Increased Expression of Elastolytic Cathepsins S, K, and V and Their Inhibitor Cystatin C in Stenotic Aortic Valves


Objective—To investigate the possible role of elastolytic cathepsins S, K, and V and their endogenous inhibitor cystatin C in adverse extracellular matrix remodeling of stenotic aortic valves.

Methods and Results—Stenotic aortic valves were collected at valve replacement surgery and control valves at cardiac transplantations. The expression of cathepsins S, K, and V and cystatin C was studied by conventional and real-time polymerase chain reaction and by immunohistochemistry. Total cathepsin activity in the aortic valves was quantified by a fluorometric microassay. When compared with control valves, stenotic valves showed increased mRNA expression of cathepsins S, K, and V (P<0.05 for each) and a higher total cathepsin activity (P<0.001). In stenotic valves, cystatin C mRNA was increased (P<0.05), and cystatin C protein was found particularly in areas with infiltrates of inflammatory cells. Both cathepsin S and cystatin C were present in bony areas of the valves, whereas cathepsin V localized to endothelial cells in areas rich of neovascularization. Incubation of thin sections of aortic valves with cathepsins S, K, and V resulted in severe disruption of elastin fibers, and this cathepsin effect could be blocked by adding cystatin C to the incubation system.

Conclusions—Stenotic aortic valves show increased expression and activity of elastolytic cathepsins S, K, and V. These cathepsins may accelerate the destruction of aortic valvular extracellular matrix, so promoting the progression of aortic stenosis. (Arterioscler Thromb Vasc Biol. 2006;26:1791-1798.)

Key Words: aortic stenosis • cathepsin • cystatin C • elastin • heart valves

Aortic stenosis is an active inflammatory process resembling atherosclerosis. The pathophysiological changes in the stenotic aortic valves include inflammatory cell infiltration, neoangiogenesis, accumulation of lipids, and active mediators of calcification with extracellular matrix (ECM) degradation.1–7 Furthermore, aortic valvular myofibroblasts undergo phenotypic changes toward an osteoblast-like phenotype, and active bone formation and remodeling occur in the stenotic valves.8,9 These processes lead to loss of normal valvular geometry, disruption of the elastic fibers, and accumulation of fibrocalcific masses. This adverse remodeling of the valves is associated with the loss of their mechanical and elastic properties, leading to thickening and stiffening of the valve leaflets and to progression of aortic stenosis.

The pathophysiological mechanisms involved in the abnormal accumulation and degradation of ECM in the valves are incompletely understood. In atherosclerotic lesions, elastolytic cysteine proteases, including cathepsins S and K, are overexpressed at sites of arterial elastin damage, whereas their endogenous inhibitor, cystatin C, is severely reduced.10,11 Moreover, a marked deficiency of cystatin C expression with accentuated cathepsin S and K expression in aortic aneurysms and a negative correlation of cystatin C serum levels with the diameter of abdominal aorta has been reported.11 These observations support the role of an imbalance between cathepsins and cystatin C in diseases characterized by pathological elastin degradation. In addition, cathepsin K has a crucial role in bone matrix degradation necessary for normal bone growth and remodeling,12 whereas cystatin C inhibits both bone resorption and osteoclast formation.13 In cathepsin K–deficient mice, impaired bone resorption leads to an osteoporotic-like phenotype, and patients with single-point mutations in the cathepsin K gene, resulting in a lack of cathepsin K activity, develop severe osteopetrosis.14–16 Because cathepsin S was shown recently to participate in ECM degradation during microvessel formation,17 this cathepsin and perhaps also other cathepsins may participate in neoangiogenesis, a process critical for progression of aortic stenosis.7,18

The possible role of cathepsins and cystatin C in the pathogenesis of aortic stenosis has not been examined previously. Moreover, there are no data available on the possible role of the elastolytic protease cathepsin V in cardiovascular diseases. The aim of our study was to determine whether the
elastolytic cathepsins S, K, and V and their inhibitor cystatin C are present at stenotic aortic valves and whether they might participate in the ECM remodeling known to associate with the progression of aortic stenosis.

