

Induction of Local Angiotensin II-Producing Systems in Stenotic Aortic Valves

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OBJECTIVES	The purpose of this study was to investigate the expression of angiotensin II (Ang II)-producing enzyme systems in normal and stenotic aortic valves.
BACKGROUND	Chronic inflammation and fibrosis are involved in the pathogenesis of aortic stenosis (AS), but the detailed molecular mechanisms of this atherosclerosis-like process remain obscure. Angiotensin II, a powerful mediator of inflammation and fibrosis, may participate in AS progression.
METHODS	Stenotic aortic valves (n = 86) were obtained from patients undergoing valve replacement surgery, and control valves (n = 11) were obtained from patients undergoing cardiac transplantation. Angiotensin-converting enzyme (ACE) and mast cell (MC)-derived chymase were quantified by reverse-transcription polymerase chain reaction, autoradiography, and immunostaining. The MCs, macrophages, and T lymphocytes were detected by immunohistochemistry, and angiotensin II type 1 receptor (AT-1R) by autoradiography.
RESULTS	Compared with control valves, stenotic aortic valves showed a significant increase in both messenger ribonucleic acid (mRNA) (p = 0.001) and protein (p < 0.001) expression of ACE, which colocalized with macrophages. Similarly, the expression of AT-1R protein and chymase mRNA and protein was upregulated (p < 0.001), and the number of MCs was six-fold higher in stenotic than in normal valves. The MCs were associated with the calcified areas, and—in contrast to control valves—showed an increased degree of degranulation, a prerequisite for chymase secretion and action.
CONCLUSIONS	Angiotensin-converting enzyme and chymase, two Ang II-forming enzymes, are locally expressed in aortic valves, and owing to infiltration of macrophages and MCs, are further upregulated in stenotic valves. These novel findings, implicating chronic inflammation and an increased expression of local Ang II-forming systems, suggest that therapeutic interventions aiming at inhibiting these processes may slow AS progression. (J Am Coll Cardiol 2004;44:1859–66) © 2004 by the American College of Cardiology Foundation

Recent studies have demonstrated that stenotic aortic valves, instead of merely being the result of a passive degeneration, show similarities with the active pathobiology of atherosclerosis. These include infiltration of T lymphocytes and macrophages as evidence of inflammation (1–3), and the accumulation of oxidized lipoproteins (4) and mediators of ossification and calcification (5,6). The idea of aortic stenosis (AS) as an atherosclerosis-like process is supported by epidemiologic studies showing that the development of this disease is associated with hypertension, diabetes, smoking, male gender, advancing age, and hypercholesterolemia (7,8). There are also retrospective data suggesting that the use of statins may slow the progression of valve stenosis (9,10). Recently, the presence of angiotensin-converting enzyme (ACE) was

identified in stenotic but not in normal aortic valves (11). The authors suggest that ACE enters the stenotic valve lesions from the circulation bound to and carried by low-density lipoprotein (LDL) particles. Whether ACE is also locally produced in the valvular tissue is at present unknown.

Angiotensin II (Ang II), the enzymatic product of ACE, is expressed in strategically relevant sites of human atherosclerotic plaques and may participate in the inflammatory process within the vascular wall (12). Because Ang II is an important mediator of inflammation and fibrosis (13–17), it may play a role also in the pathogenesis of AS. In addition to ACE, the major Ang II-forming enzyme, the mast cell (MC)-derived neutral protease, chymase, also contributes to Ang II formation (18). Mast cells are bone marrow-derived inflammatory cells, originally linked to allergies and subsequently to many inflammatory processes, as well as to lipoprotein metabolism (19). The influence of MCs in the process of AS has not been evaluated previously, nor has the effect of MC chymase, which is stored in the secretory granules of these cells and released during their activation and ensuing degranulation.

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Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
Ang II	=	angiotensin II
AS	=	aortic stenosis
AT-1R	=	angiotensin II type 1 receptor
AT-2R	=	angiotensin II type 2 receptor
DNA	=	deoxyribonucleic acid
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
LDL	=	low-density lipoprotein
MC	=	mast cell
mRNA	=	messenger ribonucleic acid
RT-PCR	=	reverse transcription-polymerase chain reaction

In the present study, we sought to determine whether ACE and MC chymase are produced locally in the valvular tissue and whether the Ang II-forming potential in stenotic aortic valves is increased.

