Regession of Left Ventricular Hypertrophy After Surgical Therapy for Aortic Stenosis Is Associated With Changes in Extracellular Matrix Gene Expression

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Background—Regression of left ventricular hypertrophy (LVH) after surgical correction for aortic stenosis is not fully understood on the molecular level. The aim of this study was to examine whether there is an association between LVH regression and extracellular matrix (ECM) gene expression.

Methods and Results—A standard model of controlled LVH induction by supracoronary banding (A = baseline) was applied in 44 growing sheep (age, 6 to 8 months). Surgical correction to release the pressure gradient was performed 8.3 ± 1 months later (B). The animals were killed after another 10.1 ± 2 months (C). At all time points, hemodynamic evaluations and quantitative analysis of mRNA and protein expression for matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) was performed. Left ventricular mass index was 82 ± 6 vs. 150 ± 33 g/m² (B), P < 0.01, and 78 ± 18 g/m² (C), P < 0.01. Left ventricular function and cardiac index remained stable. Myocardial fiber diameter index was 9.1 ± 1.2 vs. 12.3 ± 1.4 (B), P < 0.01, and 8.4 ± 1.3 µm/m² (C), P < 0.01. In parallel to the development of LVH at B, gene expression was increased significantly for MMP-1, MMP-2, MMP-3, and MMP-9 and for TIMP-1 and TIMP-2 and decreased significantly for TIMP-3. After surgical correction (C), there was a complete regression of gene expression to baseline measures.

Conclusions—Controlled induction of compensated LVH leads to significant increase in ECM gene expression. The regression of LVH after surgical therapy is associated with complete regression of ECM gene expression. However, no cause-and-effect relation could be demonstrated. (Circulation. 2001;104[suppl I]:I-54-I-58.)

Key Words: stenosis I hypertrophy I aorta I genes

Left ventricular hypertrophy (LVH) is an independent cardiac risk factor. In patients with aortic stenosis, it usually develops as the result of the increase in pressure load. After aortic valve replacement regression of LVH has been documented in clinical studies. However, the underlying cellular and molecular changes are not yet completely understood.

The development of LVH and its regression after therapeutic interventions are accompanied by changes of the cardiac extracellular matrix (ECM). The ECM is a 3D collagen network, sustaining myocardial structure and functional properties. Balanced collagen turnover is controlled by specific matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of MMPs, TIMPs) under normal hemodynamic conditions. Changes in ECM composition have been shown to coincide with myocardial ischemia and dilatative cardiomyopathy. However, changes in MMP and TIMP gene expression have not been examined in parallel to induction and regression of LVH. Therefore aim of this study was to assess ECM gene expression after controlled induction of compensated LVH and after regression of LVH after complete correction by surgical therapy.

Experimental Model

The study was performed with 44 growing female sheep (Merino). All animals received human care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication 85-23, revised 1985). Approval was obtained from the German governmental offices according to the “Tierschutzgesetz.” General anesthesia was applied at all surgical interventions. For induction, scopolamine (0.03 mg/kg), xylazine (0.22 mg/kg), and ketamine (11 mg/kg) were given intramuscularly. After endotracheal intubation, isoflurane at 0.5 to 2 vol%, 0.1 mg fentanyl, and pancuronium were used.

Baseline (A) operations for controlled induction of LVH were performed at the age of 6 to 8 months. A left lateral thoracotomy in the 4th intercostal space was performed to apply supracoronary banding of the ascending aorta. The second operation was performed 8.3 ± 1 months later, after significant LVH had developed (B). Through a median sternotomy, the banding was relieved and normal ascending aortic blood flow was restored. Another 10.1 ± 2 months later, after complete regression of LVH and normalization of left
ventricular mass (C), final examinations were performed and the animals were euthanized. During all interventions A, B, and C hemodynamic and standard echocardiographic examinations with transthoracic echocardiography and transesophageal echocardiography were performed to assess left ventricular morphology and hemodynamics. Sections of the left ventricular free wall were snap-frozen in liquid nitrogen and stored at −80°C until molecular biological analyses were performed. Each animal served as its own individual control. For quantitative histological analyses after hematoxylin and eosin staining, an Axioplan2 (Carl Zeiss GmbH, Jena, Germany) microscope at ×400 enlargement and the KS 300 Imaging System 3.0 (Carl Zeiss Vision GmbH) were used.