Methods

For Methods, please see the online supplement, available at http://atvb.ahajournals.org.

Results

Levels of mRNA Expression in Control and Stenotic Valves

RT-PCR revealed that cathepsins S and K and cystatin C were produced locally in both stenotic and control aortic valves. The mRNA expression levels of cathepsins S and K were significantly higher in the stenotic than in the control valves (4.5 [0.4 to 79.1] versus 0.9 [0.03 to 14.5] AU; P<0.01; Figure 1A). A positive correlation appeared between the mRNA expression levels of cathepsin S and K (r=0.86; P<0.001) as well as between the mRNA levels of cathepsins V and S (r=0.86; P<0.001), and cathepsins K and V (r=0.81; P<0.001). Moreover, the mRNA expression levels of all 3 cathepsins correlated positively with those of cystatin C (r=0.91 for cathepsin S, r=0.87 for cathepsin K, and r=0.86 for cathepsin V; P<0.001 for all). The mRNA expression levels were similar in both tricuspid and bicuspid stenotic aortic valves. In addition, patients with aortic stenosis who received statin treatment (n=9) had a tendency toward lower mRNA expression levels of cathepsins S and K and also of cystatin C (data not shown).

Immunohistochemical Detection of Cathepsin S, Cathepsin V, and Cystatin C

Immunohistochemistry showed that cathepsin S was more abundant in the stenotic than in the control aortic valves (Figure 2A and 2B). In the stenotic valves, positive staining of cathepsin S was found in superficial endothelium lining the valves and also in the deeper areas rich of inflammatory cells. In contrast, in the control aortic valves, cathepsin S protein was detected only in the superficial endothelium lining the valves. Double immunofluorescence stainings demonstrated that cathepsin S colocalized with macrophages in the inflammatory areas of the stenotic valves (Figure 2C through 2E), and large regional differences in the amount of cathepsin S–positive macrophages between the different valvular regions were observed. In addition, cathepsin S protein was detected in the ossified areas of the valves, where it appeared as a perinuclear rim in the majority of the S100-positive chondroblast-like cells, suggesting active cathepsin S synthesis by these cells (Figure 2F through 2H). Colocalization of cathepsin S and cystatin C was detected in the superficial endothelium lining the valves as well as in a fraction of macrophages in the stenotic valves (Figure 2I through 2K).

Cathepsin V was present in the endothelium lining the valve leaflets of both stenotic and control valves (Figure 3A and 3B). Importantly, in stenotic aortic valves, strong cathepsin V staining was also detected in the endothelial cells forming the neovessels within the valves (Figure 3C and 3D). Double immunofluorescence stainings (Figure 3E through 3J) confirmed colocalization of cathepsin V and endothelial cells, both in thick-walled (Figure 3E through 3G) and thin-walled (Figure 3H through 3J) neovessels. In the thick-walled vessels, some cathepsin V staining was also observed in the surrounding pericytes (Figure 2F).

Cystatin C protein was detected both in the stenotic and in the control valves. However, the staining intensity of cystatin C appeared stronger in the stenotic than in the control aortic valves. In control valves, most of the cystatin C staining was present in acellular areas (Figure 4A), whereas in stenotic valves, cystatin C was typically present both intracellularly and extracellularly in cell-rich areas (Figure 4B). Similar to
cathepsin S, cystatin C was also found in the majority of the chondroblast-like cells in bony areas of the stenotic valves (Figure 4C and 4D). As shown in Figure 5, strong cystatin C staining was detected in areas rich of inflammatory cells, notably macrophages (Figure 5A and 5B). Moreover, in stenotic leaflets, cystatin C protein was found in the areas where elastin fibers were preserved (Figure 5B and 5C). Double immunofluorescence stainings revealed the colocalization of cystatin C and macrophages, with approximately one third of the macrophages showing positive signals. (Figure 5D through 5I). As shown in Figure 5J through 5L, cystatin C was detected in the superficial endothelium lining the valve leaflets. In contrast, cystatin C was absent from the endothelial cells forming the neovessels of the stenotic aortic valves (Figure 5M through 5O). Negative controls of all immunohistochemical and double immunofluorescence stainings are shown in supplemental Figures I and II (available online at http://atvb.ahajournals.org).

