited in the presence of EDTA.

Validation of CABG Patients as a Control Group

To ensure that heart tissue obtained during CABG indeed reflects relatively “healthy” myocardium, we performed additional structural analysis in CABG compared with AS tissues and also determined fibrosis, MMP and TIMP immunoreactivity, and MMP immunoblotting in CABG compared with nonused donor hearts.

Electron microscopy revealed pronounced interstitial fibrosis and slight degeneration of cardiomyocytes in AS but a normal appearance in CABG patients (Figure 7, A and B). Immunoreactivity of ubiquitin, a ubiquitous polypeptide with a pivotal role in intracellular protein degradation, was significantly increased in AS compared with control patients.
distribution of desmin was disturbed in AS but more homogeneous in CABG patients (Figure 7, E and F). These data thus confirmed that the selected control CABG patients did not show signs of ongoing myocyte ischemia, suffering, or degeneration, in contrast to pronounced myocyte degeneration, cell death, and fibrosis in AS patients.

When comparing nonused donor samples with CABG samples, collagen deposition, immunoreactivity of MMPs and TIMPs, and MMP immunoblotting did not differ significantly (Table 3; Figure 8, A–L) (% immunoblotting in nonused donor relative to CABG LV: MMP-9, 96±11% in donor versus 100±27% in CABG; for MMP-2, 88±9% in donor versus 100±27% in CABG; P=NS), indicating that LV tissue as obtained during CABG does not differ from myocardial tissue of nonused donor hearts with respect to the levels of TIMPs, MMPs, and fibrosis.

### Discussion

The present study investigates the expression of MMP/TIMP in cardiac transmural biopsies of patients with pronounced LV hypertrophy and fibrosis caused by AS. Fibrosis is a crucial determinant of cardiac dysfunction during pressure overload caused by AS, which highlights the need for a better understanding of the mechanisms contributing to fibrosis during chronic pressure overload by AS.

Microarray analysis reveals significant upregulation of transcript levels of collagens and enzymes involved in collagen synthesis in AS patients compared with control patients.

### Table 3. Histological Analysis of TIMP and MMPs in CABG Compared With Nonused Donor Hearts (See Figure 8)

<table>
<thead>
<tr>
<th></th>
<th>CABG Hearts</th>
<th>Nonused Donor Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total collagen, %</td>
<td>7.2±0.3</td>
<td>6.9±2.4</td>
</tr>
<tr>
<td>TIMP-1, % immunoreactivity</td>
<td>6.1±0.3</td>
<td>4.0±2.1</td>
</tr>
<tr>
<td>TIMP-2, % immunoreactivity</td>
<td>2.5±0.4</td>
<td>1.9±1.4</td>
</tr>
<tr>
<td>MMP-1, % immunoreactivity</td>
<td>2.2±0.2</td>
<td>1.4±1.0</td>
</tr>
<tr>
<td>MMP-2, % immunoreactivity</td>
<td>2.4±0.3</td>
<td>3.6±0.9</td>
</tr>
<tr>
<td>MMP-9, % immunoreactivity</td>
<td>2.9±0.1</td>
<td>2.4±0.9</td>
</tr>
</tbody>
</table>

P=NS for all comparing CABG with nonused donor LV.
In the present study, we observed increased transcript levels of TIMP-1 and -2 in AS compared with control patients, which was most pronounced for TIMP-2. TIMP-2 plays a regulatory role in the proteolytic activation of proMMP-2. At low concentration, TIMP-2 serves as a receptor for proMMP-2, resulting in increased activation of proMMP-2 by MT1-MMP. However, at high concentration, TIMP-2 neutralizes MT1-MMP and prevents MMP-2 activation, indicating that preferential inhibition of MMP-2 by high levels of TIMP-2 might facilitate interstitial fibrosis.
The primary action of TIMPs is to inhibit matrix metalloproteinases, but numerous studies have reported cell growth-promoting, antiapoptotic, steroidogenic, and antiangiogenic activities reviewed previously. A part of these functions is attributed to MMP inhibition, but TIMPs also exhibit cellular activities that seem to be independent of MMP inhibition. Importantly, both TIMP-1 and TIMP-2 may stimulate the growth of fibroblasts in vitro, apart from their MMP inhibition. Lovelock et al clearly demonstrated a predominant role of TIMP-2 over TIMP-1 in stimulation of collagen production by cardiac fibroblasts, independent of its ability to inhibit MMPs. Thus, it remains possible that the upregulation of TIMPs increases cardiac fibrosis not just by inhibiting MMPs but also by separate direct profibrotic mechanisms.

Fibrosis is an important structural substrate for cardiac failure and sudden death. A major result of this study was to reveal that increased expression of cardiac TIMP-1 and TIMP-2 in AS compared with control patients was related to the degree of interstitial fibrosis. Therefore, TIMP-1 and -2 deserve further investigation as potential targets for therapeutic interventions in AS patients.

**Study Limitations**

CABG patients without cardiac dysfunction, unstable angina, ischemic heart disease, or LV hypertrophy were used as control subjects. Coronary artery disease has been associated with increased plasma levels of MMPs/TIMPs. However, altered cardiac MMP/TIMP expression levels in patients with coronary artery disease but without LV dysfunction, ischemia, or hypertrophy have, to the best of our knowledge, never been demonstrated. Nonused donor hearts, as an alternative for control hearts, are also not likely to be completely normal. Confounding factors linked to brain death cause of death (trauma), age, cardiac ischemia, and drug administration (inotropes) have been described to affect myocardial function and structure. In our study, we showed that myocardial tissue as obtained during CABG did not differ from myocardial tissue of nonused explanted donor hearts with respect to the levels of TIMPs, MMPs, and fibrosis.

The duration of AS-induced chronic pressure overload before aortic valve surgery could not be evaluated in the present study, because most of the patients presented only when severe AS resulted in dyspnea, angina, or syncope. Because of ethical considerations, follow-up biopsies could not be taken.

Use of β-blockers was different in control and AS patients. Whereas β-blockade reduces MMP-9 activity after acute myocardial infarction in rats, an effect of β-blockade on mRNA expression of cardiac TIMPs/MMPs has never been demonstrated. In the present study, TIMP/MMP transcript levels did not differ in AS or CABG patients with or without β-blockade (Data Supplement Table II).

**Acknowledgments**

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**References**

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