MMP/TIMP Measurements

Subtractive hybridization technique (MMP-9; TIMP-1 and TIMP-2) and alignment between different species (human, bovine, pig, rat, mouse) (MMP-1, MMP-2, and MMP-3; TIMP-3) with a reference data bank (National Center for Biotechnology Information, USA) were used to specifically delineate the relevant sequences. RNA analyses were performed semiquantitatively. At invariant positions, sense and antisense primer were determined and applied. The amplified sequences were cloned with TOPO-TA cloning vector (Invitrogen) and sequenced with an ABI 377 sequencer.

Frozen left ventricular tissue samples were prepared with an ultraturrax, with Trizol reagent (Life Technologies). An aliquot of total RNA (500 ng) was reversely transcribed into cDNA with reverse transcriptase (Superscript II plus, Life Technologies) and random primers (Life Technologies). To quantify mRNA expression levels, an aliquot of the cDNA was used in the PCR reaction containing the gene-specific primers and ReadyMix REDTaq PCR reaction mix (Sigma). After PCR, the products were separated on a 1% agarose gel and stained with ethidium bromide. The fragment intensity was analyzed by densitometry, and the amount of specific gene product was expressed as a ratio of specific gene product intensity/GAPDH intensity in relative units. Every PCR was performed 3 times, resulting in a variability of <10%. PCR conditions were as follows: annealing temperature 57°C to 65°C and elongation time 30 to 40 seconds at 21 to 40 PCR cycles.

For protein analyses, the frozen tissue samples were homogenized with an ultraturrax, with the use of a specific buffer. Protein expression was analyzed by Western blot. 20 μg of left ventricular tissue protein was separated on a 10% SDS-PAGE and blotted on 10% agarose gel and stained with ethidium bromide. The fragment intensity was analyzed by densitometry, and the amount of specific gene product was expressed as a ratio of specific gene product intensity/GAPDH intensity in relative units. Every PCR was performed 3 times, resulting in a variability of <10%. PCR conditions were as follows: annealing temperature 57°C to 65°C and elongation time 30 to 40 seconds at 21 to 40 PCR cycles.

For protein analyses, the frozen tissue samples were homogenized with an ultraturrax, with the use of a specific buffer. Protein expression was analyzed by Western blot: 20 μg of left ventricular tissue protein was separated on a 10% SDS-PAGE and blotted on polyvinylidene fluoride membranes (PVDF, Serva Biochemicala). The protein blots were incubated with specific polyclonal, anti-human antibodies (MMP-1, AB806, 20 seconds; MMP-2, AB809, 20 seconds; MMP-3, AB811, 1 minute; MMP-9 AB805, 1 minute; TIMP-1, AB 800, 1 minute; TIMP-2, AB801, 5 minutes; TIMP-3, AB802, 5 minutes, Chemicon) at a dilution of 1:1000. All polyclonal antibodies were produced in rabbits. These antibodies were applied on the xenogenic tissue because of the high degree of conservation of the MMP/TIMP proteins and the unavailability of specific anti-sheep MMP/TIMP antibodies. To ensure correct identification of protein bands, human and sheep samples were tested on the same blot showing bands of same molecular weight. For standard bands, recombinant MMPs and TIMPs from cell culture media were used (AG770, Chemicon). For staining, 1:5000 diluted IgG secondary antibody (peroxidase-conjugated AffiniPure goat anti-rabbit, Di-anova) was used. Detection was performed with the Supersignal West Pico Chemiluminescent Substrate-Kit (Pierce). To quantify proteins, the stained Western blots were analyzed with a 1D analysis software package (One-Disc Scanalytics). Every Western blot was performed 2 times, with a variability between measurements of <10%.