Degradation of the Aortic Valvular Elastin Fibers by Cathepsins S, K, and V Ex Vivo
Histological staining of elastin and collagen fibers revealed a pattern of degradation and disorientation of elastin fibers in the stenotic aortic valves, whereas the majority of the elastin fibers in the control aortic valves were intact. The observed in vivo pattern of elastin degradation could be mimicked in vitro by incubating cryostat sections of control aortic valves with recombinant human cathepsin S, K, and V. Figure 6A demonstrates the quantitative results of the analysis of valvular elastin fibers by computer-assisted morphometry. The average content of elastin fibers in the control aortic valves was 45±17% before incubation and 45±16% after incubation (24-hour) of the sections with a control buffer and 56±3% after incubation with recombinant cystatin C. Incubating frozen tissue sections of control aortic valves with cathepsin S decreased the valvular elastin content to 25±12% (P<0.01). Similarly, incubating sections with cathepsin K and cathepsin V decreased the valvular elastin content to 33±13% (P<0.01) and 34±13% (P<0.01), respectively. The elastin degradation effects of all cathepsins tested were inhibited by exogenous cystatin C (Figure 6A). Histological stainings of the sections demonstrating degradation of the elastin fibers (dark purple) by the studied cathepsins are shown in supplemental Figure III.

Determination of Cathepsin Activity in the Aortic Valves
Direct determination of total cathepsin activity in tissue sections using a fluorometric microassay showed the pres-
ence of enzymatically active cathepsins in both control and stenotic aortic valves. However, in stenotic aortic valves, the level of enzymatically active cathepsins was significantly higher than in the control valves ($P<0.001$; Figure 6B). The level of total cathepsin activity in the tissue sections from the stenotic valves equaled the activity of 20 nmol/L recombinant human cathepsin V and was 3.4-fold higher than the activity of 20 nmol/L recombinant cathepsin S. Degradation of the cathepsin-specific substrate Z-Phe-Arg-AMC, reflecting cathepsin activity, could be completely inhibited by adding the cathepsin inhibitor E-64 to the incubation system (Figure 6B).

**Discussion**

This is the first study to suggest a potential involvement of cathepsins S, K, and V in the pathogenesis of aortic stenosis. The increased levels of these elastolytic cathepsins in stenotic aortic valves and their ability to degrade aortic valvular elastin suggest that they may disturb the balance between matrix synthesis and degradation in the diseased valves. Such disequilibrium between the synthesis and degradation of ECM components, notably collagen and elastin, would then lead to pathological remodeling and stiffening of the valve leaflets. The degree of cathepsin-mediated matrix degradation is regulated by their natural inhibitor, cystatin C, and, accordingly, we found that its expression was increased in the stenotic aortic valves. The factors, which stimulate cystatin C expression in the stenotic valves, are unknown. However, cystatin C secretion is induced by transforming growth factor-$\beta$1, which is abundantly expressed in stenotic valves,\textsuperscript{11,19} so rendering it possible that transforming growth factor-$\beta$1 acts as a stimulator of cystatin C expression in the valves. The present observation that an upregulation of cathepsins in stenotic valves is accompanied by an elevated level of cystatin C differs from that of atherosclerotic lesions, in which increased cathepsin activity has been found to associate with decreased cystatin C expression.\textsuperscript{11} Whether the increased amounts of cystatin C in stenotic aortic valves are sufficient to inhibit the elevated levels of elastolytic cathepsins in the valves is presently unknown. Our observation that tissue sections of the stenotic aortic valves contained active...
Cathepsins would suggest that the increased expression of cystatin C is not sufficient to fully inhibit the increased cathepsin activity. Also, the differential localization of cathepsins and cystatin C in the diseased aortic valves reveals that local areas expressing active cathepsins but lacking cystatin C exist and so provide potential explanation for the observed destruction of the valvular elastin fibers despite upregulation of cystatin C. Importantly, by measuring mRNA expression, we observed local production of the 3 cathepsins and cystatin C in the aortic valves. Moreover, immunostainings showed cathepsin-positive endothelial cells, macrophages, and chondroblast-like cells in the stenotic valves, suggesting local secretion of the studied cathepsins. However, we cannot rule out the possibility that a fraction of these molecules have entered the valve leaflets from the circulation.