METHODS

Samples and study population. The stenotic aortic valves were obtained from 86 patients aged on average 67 years (range 39 to 82 years) undergoing valve replacement surgery. All had pure AS in that patients with more than mild aortic or mitral regurgitation were excluded, as were patients with any proximal coronary artery stenosis exceeding 50% of luminal diameter at angiography. Individuals with complicated diabetes and renal insufficiency (serum creatinine >170 $\mu\text{mol/l}$) were also excluded. Twenty percent of the patients were receiving statins, 22% used either ACE inhibitors or angiotensin II type 1 receptor (AT-1R) antagonists, and 3% received combined therapy consisting of a statin and ACE inhibitors or an AT-1R antagonist. The average aortic valve area at cardiac catheterization was 0.67 cm^2 (range 0.30 to 1.1 cm^2). At surgery, 72 valves were tricuspid, 13 were bicuspid, and 1 valve had four cusps. The control valves were obtained from patients undergoing cardiac transplantation owing to dilated ($n = 8$) or ischemic ($n = 1$) cardiomyopathy or from organ donors without cardiac disease whose hearts could not be used as grafts ($n = 2$). Of the nine patients undergoing transplantation, seven had been on ACE inhibitor therapy for prolonged periods. The protocol was approved by the ethics committee of Helsinki University Central Hospital, and the participating patients gave their informed consent to the study.

Autoradiography of ACE and AT-1R. Sections of frozen aortic valves (20 μm thick) of 11 randomly selected patients and 11 control subjects were cut on a cryostat at -17°C , and thaw mounted onto Super Frost Plus slides (Menzel-Glaser, Germany). For ACE autoradiography, a tyrosyl residue of the ACE inhibitor lisinopril, compound 351A (Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania), was iodinated by the chloramine T method and purified on a SP-Sephadex C-25 column (Pharmacia, Rockville, Maryland). For quantitative in vitro ACE auto-

radiography, a previously described technique (20) was applied. Briefly, aortic valve sections were pre-incubated for 15 min at room temperature in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.2% bovine serum albumin, followed by incubation for 1 h at room temperature in a fresh volume of the same buffer containing 0.3 $\mu\text{Ci/ml}$ of ^{125}I -351A. Non-specific binding was determined in parallel incubations in the same buffer containing 1 mM Na_2 -ethylenediaminetetraacetic acid. After incubation, the sections were washed four times for 1 min in ice-cold buffer without bovine serum albumin and ^{125}I -351A to remove unbound radioligand, and dried under a stream of cool air. For quantification of ACE binding, the sections were placed on a Fuji Imaging Plate BAS-TP2025 (Tamro, Finland) for 3 h. The optical densities were quantified by an AIDA computer image analyzing system (AIDA 2D densitometry, Paris, France) coupled to a Fujifilm BAS-5000 phosphoimager (Tamro). Specific binding was calculated as total binding minus non-specific binding. To study if angiotensin receptors also are upregulated in stenotic aortic valves, we performed in vitro autoradiography of AT-1R and angiotensin II type 2 receptor (AT-2R) from frozen sections using a previously described method (21).

Competitive reverse-transcription polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) was isolated from the aortic valves of 84 patients (RNA isolation from two patient samples failed because of a high degree of calcification) and 11 control subjects. Isolation was performed with an ultra-pure TRIzol reagent (Gibco BRL, Gaithersburg, Maryland), and a RNeasy Fibrous Tissue Mini Kit (Qiagen) including deoxyribonuclease digestion (Qiagen, Hilden, Germany); 0.25 μg of purified total RNA was transcribed into complementary deoxyribonucleic acid with a Superscript TM pre-amplification system (Gibco BRL). The primers were as follows: ACE: 5'-GGTGCCT-TTCCCAGACAAGC (S), 5'-AGAGCTGGTCCAT-CGTGACC (AS); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-ACCACAGTCCATGCCATCAC (S), 5'-TCCACCACCCTGTTGCTGTA (AS); chymase: 5'-TCCCACCTGGGAGAATGTGC (S), 5'-TGCA-TCCGACCGTCCATAGG (AS).

The competitor deoxyribonucleic acid (DNA) for ACE was obtained by insertion of a 380 bp external DNA fragment into the SacI site and for chymase by insertion of a 344-bp external DNA fragment into the ApaI site. The polymerase chain reaction product was verified, by DNA sequencing, to represent the corresponding target. The use of equal amounts of messenger ribonucleic acid (mRNA) in the RT-PCR assays was confirmed by analyzing the expression levels of GAPDH (data not shown). The polymerase chain reaction products were quantified with a Gel Doc 2000 gel documentation system (Bio-Rad), and the logarithm of the competitor-to-target ratio was plotted against the logarithm of the competitor DNA molecules (22).