Data Analysis

Results are given as mean ±SD. All statistical analyses were performed with SPSS software (SPSS Inc). All results were tested for normal distribution. For comparison of mean values for the different time points, 1-way ANOVA was performed. For post hoc analysis, the Bonferroni test was applied. A probability value of 0.05 or lower on 2-tailed testing was considered to indicate statistical significance.

Table 1. Morphological, Functional, and Hemodynamic Results at Baseline, After Development of LVH, and After Corrective Surgical Therapy

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Baseline</th>
<th>After LVH</th>
<th>After Corrective Surgical Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVM, g/m²</td>
<td>82±21</td>
<td>150±33*</td>
<td>78±23*</td>
</tr>
<tr>
<td>Fiber index, μm/m²</td>
<td>9.1±1.2</td>
<td>12.3±1.4*</td>
<td>8.4±1.3*</td>
</tr>
<tr>
<td>Function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF, %</td>
<td>66±6</td>
<td>69±10</td>
<td>62±8</td>
</tr>
<tr>
<td>CI, L/min per m²</td>
<td>3.5±1.5</td>
<td>4.2±1.3</td>
<td>4.2±1.4</td>
</tr>
<tr>
<td>Wall stress, dyne/cm²</td>
<td>55±19</td>
<td>47±35</td>
<td>57±18</td>
</tr>
<tr>
<td>Aortic blood flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vmax, m/s</td>
<td>0.7±0.1</td>
<td>3.7±0.5*</td>
<td>0.9±0.1*</td>
</tr>
<tr>
<td>Pmax, mm Hg</td>
<td>2.7±0.1</td>
<td>56.8±4.7*</td>
<td>3.2±0.2*</td>
</tr>
</tbody>
</table>

Fiber index indicates myocardial fiber diameter index; EF, left ventricular ejection fraction; CI, cardiac index; wall stress, circumferential wall stress; Vmax, maximum transaortic blood flow velocity; and Pmax, maximum pressure gradient.

*P<0.01 vs previous time point.

Results

Banding (A) resulted in a reduction of the cross-sectional area of the ascending aorta by 25%, leading to a systolic pressure gradient of 25.8±6.3 mm Hg. Serial echocardiographic control examinations revealed a stepwise increase in gradients in parallel to growth.

During the time interval after banding (A to B) 8 of the 44 sheep (18.2%) died as the result of perforation of the ascending aorta (n=3), heart failure (n=3), a pericardial cyst (n=1), and respiratory infection (n=1). Corrective surgery (B) was performed on the surviving 36 sheep, 9 of which died during the perioperative and postoperative course after the second operation as the result of bleeding (n=3), low cardiac output syndrome (n=2), sternal wound infection (n=5), or endocarditis (n=1). Thus, 27 animals were available for final analysis (C).

Body weight was 35.9±5.5 kg (A) versus 54.1±6 kg (B) and 59.3±7 kg (C); body surface area was 1.1±0.1 m² versus 1.4±0.1 m² (B) and 1.5±0.2 m² (C). Normal left and right ventricular function was found after induction of uncompensated concentric LVH (B) and after complete regression of LVH at C. There were no significant changes in circumferential wall stress throughout the study. Detailed morphological, functional, and hemodynamic results are given in Table 1.

The unknown sequences of MMPs and TIMPs were identified with the methods described. Gene-specific primers feasible for human tissue also were then selected. After development of LVH (B), there was a significant increase in MMP-1, MMP-2, MMP-3, and MMP-9 as well as TIMP-1 and TIMP-2 and a significant decrease in TIMP-3 mRNA expression. These changes were also observed on the protein level. Complete regression of all these changes was observed after surgical therapy (C). Detailed results on MMP and TIMP
mRNA and protein expression are given as relative units in relation to the individual GAPDH standard in Table 2.