In addition to the cathepsins, matrix metalloproteinases (MMPs) may contribute to the degradation of ECM components in aortic valves. Indeed, an increased expression and activity of MMPs in stenotic aortic valves have been demonstrated.\textsuperscript{20-22} Locally produced cytokines, such as tumor necrosis factor-\(\alpha\), may stimulate the expression and activation of MMPs in stenotic valves\textsuperscript{22} and induce secretion of cathepsin S by endothelial cells and smooth muscle cells.\textsuperscript{10,17} Thus, an increased production of proinflammatory cytokines by infiltrating inflammatory cells may be responsible for the local upregulation of both cathepsins and MMPs in stenotic aortic valves. However, increased expression of MMPs in the stenotic valves is also accompanied by overexpression of their natural inhibitors, the tissue inhibitors of MMPs.\textsuperscript{21} Actually, Fondard et al reported that the tissue inhibitor of MMP-1/MMP-9 ratio was significantly increased in stenotic aortic valves.\textsuperscript{21} Therefore, alternative pathways of matrix degradation independent of MMPs are likely to contribute to the adverse remodeling of the valves. In the present work, frozen aortic valves were incubated with recombinant cathepsins in the presence of EDTA, which, by chelating divalent cations, efficiently blocks MMP activity, and therefore, the elastin degradation presented in Figure 6A has occurred largely without the action of MMPs.

Cathepsin V, a novel member of the cysteine protease family, possesses by far the most potent elastase activity described.\textsuperscript{23} Although the presence of cathepsin V protein in atheroma tissue has been suggested,\textsuperscript{23} its role in the pathogenesis of cardiovascular diseases is unknown. Cathepsin V is closely related to cathepsin L, which is expressed abundantly in aortic aneurysms and atherosclerotic tissue, and the serum levels of which are elevated in patients with coronary artery stenosis.\textsuperscript{24} Previous reports of the strong elastolytic activity of cathepsin V\textsuperscript{23} and our present findings of increased cathepsin V mRNA expression in stenotic aortic valves suggest a role for this enzyme in the degradation of valvular elastin. The strong immunoreactivity of cathepsin V in the endothelial cells lining the neovessels of the stenotic valves renders it possible that this cathepsin, like cathepsin S, may promote invasion of endothelial cells and neovessel growth.\textsuperscript{17} Indeed, neovascularization of the normally avascular valve tissue is a prominent feature of aortic stenosis.\textsuperscript{7,18} Interestingly, in the stenotic valves, the cells surrounding the neovessels were cystatin C positive, but the neovessels themselves were cystatin C negative (Figure 5M through 5O). These findings suggest that cathepsins and cystatin C may regulate the growth of microvessels during the progression of aortic stenosis and that a local imbalance of elastolytic cathepsins and their inhibitor cystatin C may have promoted neovessel formation in the stenotic aortic valves. Because microvessel formation is likely to facilitate recruitment of inflammatory cells and accumulation of plasma lipids into the aortic valve leaflets, it may also, via these actions, promote fibrocalcific thickening of the valves. Accordingly, local inhibition of cathepsins could reduce valvular neoangiogenesis and retard aortic stenosis progression.