To correct for potential variances in mRNA extraction

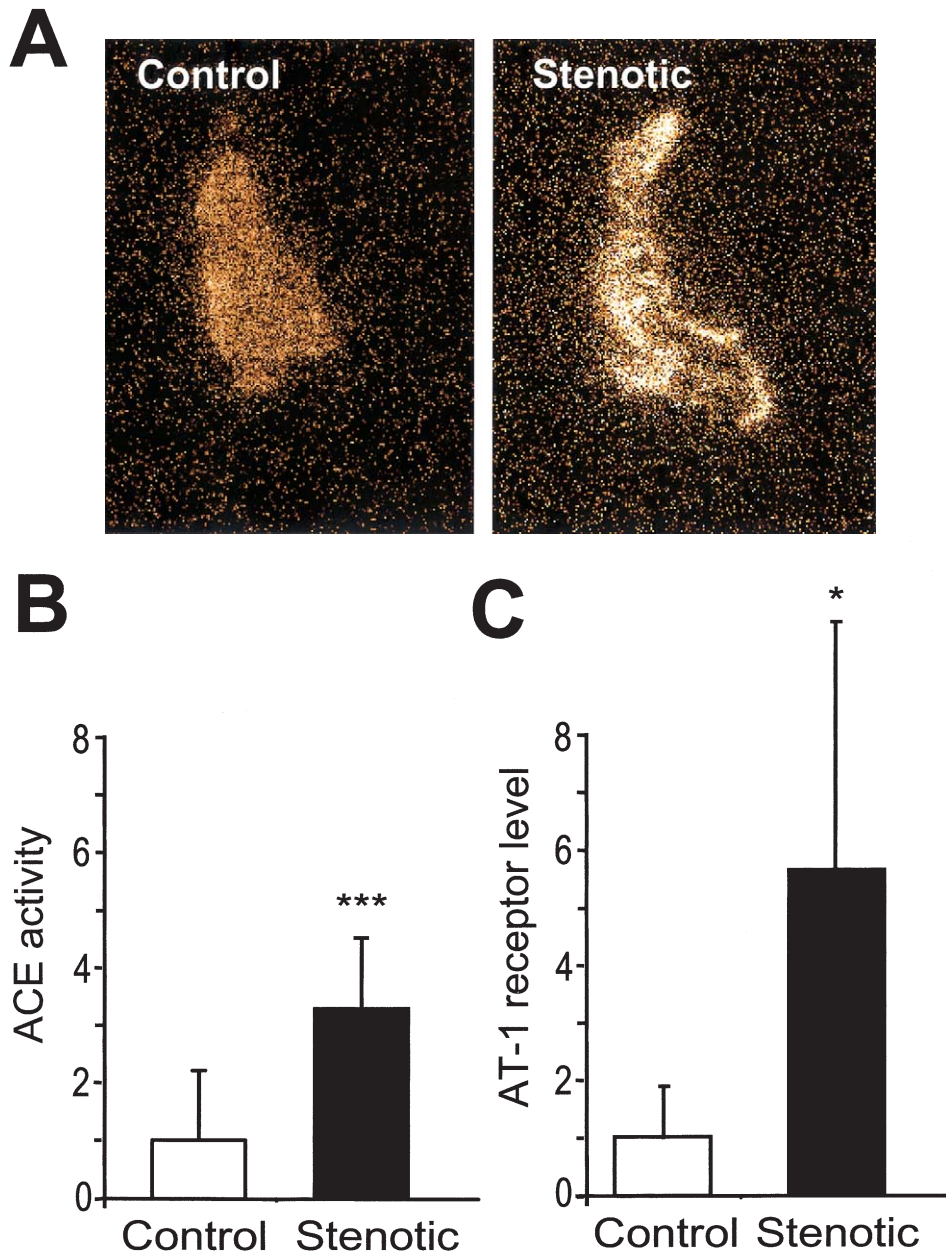


Figure 1. Local angiotensin-converting enzyme (ACE) and angiotensin II type 1 receptor (AT-1R) activities are increased in stenotic aortic valves. **(A)** ACE autoradiography images of a normal and a stenotic aortic valve. **(B)** Quantitative analysis of ACE autoradiography levels in normal (n = 11) and stenotic (n = 11) aortic valves. **(C)** Quantitative analysis of AT-1R autoradiography showed 5.5-fold increase (p < 0.05) in the level of AT-1R in stenotic aortic valves (n = 11) compared with control valves (n = 11). Results are mean values ± SD. *p < 0.05; ***p < 0.001.

and reverse-transcription efficacy between samples and for variances in polymerase chain reaction pipetting, the competitive RT-PCR assay was standardized to the expression level of GAPDH.