The association between left ventricular mass index (LVMI) as the most important morphological parameter to quantify LVH and MMP gene expression is shown in Figure 1, and the association between LVMI and TIMP gene expression in Figure 2, respectively. Between A and B, there was a parallel increase of LVMI as well as MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 and TIMP-2 gene expression; between B and C there was a parallel decrease of all these parameters. For TIMP-3, an inverse relation was found.

**Discussion**

Left ventricular hypertrophy occurs and eventually regresses on the basis of pressure load conditions. Because LVH is associated with increased cardiac morbidity and mortality, its regression may have important implications for patient outcome. In LVH, geometric chamber alterations, changes in ventricular mass, and myocardial cellular structure and increased fibrosis may be present. The transition from increased load to structural changes of the myocardium is considered to be multifactorial. In the presence of LVH, myocardial cellular hypertrophy and interstitial fibrosis associated with an unbalanced collagen turnover have been diagnosed. However, ECM gene expression during induction and regression of LVH is poorly understood at the moment. Therefore, the aim of this study was to focus on the role of collagenases, especially MMPs and TIMPs during controlled induction of LVH and after regression of LVH after corrective surgical therapy.

The experimental model chosen effectively mimicked the clinical situation of increased hemodynamic load associated with aortic stenosis. This model does not completely reflect aortic stenosis, especially with respect to coronary perfusion. However, it is an easy model in comparison to using an aortic valve cusp plication technique, thus allowing repeated surgical interventions. It was important to use growing sheep for banding to achieve gradually increasing pressure gradients that led to compensated LVH rather than to an acute pressure overload. Corrective surgery led to complete relief of the hemodynamic load as with aortic valve surgery. A selection process throughout the study may have influenced the outcome. However, the group of sheep was relatively homogeneous, the observed morbidity and mortality was caused by the surgical interventions, and, as mentioned in the results, the overall mortality rate after banding was comparable to what has been reported in the literature.

There were 3 important findings in the current study regarding MMP and TIMP gene expression and LVH: First, gene expression was significantly increased (MMP-1, MMP-2, MMP-3, MMP-9; TIMP-1, -2) or decreased (TIMP-3) after standard induction of controlled LVH, indicating an increased collagen turnover. Second, after complete corrective surgical therapy leading to regression of LVH, mRNA and protein expression are given as relative units in relation to the individual GAPDH standard in Table 2.

**TABLE 2. MMP 1, 2, 3, and 9 and TIMP 1, 2, and 3 Abundance at Baseline, After Development of LVH, and After Corrective Surgical Therapy**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Baseline mRNA</th>
<th>Baseline Protein</th>
<th>After LVH mRNA</th>
<th>After LVH Protein</th>
<th>After Corrective Surgical Therapy mRNA</th>
<th>After Corrective Surgical Therapy Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>0.15</td>
<td>101</td>
<td>0.2*</td>
<td>136*</td>
<td>0.16*</td>
<td>103*</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.29</td>
<td>101</td>
<td>0.39*</td>
<td>143*</td>
<td>0.24*</td>
<td>118*</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.49</td>
<td>100</td>
<td>0.99*</td>
<td>161*</td>
<td>0.5*</td>
<td>123*</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1</td>
<td>102</td>
<td>1.26*</td>
<td>135*</td>
<td>0.69*</td>
<td>99*</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.52</td>
<td>101</td>
<td>1.1*</td>
<td>145*</td>
<td>0.62*</td>
<td>124*</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.67</td>
<td>99</td>
<td>0.9*</td>
<td>142*</td>
<td>0.77*</td>
<td>117*</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.17</td>
<td>101</td>
<td>0.15*</td>
<td>91*</td>
<td>0.18*</td>
<td>115*</td>
</tr>
</tbody>
</table>

mRNA measurements are given in relation to GAPDH in relative units and protein analyses are given in relative units normalized to baseline = 100%.