Active bone formation, expression of osteogenic factors, and conversion of valvular myofibroblasts into an osteoblastic phenotype with the development of calcific nodules are
typical features of aortic stenosis. Indeed, calcification is a major determinant of leaflet stiffness in severe aortic stenosis, and it is associated with rapid hemodynamic progression of the disease. Dystrophic calcification and even endochondral bone formation and bone remodeling occur in the stenotic aortic valves. In the present study, cathepsin K, the major protease involved in bone matrix remodeling, was highly upregulated in the calcified stenotic valves when compared with the control valves. In addition, both cathepsin S and cystatin C protein were present in the chondroblast-type cells in the samples of stenotic valves that contained bone tissue. The present findings suggest that similar to their roles in the skeleton, cathepsins and cystatin C may also regulate calcification and bone formation in aortic valves. Indeed, cystatin C is present both in preosteoblasts and mature osteoblasts and is able to prevent the osteoclastic degradation of bone matrix proteins induced by cathepsin K. Thus, in stenotic aortic valves, cathepsins could degrade matrix at sites of bone formation, so reducing ossification of the valves, whereas cystatin C could accelerate bone formation by inhibiting these processes in the valves. Currently, in atherosclerotic plaques and aortic aneurysms, whereas their inhibitor cystatin C is considered to be a protective factor. However, in the pathogenesis of aortic stenosis, this simple dichotomy may not apply, and dual roles of both cathepsins and cystatin C merit consideration (ie, elastin degradation and neovascularization being harmful, and resorption of the ossified structures of the end-stage stenotic valves representing a mechanism ultimately limiting the disease progression). Finally, in statin-treated patients, there was a tendency toward lower expression levels of cathepsins S and K, and also of cystatin C, a finding that may partly explain the previous observation of statin treatment being able to inhibit calcification of the aortic valves.

In summary, here, we report an association of cathepsins S, K, and V and their inhibitor cystatin C with calcific aortic valve disease and suggest that these enzymes may participate in the adverse ECM remodeling of stenotic aortic valves. In addition, intense association of cathepsin V with neovessels of the stenotic aortic valves suggests a role for this enzyme in the neovascularization of the valves. Because both cathepsin S and cystatin C were detected in chondroblast-like cells of the valves, these molecules may also participate in bone formation and remodeling observed in the stenotic valves. These findings urge for a more precise definition of the role

Figure 5. In adjacent sections of stenotic aortic valves (A through C), macrophage-rich areas (A) contained cystatin C-positive cells (B). Elastin fibers (C; dark purple; arrows) could be seen in areas in which cystatin C-positive macrophages were also present (compare C with A and B). D through F (high power) and G through I (low power) images of double immunofluorescence of macrophages (green) and cystatin C (red) in stenotic aortic valves. Cystatin C-positive staining was found in macrophages (F and I; examples of colocalization are shown with arrows in I; arrowheads indicate endothelium lining the valves). J through O, Double immunofluorescence of CD31–CD34 (green) and cystatin C (red) revealed colocalization of cystatin C with endothelial cells lining the valves (J through L; arrows) but not with the endothelial cells forming the neovessels of the stenotic valves (M through O).
of the cathepsins and cystatin C in the pathophysiology of aortic stenosis. If clinically significant, elastolytic cathepsins may represent novel therapeutic targets in the so far unsuccessful pharmacological prevention of aortic stenosis.30

Study Limitations
Because normal, fresh surgical samples of aortic valves are rarely available, the control group consisted of both organ donors without cardiovascular diseases and patients undergoing cardiac transplantation. However, only macroscopically and microscopically normal valves were accepted in the control group, and no statistically significant differences in any of the investigated parameters were observed between these 2 control subgroups.

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Disclosures
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References


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METHODS

Samples and study population

Stenotic aortic valves were obtained from 32 patients undergoing valve replacement surgery. All patients had isolated non-rheumatic aortic stenosis, and those with more than mild aortic or mitral regurgitation were excluded, as were those with either a history of myocardial infarction or any proximal coronary artery stenosis exceeding 50% of the luminal diameter at angiography. Individuals with complicated diabetes and renal insufficiency (serum creatinine >170 µmol/l) were also excluded. Healthy control valves (n=13) were obtained from patients undergoing cardiac transplantation due to dilated or ischemic cardiomyopathy, or from organ donors without cardiac disease whose hearts could not be used as grafts. The characteristics of the patients and controls are shown in Table I and Table II, respectively. The investigation conforms to the principles outlined in the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Helsinki University Central Hospital. All participants signed an informed consent document.