Immunohistochemistry of MCs, ACE, T lymphocytes, and macrophages. Frozen aortic valves were divided into four sections from base to tip, and immunohistochemistry of MCs was performed in all sections (of 86 patients and 11 control subjects) by a commercially available monoclonal anti-tryptase antibody (clone AA1, Dako, Glostrup, Denmark) at a concentration of 0.11 μg/ml. A randomly selected subpopulation of the stenotic valves (11 patients)

and all normal valves were also stained with monoclonal anti-chymase antibody (concentration 10 μg/ml, Serotec, Hanar, Germany) for chymase-positive MCs. Briefly, after incubation in methanol for 10 min, the endogenous peroxidase activity was blocked by incubation in 2% H₂O₂ in methanol for 30 min. The slides were washed in phosphate-buffered saline, incubated with blocking serum (normal horse serum for tryptase, and rabbit serum for chymase; Vectastain Elite Kit, Vector Laboratories, Burlingame, California) for 30 min and then with primary antibody diluted in blocking serum overnight. After washing of the sections with phosphate-buffered saline, a biotinylated secondary

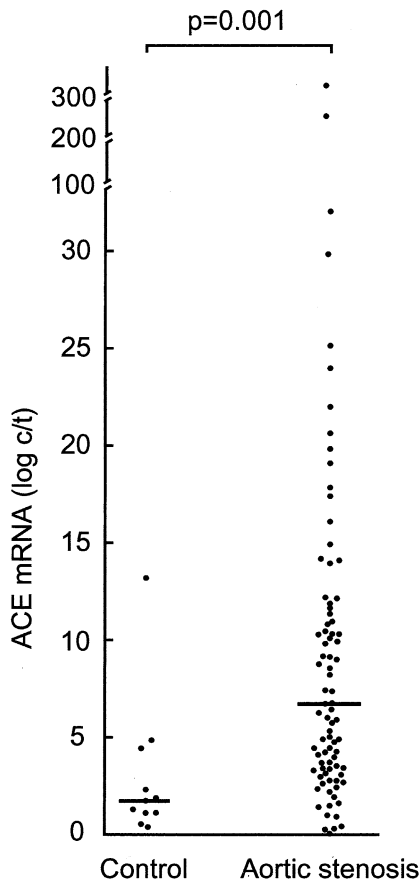


Figure 2. Angiotensin-converting enzyme (ACE) messenger ribonucleic acid (mRNA) expression is higher in stenotic aortic valves (n = 84) than in control valves (n = 11). Individual data points and medians are shown.

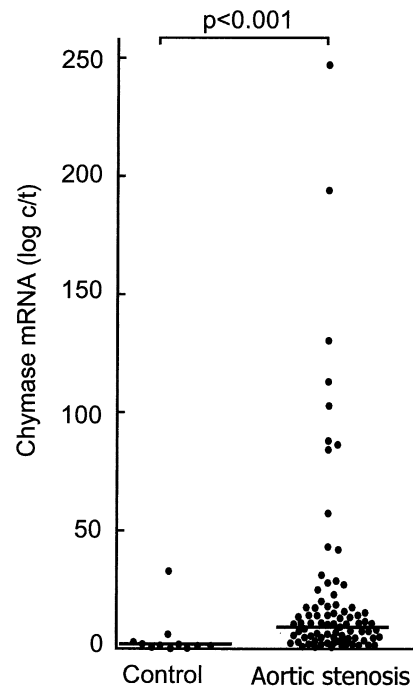


Figure 3. Chymase messenger ribonucleic acid (mRNA) expression is increased in stenotic aortic valves (n = 84) over that of control valves (n = 11). Lines denote medians.

antibody (Vectastain Elite Kit) against tryptase or a horseradish peroxidase-F(ab)-conjugated secondary antibody (Serotec) against chymase was applied for 30 min, followed by washes with phosphate-buffered saline and avidin-biotin-peroxidase conjugate (ABC Elite, Vector Laborato-

