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# Could activated tissue remodeling be considered as early marker for progressive valve degeneration? Comparative analysis of checkpoint and ECM remodeling gene expression in native degenerating aortic valves and after bioprosthetic replacement

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**Summary.** *Objectives.* Aortic stenosis is the leading cause of heart valve disease in elderly. Little is known about molecular mechanisms leading to altered left ventricular geometry generally and, particularly, to remodeling of degenerating aortic valve. Alterations in native degenerating aortic valves and valvular tissue after replacement might result from a stage specific tissue remodeling protein core induced by stress responsible factors. Here we were looking for a possible stage specificity of tissue remodeling and stress responsive checkpoint gene activation in native degenerating human aortic valves and bioprosthetic valvular tissue after replacement.

*Materials and methods.* Specimens of native degenerating aortic valves as well as bioprosthetic valves after replacement were tested for their morphological properties. Native degenerating valves were selected for two groups: non-calcified (7 samples) and calcified (5 samples) one; the third group (5 samples) was consisting of bioprosthetic valve samples after replacement. Individual mRNA-pools were isolated from each tissue sample, and semi-quantitative RT-PCR was performed. Target transcripts of $p21^{waf1/cip1}$, MT1-MMP, MMP-2, MMP-9 and TIMP-1 were measured. The specificity was controlled by restriction analysis of PCR products.

*Results and conclusions.* According to the abundant expression of $p21^{waf1/cip1}$, a highly activated stress response was found in non-calcified native degenerating aortic valves, whereas no stress response was monitored in valvular tissue after replacement. Whereas MT1-MMP expression was almost equally induced in all three groups investigated, MMP-9 was higher expressed in non-calcified *versus* calcified native valves, and was not expressed after replacement. An induced expression of MMP-2 was detected in non-calcified native degenerating aortic valves only. An abundant expression of tissue inhibitor of metalloproteinases TIMP-1 was observed in all three groups tested. Apparently, the ECM degradation potential is specifically enhanced in non-calcified native degenerating aortic valves e.g. at the early degeneration stages. In contrast, the replaced valves were found to be actively resorbing tissue with no detectable stress response, where both MT1-MMP and TIMP-1 might play the key role in geometry remodeling.

**Keywords:** Degenerating aortic valves – Bioprosthetic valvular replacement – Stress responsive checkpoints – Extracellular matrix remodeling – Metalloproteinases – Diagnostic molecular marker

## Introduction

Aortic valve degeneration is one of the leading heart diseases in the elderly, molecular pathomechanisms of which are poor understood until now. Mechanical stress is currently considered to be the main cause triggering degenerative processes. This is accompanied with a thickening of valve cusps, and remodeling of the left ventricular geometry. Clinical-pathological studies of aortic stenosis have demonstrated an abundant deposition of extracellular matrix (ECM) proteins physiologically present in bones (Srivatsa et al., 1997), and cuspal calcific deposits associated with mineralization of devitalized cells (Kim, 1995). Moreover, bone-marrow derived endothelial progenitor and dendritic cells have been recently identified in both native degenerative aortic valves and degenerative prostheses; the co-localization of those cells with inflammatory infiltrates has further been demonstrated (Skowasch et al., 2005).

At high-grade degeneration stages, aortic valve tissue becomes non-functional and should be substituted with bioprosthetic valves in time. In order to improve the quality of life after cardiac valvular surgery, new procedures and new prostheses have been developed in the past decade. Nevertheless, the provided world-wide statistics indicate that each kind of aortic valve replacement (AVR) is quite frequently followed by different metabolic impairments and physiologic complications like progressively abnormal lipid profiles, a non-specific inflammation,

blood trauma, hemorheologic changes or severe congestive heart failure and even death during individually long postoperative time (Hattori et al., 1994; Sugiyama et al., 1997; Yun et al., 1999; Chen et al., 2000; Vrandecic et al., 2000; Novaro et al., 2001; Horiguchi et al., 2001). After AVR, the wall thickness remained significantly greater than normal for patients with aortic stenosis and after 5 years of follow-up the remodeling of the left ventricular geometry is observed after AVR (Murakami et al., 2000). Although of great importance conditional alterations in gene expression by the valvular replacement are still poorly understood until now.