Conventional and real-time PCR

Total RNA was isolated from 32 stenotic aortic valves and from 13 non-stenotic control valves and RT-PCR of cathepsin S, K, and cystatin C (35 cycles) was performed as described previously.1 The PCR products were verified, by DNA sequencing, to represent the corresponding target. The RT-PCR assay was standardized to the expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR products were quantified with a Gel Doc 2000 gel documentation system (Bio-Rad). The
primers were as follows: cathepsin S: 5’-GCACAGCTGAAGCTGAAA(F), 5’-
CCCATTAAGATCCACCATAG (R); cathepsin K: 5’-GGCCAACTCAAGAAGAAA (F),
5’-GTACCCCTCTGCATTAGC (R); cystatin C: 5’-
AGACCCAGCCCAACTTGGAAC(F), 5’-CAGTCCAGGGGTTGGGAATAC (R);
GAPDH: 5’-ACCACAGTCCATGCCCATCAC (F), 5’-TCCACCACCCCTGTTGCTGTA
(R). Due to low expression of cathepsin V mRNA (40 cycles of amplification were
required in the PCR reaction) the accurate differences between control and stenotic
valves were determined by real-time PCR using a relative quantification method with
GAPDH as an endogenous control.² For primers and probes design, the annotated DNA
and mRNA sequences were retrieved from GenBank® database (National Institutes of
Health, Bethesda, MD). The software package Primer Express® (v2.0, Applied
Biosystems, Foster City, CA) was used to identify gene-specific primers and fluorogenic
probes. The probe was labeled at the 5’ end by 6-carboxyfluorescein (FAM) and the 3’
end by non-fluorescent amidite (BHQ-1, BioSearch Technologies, Novato CA) or minor
groove-binder (MGB, Applied Biosystems). Oligonucleotide sequences were verified to
amplify unique sequence using the Basic Local Sequence Alignment Tool (BLAST,
National Institutes of Health), and were as follows: cathepsin V: 5’-
GAGAAGGCCCTGATGAAAGC (F), 5’-GGTTCAAAATAATGCCTGATTTG (R)
and the fluorogenic probe 5’- FAM-CCCATCTCCGTTGCTATGGATGCAG–BHQ1 -
3’; and GAPDH: 5’-GTCAACGGATTTGGTATCAG–BHQ1 -
GGCAACATATCCACTTTACCAGAGT (R) and the probe 5’- FAM-
AAGCAGCCCTGGTTGACCA–MGB -3’. Oligonucleotides for cathepsin V were
synthesized by Oligomer Oy (Helsinki, Finland) and for GAPDH by Applied Biosystems.
Analyses were performed in 25 µl reaction volume in 96-well plates (Applied Biosystems) using TaqMan© Universal Master Mix (Applied Biosystems) with uracyl-N-glycosylase (UNG) treatment. The samples were run on ABI Prism 7500 Sequence Detection System (Applied Biosystems) using a two-step program consisting of 15 sec at 95°C and 55 sec at 60°C for 45 cycles. The threshold was set to the geometric phase of the amplification curve, which occurred between cycles 31-37, and the amount of target was calculated using the formula $2^{-\Delta\Delta C_T}$.

