Valve Disease

Activation of the Cardiac Renin-Angiotensin System and Increased Myocardial Collagen Expression in Human Aortic Valve Disease

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OBJECTIVES
We sought to determine whether the cardiac renin-angiotensin system (RAS) is activated in human aortic valve disease depending on left ventricular function, and we analyzed the concomitant regulation of the extracellular matrix components.

BACKGROUND
In animal models with pressure or volume load, activation of the cardiac RAS increases fibrosis. In human aortic valve disease, the ventricular collagen protein content is increased, but only scarce data on the activation state of the cardiac RAS and its effects on collagen and fibronectin messenger ribonucleic acid (mRNA) are available.

METHODS
In left ventricular biopsies from patients with aortic valve stenosis (AS) and aortic valve regurgitation and from control subjects, we quantitated mRNAs for angiotensin-converting enzyme (ACE), chymase, transforming growth factor-beta1 (TGF-beta1), collagen I, collagen III and fibronectin by reverse-transcription polymerase chain reaction. Proteins were localized by immunohistochemistry; ACE activity was determined by high performance liquid chromatography; and TGF-beta protein by quantitative enzyme immunoassay.

RESULTS
Protein, ACE and TGF-beta1 mRNA were significantly increased in patients with AS and AR (1.5- to 2.1-fold) and correlated with each other. The increase occurred also in patients with normal systolic function. Collagen I and III and fibronectin mRNAs were both upregulated about twofold in patients with AS and AR. In AS, collagen and fibronectin mRNA expression levels were positively correlated with left ventricular end-diastolic pressure and inversely with left ventricular ejection fraction (LVEF).

CONCLUSIONS
In human hearts, pressure and volume overload increases cardiac ACE and TGF-beta1 in the early stages. This activation of the cardiac RAS may contribute to the observed increase in collagen I and III and fibronectin mRNA expression. The increase in extracellular matrix already exists in patients with a normal LVEF, and it increases with functional impairment.

Myocardial fibrosis, characterized by an elevation of myocardial collagen and fibronectin content, appears early in animal models of mechanical overload and increases further during the transition from hypertrophy to heart failure (1–3). Increased myocardial angiotensin-converting enzyme (ACE) activity suggests that fibrosis is due to activation of the cardiac renin-angiotensin system (RAS) (4–8). Angiotensin II induces transforming growth factor-beta1 (TGF-beta1), which increases collagen synthesis in cell culture and in in vivo models (9–12). In several models, an early increase in TGF-beta1 messenger ribonucleic acid (mRNA) precedes the increase in collagen and fibronectin mRNA, suggesting that TGF-beta1 mediates the profibrotic effects of angiotensin II (13,14).

Increased myocardial fibrosis has also been observed in human hearts with aortic valve disease and contributes significantly to an increase in diastolic stiffness and impairment of systolic function with progressive disease (15–18). Morphometric studies showed an increased collagen protein content, which was slowly reversible after mechanical unloading (16,18,19). In the human heart, it is not known whether this increase in collagen occurs at the mRNA level or whether it is mainly determined by post-translation regulation (i.e., by an altered turnover rate) (20). In human end-stage heart failure, regulation of collagen synthesis at the mRNA level and a significant change in the collagen I to III mRNA ratio were reported, which suggests an independent regulation of these two genes, with potential functional consequences (21,22).

Expression and activity of ACE are increased in human heart failure, in cardiomyopathy (23) and near an infarct scar (24). Because ACE mRNA or activity and TGF-beta1 have not yet been measured in mechanically loaded human
hearts with maintained systolic function, it remains debatable whether mechanical overload by itself induces an activation of the human cardiac RAS. In addition to ACE, human heart chymase, which is mainly expressed in macrophages, and thus locally separated from myocytes and fibroblasts, is able to form angiotensin II in the human heart and to activate fibrotic processes (25,26).

In this study, we investigated whether ACE and TGF-beta1 are increased in the myocardium of patients with aortic valve disease and whether their regulation is associated with an increase in collagen I and III and fibronectin mRNA and with cardiac function.