Tissue remodeling observed in general left ventricular geometry after AVR might result from induced activity of extracellular matrix (ECM) remodeling proteins. Matrix metalloproteinases (MMPs) play the key role in the tissue remodeling under both physiologic and pathologic conditions. MMPs are produced as zymogens (pro-MMPs) that require proteolytic activation through the elimination of the N-terminal propeptide via membrane-type matrix metalloproteinases (MT-MMPs) activity. Tissue inhibitors of metalloproteinases (TIMPs) act to inhibit metalloproteinase activity by forming a non-covalent irreversible complex with MMPs. A shift in resulting MMPs activity is well documented under stress conditions (Chen et al., 2001). However, less is known about the regulation of ECM degrading enzymes in native degenerating aortic valves and in valvular tissue after replacement. Insights in the mechanism of valvular tissue remodeling are essential for further development of this scientific and clinical branch.

The goal of this study was a comparative expression analysis of selected tissue remodeling genes in native degenerating aortic valves *versus* those in valvular tissue after AVR. Additionally, an impact of stress responsible cell cycle checkpoint became evaluated in valvular specimens of comparison.

## Materials and methods

### *Sample preparation*

Aortic valve specimens were collected from patients who underwent aortic valve replacement for the first and second times for calcific stenosis. Aortic valve replacement was performed by usual procedure using porcine bioprosthetic valves. Immediately after valvular tissue extraction an aliquot of each tissue sample was rinsed with sterile saline solution, fixed in 10% neutral buffered formalin, embedded in paraffin, and further used for the classification of terms of calcification grade. This classification was done upon microscopic analysis. Each extracted valvular sample was shock frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until RNA isolation. The present study was approved by the Ethics Committee of the Medical Faculty, University of Bonn, and all patients gave informed consent for the analysis of removed tissue.

### *Assessment of cell density*

Morphometric assessment of cell density in intimal regions was performed by counting hematoxylin-stained cell nuclei on a high-resolution video monitor (Endovision 534, Storz Inc., Germany). The microscopic image (Optiphot-2, Nikon, Germany) was relayed by a miniaturized video camera (Endovision 534) to a downstream monitor (final magnification, $\times 500$). Ten randomly selected tissue areas, each encompassing $0.4\,mm^2$, were assessed per each sample, and the corresponding number of cells was counted. Morphometric examination of cell density was performed by two independent investigators in a double-blind fashion.

### *Assessment of macrophages tissue detection*

Monoclonal antibodies specific for macrophages (CD68) were obtained from Boehringer Inc. (Germany). The color reaction was done with APAAP marking (Dako, Denmark) according to a standard protocol. Positive immunohistochemical reaction was marked by distinct, red to lilac signals. Nuclear counter-staining was performed by standard hematoxylin staining. Histological sections were photographed by a Nikon Optiphot-2-photomicroscope (Nikon, Germany) using Kodak Ektachrome 100 ISO color films.

### *Isolation of total RNA and mRNA*

Total RNA was extracted using the commercial RNAzol-B isolation kit (WAK-Chemie Medical GmbH, Germany), and an isolation of mRNA from the individual total RNA-pools was performed using the Oligotex® mRNA Mini Kit (Qiagen, Germany) according to protocols supplied by the manufactures.

**Table 1.** Primer design, expected length of specific PCR products, and nucleases used for individual restriction analysis

| Gene | Accession no. | Forward primer ($5' \rightarrow 3'$) | Reverse primer ($5' \rightarrow 3'$) | Expected length of PCR products, bp | Endonucleases used in restriction analysis |
|---|---|---|---|---|---|
| β-actin | BC014861 | GATGGTGGGCATGGGTCAG | TGGGGTTCAGGGGGGCCT | 209 | Alu I, Hae III, Rsa I |
| MMP-2 | NM_004530 | GCTAATGGCCCGGGGC | TGATCCTGTATGTGATCTGGTTC | 377 | Alu I, Bal I, Apa II |
| MMP-9 | NM_004994 | CTCTGGCAGCCCCTGG | ACCGTGGGGTTCGCATG | 289 | Alu I, Hpa II, Pvu II, Rsa I |
| TIMP-1 | NM_003254 | GGCCCCCTTTGAGCCC | CTCAGGCTGTTCCAGGGA | 393 | Hae III, Hind II, Hpa II, Pst I |
| MT1-MMP | U41078 | GTCTCCTGCTCCCCCT | CGAACATTGGCCTTGATCTCA | 295 | Hae III, Rsa I |
| $p21^{waf1/cip1}$ | Z85996 | CAGAACCGGCTGGGGAT | CGGCGTTTGGAGTGGTAG | 463 | Alu I, Hha I, Pst I, Pvu II |

*Reverse transcriptase PCR (RT-PCR)*

RT-PCR has been performed using specific primers designed to MMP-2, MMP-9, MT1-MMP, TIMP1, $p21^{waf1/cip1}$, and a housekeeping gene β-actin. cDNA synthesis was performed using the "First-Strand cDNA Synthesis Kit" (GE Healthcare, UK). For each cDNA synthesis, 1 µg mRNA was reverse transcribed using oligo(dT)18 primer in a final volume of 33 µl each, according to the protocol supplied by manufacturer. After heat inactivation of reverse transcriptase 2 µl of the cDNA were used for each PCR amplification. PCR primers are described in Table 1.