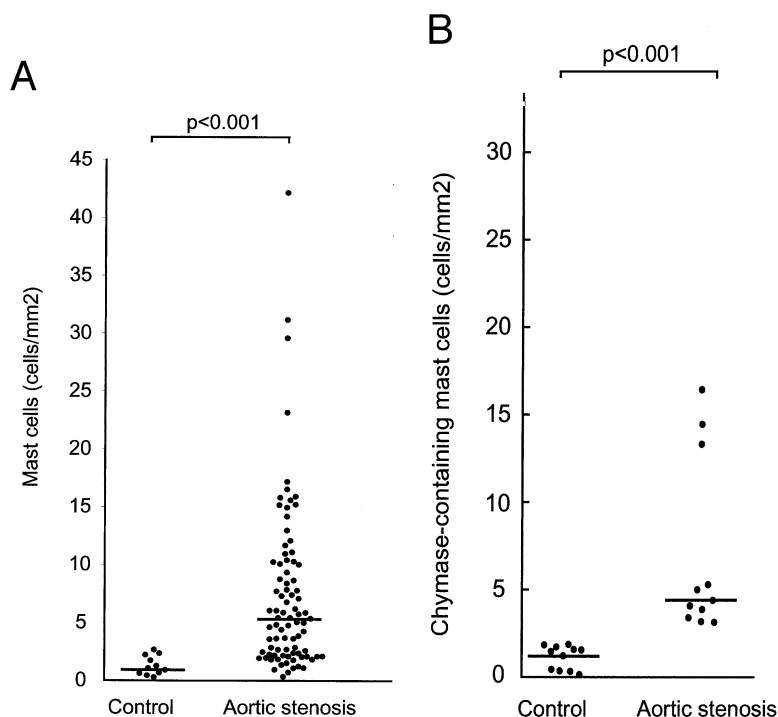


Figure 4. Mast cell (MC) numbers are higher in stenotic than in normal aortic valves. (A) The total number of MCs (cells/mm²) in stenotic (n = 86) and normal (n = 11) aortic valves. (B) The number of chymase-containing MCs in stenotic (n = 11) and normal (n = 11) aortic valves. Lines indicate medians.

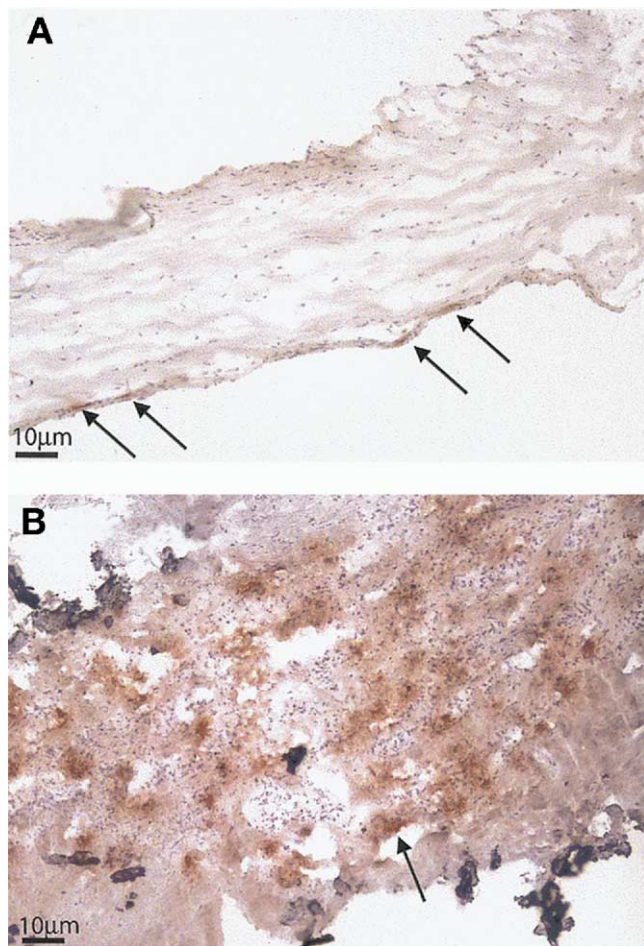


Figure 5. Immunostaining of mast cells (MC) in normal (A) and stenotic (B) aortic valves. (A) Section of a normal aortic valve showing a few MCs (arrows) in a subendothelial location. (B) Section of a stenotic aortic valve demonstrating MC distribution throughout the leaflet and association of MCs with the calcified lesions of the valves (one arrow).

ries) for 30 min. Standard peroxidase enzyme substrate, 3-amino-9-ethylcarbazole was added, and the sections were counterstained with hematoxylin (Mayer, Merck, Darmstadt, Germany) and mounted. The exact numbers of tryptase-positive and chymase-positive MCs in each section were counted by light microscopy, and the area of each section was measured by computer-assisted morphometry (Image-Pro Plus, version 4.5). For the purpose of determining the degree of MC activation, the number of degranulated MCs in relationship to the total number of valvular MCs was calculated in 11 randomly selected stenotic and in all 11 control aortic valves. The ACE was detected immunohistochemically with polyclonal antibodies (concentration 2 µg/ml, Santa Cruz, Santa Cruz, California), and macrophages and T lymphocytes were detected with monoclonal antibodies HAM-56 (Dako) and CD3 (Dako) at concentrations of 0.7 and 2.4 µg/ml, respectively.

Statistics. Statistics were calculated with the SPSS software, version 11.0. The data presented in Figure 1 were analyzed using the Student *t* test, and the results are expressed as the mean value and SD. When analyzing the

data presented in other figures (Figs. 2 to 6), the Mann-Whitney *U* test served to calculate differences between the groups. Individual data points and medians are shown in the figures. Differences were considered statistically significant when $p < 0.05$. For correlations, Spearman's correlation coefficients were calculated.