METHODS

Patients. Myocardial samples from 17 patients with predominant aortic valve stenosis (AS) (AS group I [AS-I]) and 6 patients with predominant aortic valve regurgitation (AR) were analyzed for their mRNA content. In 14 of these patients with AS and 4 with AR, enough material was available to determine myocardial ACE activity. In a second series, eight new patients with AS (AS group II [AS-II]) and six control subjects were analyzed for TGF-beta1 protein (Table 1). Tissue from the left ventricular part of the septum was obtained during cardiac surgery and frozen immediately on dry ice. For a subgroup analysis, the 17 patients in the AS-I group were classified into those with a normal (<55%) left ventricular ejection fraction (LVEF) (62 ± 4%, n = 8, left ventricular end-diastolic pressure [LVEDP] 12 ± 2 mm Hg, aortic valve pressure gradient [dp] 86 ± 11 mm Hg) and into those with impaired (<55%) LVEF (38 ± 2%, n = 9, LVEDP 22 ± 3 mm Hg and aortic valve dp ≥ 69 ± 9 mm Hg). Seven patients without aortic valve disease and normal systolic cardiac function served as the control subjects (mitral valve stenosis [n = 3], explanted hearts [n = 4] and unused donor heart [n = 1]). Written, informed consent was obtained from all patients. The study followed the rules for investigation of human subjects, as defined in the Declaration of Helsinki.

Preparation of RNA and quantitation by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA preparation, complete deoxyribonuclease (DNase)

<table>
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<th>Abbreviations and Acronyms</th>
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<tr>
<td>ACE = angiotensin-converting enzyme</td>
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<td>AS = aortic (valve) stenosis</td>
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<td>hHC = human heart chymase</td>
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<td>LVEF = left ventricular ejection fraction</td>
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<td>mRAS = messenger ribonucleic acid</td>
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<td>PDH = pyruvic dehydrogenase</td>
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<td>RAS = renin-angiotensin system</td>
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<td>TGF-beta1 = transforming growth factor-beta1</td>
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Table 1. Clinical Features and Medical Therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Functional Class</th>
<th>LVEDD (mm)</th>
<th>LVEDP (mm Hg)</th>
<th>dpmax (mm Hg)</th>
<th>ACE Inhibitors (n)</th>
<th>Calcium Antagonists (n)</th>
<th>Beta-Blockers (n)</th>
<th>Diuretics (n)</th>
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<tr>
<td>AS-I</td>
<td>17</td>
<td>15</td>
<td>75</td>
<td>69 ± 3.1</td>
<td>26 ± 0.1</td>
<td>50 ± 2</td>
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<tr>
<td>AS-II</td>
<td>8</td>
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<td>75</td>
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<td>26 ± 0.1</td>
<td>50 ± 2</td>
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<tr>
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<td>50 ± 2</td>
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Data are presented as the mean value ± SEM or number of patients, unless otherwise specified. NYHA = New York Heart Association; FS = fractional shortening, as determined by echocardiography; LVEDD = left ventricular end-diastolic diameter; LVEDP = left ventricular end-diastolic pressure; dpmax = maximal pressure gradient over aortic valve at preoperative cardiac catheterization.
digested and reverse transcription were performed as described previously (27–31). The mRNA levels for ACE, human heart chymase (hHC), TGF-beta1, collagen I and III, fibronectin and the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvic dehydrogenase (PDH) were determined by using a "hot start" PCR procedure that was validated with respect to the reproducibility and linearity within the measuring range and had comparable efficacies of ~1.9. The PCRs were run in duplicate, and the products were quantitated by reverse-phase high performance liquid chromatography, as previously described (30). The reproducibility (day to day variance <3%) and linearity (>50-fold range) of the procedure were tested by separation of a plasmid standard (PBR 322-Hae III digest, Sigma, Aldrich, Munich, Germany). The variance of the PCR/high performance liquid chromatography procedure alone was determined by repeated analysis of seven samples and was <5% in this study.

To correct for potential variances between samples in mRNA extraction and RT efficacy and for variances in PCR pipetting, the mRNA content of ACE, TGF-beta1, collagen I and III and fibronectin was related to the mRNA content of the stably expressed reference genes GAPDH and PDH from the same aliquot. The expression of GAPDH and PDH mRNA in our samples was identical in the three patient groups and correlated significantly with each other (r = 0.91, p < 0.001, data not shown). In general, GAPDH was used as a reference gene. The TGF-beta1 mRNA content was related to PDH mRNA, because the optimized RT and PCR conditions for these two genes were almost identical, but they were different for GAPDH.