The PCR mixture contained 1xPCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM $MgCl_2$/10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primer pairs (100 pM each per reaction), and cDNA-template in a final volume of 50 µl. Reactions were hot-started at 95 °C for 5 min before the addition of 1.5 units of Taq polymerase (Red-Hot®, ABgene, UK) at the annealing temperature of 56 °C followed by the polymerization at 72 °C for 1 min. Amplification was carried out in a Perkin Elmer "DNA Thermal Cycler TC480" for 45 cycles (denaturation for 45 sec at 95 °C, annealing for 45 sec at the annealing temperature of 56 °C, and polymerization time of 30 sec at 72 °C, followed by a final 7 min extension at 72 °C). Negative controls without DNA as well as positive controls with a sequenced template were performed for each set of PCR experiments. PCR products (50 µl) were directly loaded onto 3% agarose gels ("Wide Range"-Agarose for analysis of DNA fragments longer than 50 bp, Sigma-Aldrich, UK), stained with ethidium bromide after electrophoresis, and directly visualized under UV illumination.

The amplified target products were quantified using the specialized quantification program (Quantity One®, Bio-Rad, USA). For the statistical analysis the quantification of each target cDNA was done twice. The results were normalized through β-actin expression in the corresponding mRNA-pool.

*Restriction analysis*

Target PCR products were identified using site specific restriction analysis. The amplification products underwent an extraction from the agarose gel using DNA isolation kit (DNAClean™ Hybaid-AGS GmbH, Germany) before digestion. They were digested in a final volume of 50 µl with 20 units of each restriction endonuclease for 2 h, according to conditions specified by the manufacturer (Fermentas, Lithuania). For digestion of the target PCR products selected restriction endonucleases listed in Table 1 were used. Digested DNA fragments were directly loaded onto 3% agarose gels ("Wide Range" agarose for analysis of DNA fragments longer than 50 bp, Sigma-Aldrich, UK), stained with ethidium bromide after electrophoresis, and directly visualized under UV illumination.

*Statistical analysis*

All statistical analyses were done using the SPSS software (version 8.0). Statistical significance was calculated by the two-sided unpaired Student's *t*-test.

## Results

Altogether 17 tissue samples collected were grouped as following:

1. degenerating non-calcified native aortic valves (7 samples),
2. degenerating calcified native aortic valves (5 samples), and
3. bioprosthetic valves (5 samples).

The results of morphological examinations of valves are given in Table 2.

**Table 2.** Patients and valves characteristics

| Valve no. | Group | Age/gender | CD68 expression (%) | Cellular density (cells/mm$^2$) |
|---|---|---|---|---|
| 1 | 2 | 76/F | 7.2 | 1775 |
| 2 | 2 | 52/F | 4.5 | 4041 |
| 3 | 2 | 70/F | 5.9 | 2696 |
| 4 | 2 | 82/M | 14.3 | 2537 |
| 5 | 2 | 52/F | 3.6 | 2288 |
| 6 | 1 | 76/F | 1.6 | 876 |
| 7 | 1 | 71/F | 1.0 | 794 |
| 8 | 1 | 70/F | 0.8 | 569 |
| 9 | 1 | 75/M | 9.6 | 600 |
| 10 | 1 | 82/M | 10.2 | 1669 |
| 11 | 1 | 74/F | 0 | 535 |
| 12 | 1 | 54/M | 4.1 | 1121 |
| 13 | 3 | 73/M | 7.0 | 875 |
| 14 | 3 | 68/M | 10.1 | 839 |
| 15 | 3 | 71/M | 11.3 | 792 |
| 16 | 3 | 63/M | 4.6 | 645 |
| 17 | 3 | 74/M | 8.7 | 684 |

Non-calcified native degenerating valves were selected for group 1; calcified native degenerating valves were selected for group 2; valvular tissue after replacement was selected for group 3

Quantitative expression analysis was performed for altogether five target genes, and normalized *versus* β-actin. For an appropriate statistical analysis the quantification of each gene was performed two-time for each sample. Individual characteristics of RT-PCR primer sets and corresponding target products are given in Table 1.