RESULTS

Autoradiography of active ACE and AT-1R in aortic valves. Autoradiography of aortic valves with a radiolabeled inhibitor of ACE (351A) showed the presence of enzymatically active ACE in normal valves (Fig. 1A, left panel). However, in stenotic valves, the level of enzymatically active ACE was significantly increased (Fig. 1A, right panel, and Fig. 1B) and distributed throughout the aortic valve leaflet. Quantification of the autoradiographic images revealed three-fold increase in ACE activity in stenotic valves compared with that of control valves ($p < 0.001$) (Fig. 1B).

Quantitative analysis of AT-1R autoradiography showed a 5.5-fold increase ($p < 0.05$) in the level of AT-1R in stenotic aortic valves as compared with control valves (Fig. 1C). The levels of AT-2R were below the detection limit both in control and in diseased valves.

Competitive RT-PCR of ACE and chymase. The RT-PCR analyses revealed that ACE is expressed locally in both normal and stenotic aortic valves and that the expression of ACE mRNA was significantly higher in stenotic valves than in control valves (3.8-fold, $p = 0.001$) (Fig. 2). In the control valves, the ACE mRNA expression was similar in the cardiomyopathy patients who had been on chronic ACE inhibition as in patients free of cardiovascular disease or medication. Furthermore, in stenotic aortic valves, the expression levels of ACE mRNA did not differ significantly whether ACE inhibitor or AT-1R antagonist therapy was present or absent.

In addition to ACE, chymase, the other potential Ang II-forming enzyme, was also expressed in both normal and stenotic aortic valvular tissue. The expression levels of chymase mRNA were highly upregulated in stenotic versus normal aortic valves (7.6-fold, $p < 0.001$) (Fig. 3). There were no significant differences in chymase mRNA expression levels between the patients or control subjects with or without ACE inhibitor or AT-1R antagonist treatment. No statistically significant differences in the expression levels of ACE and chymase were found between bicuspid and tricuspid aortic valves. Moreover, the expression levels of ACE and chymase did not correlate with the age of the patients or control subjects. A positive correlation appeared between the expression levels of chymase and ACE mRNAs ($r = 0.325$; $p = 0.001$).

Immunohistochemical detection of MC-specific tryptase and chymase. Immunohistochemistry using anti-tryptase antibodies, a specific marker of MCs, revealed that MCs are present in aortic valves, and that their number is significantly increased in stenotic valves over that of control valves

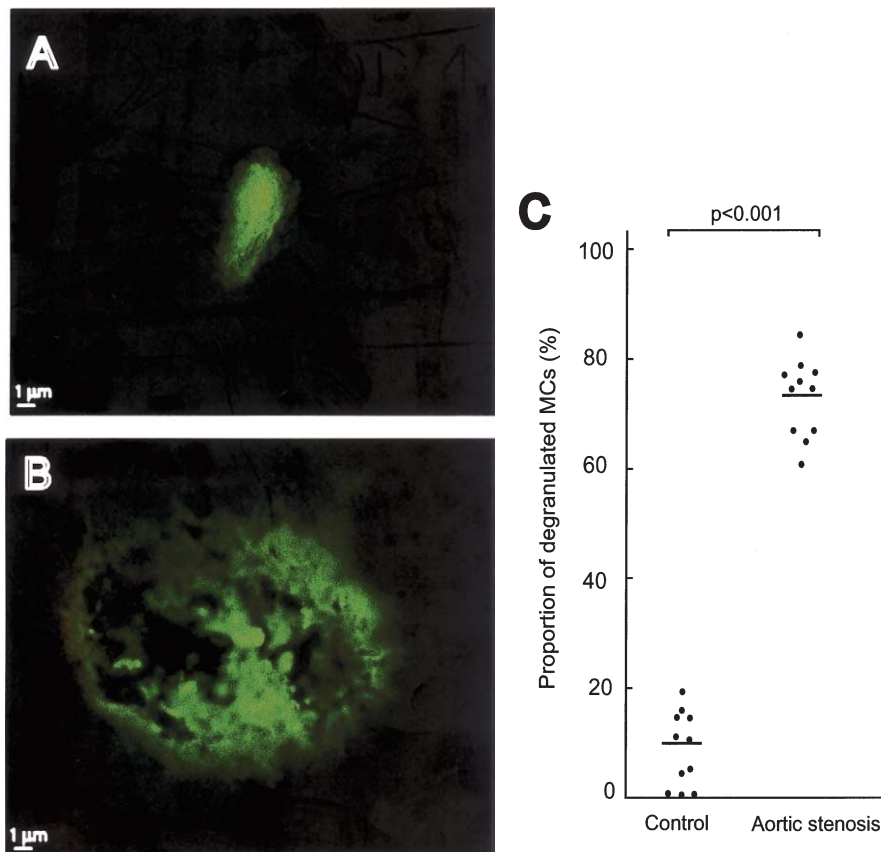


Figure 6. A resting mast cell (MC) in a control valve (A) and an activated mast cell in a stenotic aortic valve (B). (C) Quantification of the degree of MC degranulation (i.e., activation) in normal (n = 11) and stenotic (n = 11) aortic valves. Lines denote medians.