**Immunohistochemistry and double immunofluorescence**

Cryostat sections of aortic valve leaflets were immunostained for cathepsins S and V, and cystatin C with commercially available mouse-anti-cathepsin S (concentration 1 µg/ml, Abcam Ltd, Cambridge, UK), goat-anti-cathepsin V (1 µg/ml, R&D Systems, Minneapolis, MN), and goat-anti-cystatin C antibodies (4 µg/ml, R&D Systems) using standard ABC-technique as described previously.1 Macrophages were detected with monoclonal mouse-anti-human CD68 or CD163 antibodies (1 µg/ml, DAKO, Glostrup, Denmark) and endothelial cells with a mixture of monoclonal mouse-anti-human CD31 (10 µg/ml, DAKO) and CD34 antibodies (0.5 µg/ml, Novocastra, Newcastle upon Tyne, UK).3 Identification of chondroblast-like cells in bony areas of the valves was performed with polyclonal rabbit-anti-S100 antibody (9 µg/ml, DAKO). Immunohistochemistry for cathepsin K was tried with the two commercially available antibodies against this enzyme, but these antibodies did not work reliably and no specific staining could be achieved.
Double immunofluorescence stainings were performed using Alexa rabbit-anti-goat 594 IgG (red) and Alexa rabbit-anti-mouse 488 IgG1 (green) (Molecular Probes Europe BV, Leiden The Netherlands) as secondary antibodies at concentrations of 10 µg/ml. In brief, after fixation in methanol for 10 minutes, the slides were washed with PBS. Blocking serum (3% normal rabbit serum, Vector Laboratories, Burlingame, CA) was added and incubated for 30 minutes, followed by incubation with primary antibody diluted in 1% milk (Valio, Helsinki, Finland) in PBS overnight. After vigorous washing, a mixture of secondary antibodies was added and incubated for 60 minutes. The slides were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, St. Louis, MO) before mounting. Tyramide signal amplification staining for cathepsin S was performed using TSA™ Kit #22 (Molecular probes) according to instructions provided by the manufacturer. In all immunohistochemical and double immunofluorescence stainings, nonimmune mouse and goat immunoglobulins served as negative controls.

Cathepsin incubation and analysis of the elastin and collagen fibers

To determine whether cathepsins S, K, and V can degrade elastin fibers in aortic valves in vitro, and if they are inhibited by cystatin C, cryostat sections of normal aortic valve leaflets (n=3) were incubated with 1 µM human recombinant cathepsin S, cathepsin V (R&D Systems), or cathepsin K (a kind gift from Professor Dieter Brömme, The University of British Columbia, Vancouver, Canada) in the presence or absence of cystatin C (R&D Systems). All enzymes were diluted in a buffer containing 20mM MES, 150mM NaCl, and 1mM DTT, pH 6.5. In addition, 2.5 mM EDTA was added to inhibit MMP activation. After incubation in a moist chamber at +37°C for 24 h, the slides were
fixed in methanol, and stained for elastin and collagen with elastic stain (Accustain Elastic stain kit, Sigma-Aldrich Co., St. Louis, MO). Relative proportions of elastin and collagen fibers in the valvular sections were quantified and the integrity of the fibers were analyzed from light microscopy images using computer-assisted morphometry (Image-Pro Plus, version 4.5) as previously described.5

**Determination of total cathepsin activity in the aortic valves**

For determination of cathepsin activity in frozen sections of aortic valves, fluorometric microassay was performed as described previously.6 For this purpose, freshly cut 10 µm-thick cryostat cross-sections (from base to tip of the valve leaflets) from control (n=7) and stenotic (n=7) aortic valves were used. Frozen cross-sections of the valves were placed into 96-well microtiter plates (1 section/well) and 50 µl of 20 mM MES buffer pH 6.0, containing 2.5 mM EDTA, 150 mM NaCl, 0.035% Brij, and 2.8 mM freshly prepared DTT was added. In inhibition experiments, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) (Sigma), a specific inhibitor of cysteine protease-cathepsins, was added to give a final concentration of 20 µM. The reaction was started by adding 50 µl substrate solution containing 3.2 µM of the synthetic cathepsin substrate Z-Phe-Arg-7-amido-4-methylcoumarin (AMC) (Sigma) and fluorescence was measured kinetically at 37°C for 50 minutes using a microplate reader (HTS 7000 Plus, Bio Assay Reader, Perkin-Elmer, Germany) with excitation wave length of 360 nm and emission wave length of 465 nm. Enzyme activities in the samples were calculated using a standard curve established with free AMC. Human recombinant cathepsin S and cathepsin V were used as positive controls at concentrations of 10-20 nM.
Statistics

Group differences were analyzed using Mann-Whitney U test for skewed data distribution (Figure 1A and 1B) and Student’s t test for normally distributed data. The group data are summarized as medians and ranges or mean values ± SD. In figure 8, the results are expressed as the mean value and SEM. For correlations, Spearman’s coefficients were calculated. The analyses were performed using SPSS (version 11.0) software system. P-values <0.05 were considered statistically significant.