*P < 0.01 vs previous time point.
complete regression of MMP and TIMP gene expression was observed as well. Third, an association between changes in LVMI and MMP/TIMP gene expression was found. However, it must be mentioned that no cause-and-effect relation was demonstrated. The experimental results cannot be completely transferred to humans because the course of LVH, its induction, and eventually its regression may be affected by multiple factors other than those examined, for example, patient age and sex or the presence of other risk factors such as coronary artery disease and diabetes. However, in this model, after corrective surgical therapy for compensated experimental aortic stenosis, regression of LVH almost leading to “restitutio ad integrum” was seen on the cellular and molecular levels.

An upregulation of MMP gene expression and consecutive degradation of the myocardial matrix have been shown to be associated with myocardial infarction and dilatative cardiomyopathy.6,7,12–14 Recently, inhibition of MMPs led to a decrease in left ventricular dilation and wall stress.7,12 In experimental models of LVH, dynamic alterations of MMP and TIMP gene expression have been documented as well.15,16 Thus in spontaneously hypertensive rat hearts, increased mRNA levels for MMP-1 and MMP-2 were found.17,18 Our results are in accordance with these findings. Changes in ECM gene expression were induced in a chronically developing model of aortic stenosis that closely resembles the clinical situation of compensated LVH. The finding of a balanced increase in ECM gene expression is in accordance with the hemodynamic findings of compensated LVH. From the increase in gene expression at that stage, it must be assumed that during induction of LVH there is an increased ECM turnover.

Thus far, regression of LVH after aortic valve replacement has not yet been examined on the molecular level. Therefore a major result of this study was to prove that the regression of LVH that was diagnosed by echocardiographic and histological evaluations goes along with complete regression of ECM gene expression. Thus ECM regulatory mechanisms do not only play an important role in the development of LVH but may also have an impact on the regression of LVH after surgical correction for aortic stenosis. This may have implications for the treatment of patients with LVH in future.

In this experimental study, regression of LVH back to normal values was seen after debanding. This is different from clinical findings in which the presence of fibrosis is thought to be the reason for incomplete regression of LVH.4 Our results must be evaluated in relation to the compensated LVH seen in all animals, whereas patients with aortic stenosis may have episodes of decompensation before surgical therapy is performed. Furthermore, in our study, the time interval between banding and reoperation may have been too short to cause sufficient fibrosis. The age of the animals may be an explanation for this finding as well: At reoperation, even in relation to the life expectancy of sheep of up to 15 years, it was much less than for patients receiving aortic valve replacement in their 60s and 70s. Thus the finding of complete LVH regression implies that no significant fibrosis has occurred. Eventually, the result of complete LVH regression could also be animal-specific and thus not be related to findings in humans.

The differences of TIMP-1 and TIMP-2 in comparison to TIMP-3 gene expression can be attributed to differences in structure and biochemical and functional properties.19,20 TIMP-1 and TIMP-2 are soluble and closely related to all MMP activities, functioning as their inhibitors, whereas TIMP-3 has been found exclusively in the ECM and serves as a marker for terminal differentiation.21 However, the MMP levels measured do not imply activity or activation, and the differences in TIMP levels observed may or may not lead to reduced inhibitory control.

Control of MMP gene expression parallel to and after treatment of the underlying cause of LVH may be a therapeutic option in the future. The use of currently developed MMP inhibitors or ACE-inhibitory therapy in addition to the surgical correction may be of benefit for the treatment of severe LVH. Further trials will be necessary before definite conclusions can be drawn. Thus far, the development of LVH must be regarded as a protective mechanism of the left ventricle caused by alterations in loading conditions. Therefore, at present, for patients with aortic stenosis, conventional valve replacement remains the therapy of choice to achieve unloading of the ventricle and thus regression of LVH. In addition, regression of the altered ECM gene expression may then occur.

In summary, LVH is reversible after corrective surgical therapy as long as fibrosis and cell death have not occurred. In this model of experimental aortic stenosis, complete regression of LVMI and of ECM gene expression was found.
after surgical therapy. However, no cause-and-effect relation has been demonstrated. The change in ECM gene expression can be considered part of the adaptive process of the left ventricle.

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References
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