(Fig. 4A). The degree of MC infiltration was similar in bicuspid and tricuspid stenotic aortic valves. The accumulation of MCs in stenotic valves was evident in all four sections, that is, from leaflet base to tip. The difference in MC distribution density between the stenotic and control valves was, however, most obvious in the two middle sections of the valves (5.1 [0.6 to 41.9] MCs/mm² vs. 0.9 [0 to 2.4] MCs/mm², median [range], $p < 0.001$) (Fig. 4A). Immunohistochemistry using anti-chymase antibodies showed that the MCs present in stenotic and control aortic valves also contain chymase (3.6 [2.5 to 16.5] vs. 1.5 [0.4 to 2] chymase-positive MCs/mm², $p < 0.001$) (Fig. 4B). In control valves, the MCs (reddish-brown staining) were localized to the subendothelial space, whereas in stenotic valves, they were distributed throughout the leaflet and were associated especially with the calcified lesions of the stenotic valves (Fig. 5). The degree of MC degranulation, a prerequisite for chymase secretion and action, was enhanced in stenotic valves, appearing as an extracellular rim of granules and granule mediators around the activated MCs (Fig. 6B). In contrast, most of the MCs present in normal valves were intact, that is, not degranulated (Fig. 6A). In stenotic leaflets, the proportion of MCs activated was 75% (range, 60% to 83%), whereas in normal valves only 9 (0% to 19%) of the MCs showed signs of activation and degranulation ($p < 0.001$) (Fig. 6C).

Immunohistochemical detection of macrophages, T lymphocytes, and ACE. Immunohistochemistry of both macrophages and T lymphocytes showed a higher number of these inflammatory cells in stenotic valves than in control valves (data not shown), consistent with previous findings (1–3,23). Immunohistochemistry of ACE and macrophages in adjacent sections of an aortic valve showed that ACE and macrophages colocalized in the valvular tissue (Figs. 7A and 7B). The specificity of the polyclonal goat anti-ACE antibody was verified by substituting for the primary antibody with an irrelevant goat isotype-specific immunoglobulin G (Fig. 7C).

DISCUSSION

The present study is the first to reveal that aortic valves contain two parallel Ang II-forming enzyme systems: ACE and MC-derived chymase, both of which are upregulated in stenotic valves. The presence of ACE in stenotic aortic valves has been recently demonstrated by O'Brien et al. (11), but until our work, no local production of ACE has been demonstrated in the valvular tissue. Angiotensin II, the enzymatic product of angiotensin I degradation by ACE and chymase, has a number of proinflammatory and profibrotic effects that potentially contribute to the pathogenesis of calcific aortic valve disease. These include its function as