Activity of ACE and TGF-beta1 ELISA. Myocardial ACE activity was determined from 15 to 30 mg of tissue, by the formation of angiotensin II from angiotensin I in the presence or absence of 0.1 mmol/liter of captopril. The angiotensin II formed is measured by high performance liquid chromatography (25). Activity of ACE is calculated in nmol/liter of angiotensin II per min per g wet weight. The coefficient of variance was 4%.

To estimate the total (latent and active) TGF-beta1 protein content in the myocardial samples (in triplicates), we used a quantitative sandwich enzyme immunoassay technique (Quantikine, R&D Systems, Minneapolis, Minnesota), according to the manufacturer's protocol, as described previously (32).

Immunohistochemistry. Cryosections of tissue samples were air dried and fixed with acetone. The following antibodies were used for the staining of actin: collagen I (mouse, dilution 1:50 [Sigma-Aldrich]), fibronectin (rabbit, dilution 1:100 [ICN Biomedicals, Aurora, Ohio]), TGF-beta1 (mouse, dilution 1:100 [R&D Systems, Minneapolis, Minnesota]), ACE (mouse, dilution 1:1000 [Chemicon, Temecula, California]) and phalloidin (dilution 1:1000 [Sigma]). Incubation with phosphate-buffered saline instead of the first antibody served as a negative control study. Nuclei were stained with actinomycin D (Molecular Probes, Eugene, Oregon). The sections were viewed in a confocal laser microscope (Leica, Salms, Germany). The optical confocal sections taken through the depth of tissue samples at 0.5- to 1.0 μm intervals were viewed and photographed individually, or were superimposed for reconstruction in a three-dimensional mode using a Silicon Graphics Indy workstation and a three-dimensional multichannel image processing software (Bitplane, Zurich, Switzerland).

Statistics. Statistics were calculated with the SPSS software, version 10.0. Results are expressed as the mean value and SEM. The Mann-Whitney U test was used to calculate differences between the groups. Analysis of variance corrections were used for multiple testing. In the case of equal variances, the Bonferroni procedure was used, and in the case of unequal variances, the Tamhane-T2 procedure was used, as provided by the SPSS program. A corrected p value <0.05 was considered to be statistically significant. Regression analysis was prespecified for the whole group of patients to detect correlations between mRNA expression levels for genes encoding matrix proteins, ACE and TGF-beta1 and for the patients with AS whenever hemodynamic variables were included. Pearson’s correlation coefficients were calculated with SPSS for Windows.

RESULTS

Regulation of myocardial ACE, TGF-beta1 and hHC. Expression of ACE mRNA was significantly increased in patients with AS (1.6-fold, p < 0.05). This includes the subgroup with normal left ventricular function (LVEF >55%, n = 8, 1.7-fold increase, p < 0.05) (Figs. 1, 2). Expression of ACE mRNA was also significantly increased in patients with AR (1.8-fold, p < 0.01). Activity of ACE was increased in patients with AS (39.7 ± 5 nmol/min/g wet weight; n = 14; p = 0.05) and in the whole group of patients with aortic valve disease versus the control subjects (43.1 ± 5.2 vs. 29.8 ± 6.0 nmol/min per g weight; p = 0.01). Immunohistochemistry confirmed an increase in ACE expression in capillaries as well as in myocytes (Fig. 3, A and B, row IV). Chymase mRNA was downregulated in patients with AS to about one-third of the control levels (p < 0.001), and comparably in patients with normal or impaired systolic function (Fig. 2), whereas patients with AR had unchanged mean values, together with a very large variability (data not shown).