Reference List


Table I. Characteristics of the Patients with Aortic Valve Stenosis (n=32)

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<th>Characteristic</th>
<th>Mean ± SD or n of patients</th>
<th>Range</th>
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<td>Age, yr</td>
<td>63 ± 10</td>
<td>39 - 82</td>
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<tr>
<td>Sex, male/female</td>
<td>17/15</td>
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<tr>
<td>NYHA class, 1/2/3/4</td>
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<td>Aortic valve area index, cm²/m²</td>
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<td>0.16-0.57</td>
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<td>Mean pressure gradient, mmHg</td>
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<td>17 - 80</td>
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<td>LV ejection fraction, %</td>
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<td>LV mass index, g/m²</td>
<td>155 ± 37</td>
<td>83 - 235</td>
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<tr>
<td>Pulmonary wedge pressure, mmHg</td>
<td>13 ± 7</td>
<td>4 - 36</td>
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Prevalence of

- LV hypertrophy* 25 (78 %)
- Hypertension† 8 (25 %)
- Bicuspid‡/Tricuspid valve 8/24
- LDL-cholesterol (mmol/l) 3.2 ± 0.9 1.9 – 5.0

Medication

- ACE inhibitor/ AT1 blocker 9 (28 %)
- β-blockers 17 (53 %)
- Diuretics 8 (25 %)
- Statins 9 (28 %)
- Digitalis 2 (6 %)
* echocardiographic left ventricular mass index exceeding 110 g/m² in women or 134 g/m² in men

† History of antihypertensive treatment

‡ Congenitally bicuspid valves

Reference List

Table II. Characteristics of the control group (n=13)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD or n of patients</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>52 ± 10</td>
<td>34 - 63</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>9/4</td>
<td></td>
</tr>
<tr>
<td>NYHA class, 1/2/3/4</td>
<td>4/0/8/1</td>
<td></td>
</tr>
<tr>
<td>Transplantation/Organ donor</td>
<td>9/4</td>
<td></td>
</tr>
<tr>
<td>Bicuspid/Tricuspid valve</td>
<td>0/13</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor/AT1 blocker</td>
<td>9 (69 %)</td>
<td></td>
</tr>
<tr>
<td>β-blockers</td>
<td>8 (62 %)</td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>9 (69 %)</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>1 (8 %)</td>
<td></td>
</tr>
<tr>
<td>Digitalis</td>
<td>8 (62 %)</td>
<td></td>
</tr>
</tbody>
</table>
Figure I: Negative controls for immunohistochemistry and double immunofluorescence

**Cathepsin S**

- Mouse IgG₁
- HAM-56
- Merge

**Cathepsin V**

- Cathepsin V
- Goat IgG

**CD-31 & CD-34**

- Goat IgG
- Merge

**Cystatin C**

- Cystatin C
- Goat IgG

**CD-163**

- Goat IgG
- Merge
Figure II: Negative controls

**CD-31 & CD-34**

Mouse IgG1

**CD-68**

Mouse IgG1

**CD-163**

Mouse IgG1

**HAM-56**

Mouse IgM

Cathepsin S

Merge
Figure III

A. Buffer

B. Cystatin C

C. Cathepsin S

D. Cathepsin S + Cystatin C

E. Cathepsin K

F. Cathepsin K + Cystatin C

G. Cathepsin V

H. Cathepsin V + Cystatin C
**Figure III.** Ex vivo incubation of cryostat sections of aortic valves with cathepsins S, K, and V in the presence or absence of cystatin C. Histological stainings of collagen (pink) and elastin (dark purple) fibers after incubation of sections of control aortic valves with buffer (A) or cystatin C (B) showed that elastin fibers were preserved. In contrast, incubation of the same sections with cathepsin S (C) resulted in extensive elastin disruption, which could be inhibited by exogenous cystatin C (D). E-H, Similarly, cathepsins K and V disrupted elastin fibers in aortic valves in vitro (E and G) and this disruption was blocked by cystatin C (F and H).