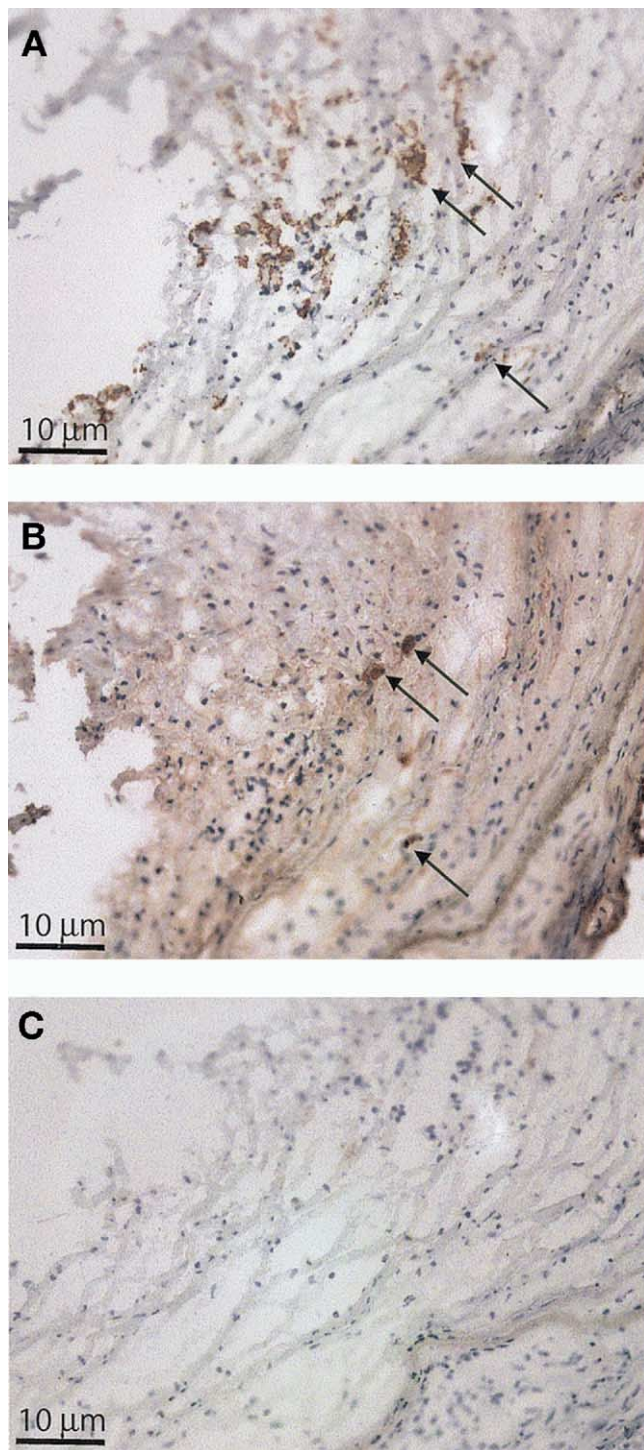


Figure 7. Adjacent sections of an aortic valve showing colocalization (arrows) of macrophages (A) and angiotensin-converting enzyme (ACE) (B). Control staining with unspecific goat immunoglobulin G shows no immunoreactivity (C).

a chemotactic factor for monocytes (13,14) as well as its capacity to enhance collagen synthesis by stimulating transforming growth factor-beta (16). The assertion of ACE-induced tissue fibrosis in stenotic aortic leaflets is supported by the finding in the myocardium of patients with AS demonstrating increased collagen and fibronectin expression

in parallel with activation of the cardiac renin-angiotensin system (24).

The presence of MCs in aortic valves, and in particular, their high number in the calcified areas of the stenotic leaflets, is an intriguing, novel finding. Moreover, a striking contrast existed between normal and stenotic valves, in that the former contained only a few resting MCs, whereas the latter showed numerous MCs in an activated, degranulated state. Activated MCs are present in atherosclerotic plaques of human coronary arteries, where they promote foam cell formation and may trigger degradation of the extracellular matrix and plaque rupture (25–27). Upon activation, MCs are capable of releasing a number of proinflammatory and profibrotic mediators, such as tumor necrosis factor-alpha and transforming growth factor-beta (28–30). The presence of activated and degranulated MCs in the fibrotic lesions of the aortic valves suggests that they may participate in the induction of fibrosis and calcification with ensuing valve stiffening. Thus, both chymase and tryptase, by generating Ang II and inducing collagen synthesis (28–30), may act as profibrotic mediators in the stenotic valves.

Currently, there exists no approved pharmacologic treatment for aortic valve stenosis, the inevitable outcome of the disease being valve replacement surgery. Present and previous findings raise the possibility that the process of AS, being a chronic inflammatory process, might be attenuated by therapeutic interventions aiming at inhibiting the activity of ACE and blocking AT-1R before the late-stage pathology of the disease, such as calcification, has become manifest. The use of AT-1R antagonists, which block Ang II-mediated effects irrespective of the Ang II-generating enzyme system, may appear as the most feasible approach. Another fascinating class of future therapies involves MC stabilizers or specific inhibitors for chymase and tryptase, which may also indirectly reduce the synthesis and secretion of MC-derived profibrotic molecules and suppress collagen synthesis independently of Ang II (31). In addition to the LDL-lowering effects of statins (i.e., less ACE-containing LDL particles entering aortic leaflets [11]), their pleiotropic anti-inflammatory effects may also be of therapeutic benefit in the treatment of AS. Indeed, recent retrospective clinical trials have demonstrated less progression of AS in patients on statin therapy. Because in the present study, the medication was not part of the study design, we were unable to draw any conclusions regarding the effects of medical therapy on AS. Large, randomized, and well-controlled clinical trials are needed to evaluate whether the novel pharmaceutical approaches will prevent the progression of AS.

Study limitations. We document here the upregulation of two separate Ang II-producing systems, ACE and chymase, in stenotic aortic valves but cannot provide information on their relative contribution to the local formation of Ang II. Moreover, the actual role of Ang II in the stenotic valves remains to be shown. Although in this study (including both patients and control subjects) the ACE or chymase

mRNA expression levels did not differ significantly between the patients with or without ACE inhibitor or AT-1R antagonist treatment, we cannot fully exclude the possibility that the results may have been affected by ACE inhibitor or AT-1R antagonist therapy.

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