Statistical analysis revealed a similar distribution of all target transcripts measured inside the selected groups; the corresponding standard deviations are shown in Figs. 1 and 2. In contrast, considerable difference in target expressions was monitored, when the groups were compared with each other. Thus, whereas abundant $p21^{waf1/cip1}$ expression was demonstrated in non-calcified native degenerating aortic valves, no traces of target transcripts could

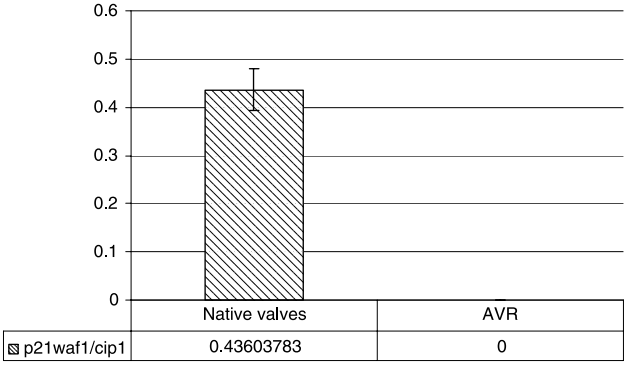
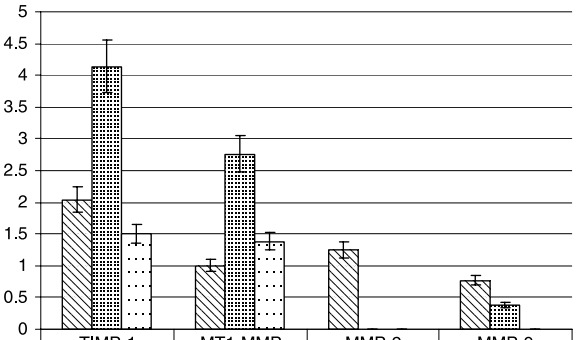



**Fig. 1.** Results of semi-quantitative RT-PCR: comparative analysis of $p21^{waf1/cip1}$ expression levels in native non-calcified degenerating aortic valves and those in valvular tissue after replacement




| | TIMP-1 | MT1-MMP | MMP-2 | MMP-9 |
|---|---|---|---|---|
| Group 1 | 2.039323046 | 1.00298656 | 1.2484879 | 0.76753507 |
| Group 2 | 4.13655914 | 2.764516129 | 0 | 0.38725307 |
| Group 3 | 1.5 | 1.381226054 | 0 | 0 |

**Fig. 2.** Results of semi-quantitative RT-PCR: comparative analysis of expression levels of tissue remodeling genes in all groups tested, where group 1 = degenerating non-calcified native aortic valves, group 2 = degenerating calcified native aortic valves, and group 3 = bioprosthetic valves

be detected in bioprosthetic valvular tissue after replacement (Fig. 1). Whereas both groups with non-calcified and calcified native degenerative valves demonstrated well expressed MMP-9, no expression of this gene was detected in the third group (Fig. 2).

Although at significantly different levels, the expression of both TIMP-1 and MT1-MMP was detected in all three groups (Fig. 2). The highest expression levels of TIMP-1 were registered in calcified native valves being about two- and three-times higher than those in non-calcified native and bioprosthetic valves respectively. Being abundantly expressed in all three groups tested, the expression levels of MT1-MMP were, however, the highest in group 2; the corresponding expression levels were about 2.7- and 2-time lower in groups 1 and 3 respectively.

A very special case formed a transcription regulation of MMP-2 gene, which was highly expressed in the group of non-calcified native valves only, whereas no traces of the target expression could be detected in other two groups of comparison (Fig. 2).

## Discussion

Molecular pathomechanisms leading to the degeneration and remodeling of valvular tissue are currently not well understood. We hypothesized here that a stage specific constellation of an activated core of tissue remodeling genes might play the key role in geometric alterations of native degenerating aortic valves and of valvular tissue after replacement. The goal of this study was to estimate a potential impact of selected ECM remodeling genes in low- and high-grade native degenerative aortic valves as well as bioprosthetic valves after replacement.

Matrix metalloproteinases (MMPs) as well as their natural activators and inhibitors are considered as key enzymes for tissue remodeling in both physiological processes and pathological states. The overall tissue activity of MMPs is controlled at three levels: transcription regulation, activation of latent pro-enzymes, and inhibition of proteolytic activity. Our examination was carried out at the primary level of regulation – at the level of transcription, the evaluation of which give a good possibility to estimate an overall ECM degrading potential in a tissue characterized by considerable heterogeneity of the cellular population such as aortic valves. Endocardial, interstitial, smooth muscle cells as well as fibroblasts and myofibroblasts have been identified in highly sophisticated dynamic structures of cardiac valves (Taylor et al., 2000). The ECM is thought to be an integral component of this coordinated dynamism (Yacoub et al., 1999).

Following main findings were achieved in our study: the chore of activated ECM degrading genes differs both qualitatively and quantitatively at each stage of valvular degeneration; after AVR it is regulated qualitatively different. There is a clear tendency for a high activation of ECM degradation potential in non-calcified compared to its significant decrease in calcified valves. Whereas MMP-9 activation differs only quantitatively being higher at the early stage, the activation of MMP-2 was observed at the early stage only. This finding is well in agreement with data measured earlier at the protein level in stenotic valves (Kaden et al., 2004). Therefore, an enhanced ECM degrading potential accumulated at the transcriptional level becomes brought into action through the considerable gelatinolytic activity developed in stenotic valves. Considering non-calcified and calcified native degenerating valves as early and later stages of valve degeneration respectively, our findings indicate activated MMPs core to be specific for the early stage of native valve degeneration. The later stage, in contrast, is characterized by dropping ECM degrading potential. From viewpoint of a clinical application of these results, ex vivo evaluation of ECM degrading genes even in blood samples seems to make sense in terms of prognosis: increased quantities of MMP-9 in blood plasma have been shown to be a risk factor for fatal cardiovascular events generally (Blankenberg et al., 2003).

ECM degrading enzymatic core constellation becomes totally different after AVR. Neither MMP-2 expression nor this of MMP-9 was detected in the replaced tissue. Additionally to the MMP-9 suppression, TIMP-1 was

shown to be activated in the valves after replacement. Being the natural inhibitor of all members of the collagenase, stromelysin, and gelatinase classes, the activated TIMP-1 represents the very last step in the regulation cascade of MMPs activity. Its role as negative regulator is to build irreversible non-covalent complexes with both latent and activated forms of MMPs both of which, therefore, become inactivated. Additionally, TIMPs have been shown to prevent a post-translational activation of some cytokines – natural activators of MMPs (McGeehan et al., 1994; Gearing et al., 1994). Consequently, TIMP-1 has been found to be highly expressed in actively resorbing tissue (Leco et al., 1994). Therefore, the rearrangement in MMP-9/TIMP-1 balance – MMP-9 suppression against TIMP-1 activation – indicates a strong tendency for the suppression of the overall ECM degradation potential in the replaced valvular tissue. This is qualitatively different regulation compared to the considerably enhanced degradation potential in degenerating valves particularly at the early stage, before an extensive calcification appears. In consensus with this conclusion, MMP-2 was shown to be suppressed after replacement, whereas this gene was abundantly expressed at early valve degeneration. Taking these facts together we conclude the replaced valves to be actively resorbing tissue.

In the case of bioprosthetic valves, we found MT1-MMP to be expressed without a co-activation of MMP-2 and -9. This is a rare case of MT1-MMP regulation currently described in literature, although MT1-MMP is known to be able to degrade extracellular matrix directly (Imai et al., 1996; Lohi et al., 2000). Our finding might indicate the key role of MT1-MMP as a matrix degrading protease in the valvular tissue remodeling after AVR, and open good perspectives for new target therapy approaches in order to avoid the most usual metabolic impairments and clinical complications well known for patients after AVR.

Our further findings to the stress response regulation support well the conclusions postulated above: whereas an abundant transcription of $p21^{waf1/cip1}$ was monitored in native aortic valves at early stage of degeneration, this expression was fully suppressed in valvular tissue after replacement – typically for an actively resorbing tissue. Any stress response is physiologically well controlled during cell cycle through activation of checkpoint genes. Further, a positive correlation between an increased stress and activation of $p21^{waf1/cip1}$ in heart is well documented by recent studies (Golubnitschaja et al., 2003, 2006). Although oxidative stress has been implicated to cardiac ischemia and reperfusion in patients after AVR (Pietri et al., 1994), we found no detectable activation of $p21^{waf1/cip1}$ in replaced valvular tissue. $p21^{waf1/cip1}$ suppression in cardiac tissue might further lead to deformed nuclear architecture of cells, concomitant local necrosis/apoptosis and non-specific inflammation (Mantel et al., 1999) which are well known complications for patients after AVR.

Taking together we conclude that a highly activated ECM degradation potential might be considered as early marker for the triggered aortic valve degeneration. The replaced valves were found to be actively resorbing tissue with no detectable stress response, where both MT1-MMP and TIMP-1 might play the key role in geometry remodeling after AVR.

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