The mRNA of TGF-beta1 was significantly raised in patients with AS (1.5-fold, p < 0.05), including the subgroup with normal LVEF (n = 8, 1.4-fold, p < 0.01) (Fig. 1, 2). In samples from patients with AR, TGF-beta1 mRNA was also significantly increased (1.6-fold, p < 0.01) (Fig. 1). The TGF-beta1 protein was measured by ELISA and was significantly upregulated in patients with AS as compared with control subjects (1,230 ± 230 vs. 623.3 ± 62 pg/mg protein, p < 0.02). Immunohistochemistry confirmed an increase in TGF-beta1 expression in the hypertrophied myocardium (Fig. 2, A and B, row II).
Upregulation of collagen I and III and fibronectin. The collagen I and III and fibronectin mRNA expression levels were significantly increased in AS (2.1-fold, p < 0.001; 1.7-fold, p < 0.05; 2.3-fold, p < 0.005). Collagen I mRNA was also increased in the small subgroup of patients with AS and normal left ventricular function (LVEF > 55%, n = 8, 1.7-fold, p < 0.05) (Fig. 1, 2). In the patients with AR, increases in collagen I and III and fibronectin mRNA were found (2.4-, 1.9- and 2.6-fold, respectively), which was comparable to the changes observed in patients with AS; however, there was more interindividual variability in the former group (p = NS; data not shown) (Fig. 1). The upregulation of collagen and fibronectin protein was confirmed by immunohistochemistry (Fig. 3, A and B, rows I and III).

Relation between gene expression and hemodynamic variables. First, regression analysis of the whole group of patients and control subjects was undertaken to detect whether the observed changes in gene expression were correlated with each other. A significant correlation was found between ACE mRNA and TGF-beta1 mRNA (r = 0.55, p < 0.002). The increases in the genes’ expression for collagen (Col) I and III and fibronectin (Fn) were closely correlated with each other (Col I/III: r = 0.89; Col I/Fn: r = 0.89; Col III/Fn: r = 0.88; all p < 0.0001, data not shown).

In a second step, in the subgroup of patients with AS, we analyzed whether a significant correlation between gene expression and hemodynamic variables could be established. Significant positive correlations between collagen I and III and fibronectin gene expression and LVEDP (LVEDP/Col I: r = 0.60, p < 0.05; LVEDP/Col III: r = 0.61, p < 0.05; LVEDP/Fn: r = 0.53, p = 0.05) were observed. Significant inverse correlations existed between collagen I and fibronectin gene expression and left ventricular systolic function (LVEF/Col I: r = −0.56, p < 0.05; LVEF/Fn: r = −0.53, p < 0.01; data not shown).

Therapy with ACE inhibitors. The ACE inhibitors enalapril (10 mg/day; n = 1), ramipril (5 mg/day; n = 1), lisinopril (10 mg/day; n = 1) and captopril (12.5 mg/day; n = 2) were used in five patients in the AS-I group to treat hypertension and heart failure. Accordingly, patients treated

**Figure 1.** Expression of ACE, TGF-beta, and collagen I mRNA in patients with AS and AR. Bar graphs show ACE/GAPDH (left); TGF-beta/PDH (middle); and collagen (Col) I/GAPDH (right) expression in control hearts (Con), patients with AS and patients with AR. The expression of ACE mRNA and TGF-beta, mRNA was significantly increased in AR and AS. The collagen I mRNA was significantly increased in AS and was elevated in AR, without reaching the level of statistical significance. The mean values ± SEM are presented, and analysis of variance was performed, with the Bonferroni-corrected (Tamhane-T2 procedure) p values indicated. n.s. = not significant.

**Figure 2.** Expression of ACE, hHC, TGF-beta, collagen I and III and fibronectin mRNA in patients with AS and normal or impaired LVEF. The bar graph shows ACE/GAPDH; chymase/GAPDH; TGF-beta/PDH; collagen (Col) I/GAPDH; collagen III/GAPDH; and fibronectin (Fn)/GAPDH expression in patients with AS and normal or impaired LVEF (>55%, <55%, respectively). The ACE mRNA, hHC, TGF-beta, and collagen I mRNA expression was significantly altered in both groups, whereas the increase in collagen III and fibronectin mRNA only reached statistical significance in patients with AS and impaired LVEF. The mean values ± SEM are presented, and analysis of variance was performed, with the Bonferroni-corrected (Tamhane-T2 procedure) p values indicated. n.s. = not significant. *p < 0.05. **p < 0.01.
Figure 3. Immunofluorescence for collagen, fibronectin, ACE and TGF-beta. Row I (top) = immunofluorescence for collagen I (green, nuclei are red). (A) Normal myocardium with fine septa surrounding the myocytes and capillaries. (B) In hypertrophied myocardium, the amount of collagen I between the myocytes is increased. Row II = immunofluorescence for TGF-beta (green, myocytes are red by phalloidin). (A) In normal myocardium, only a few cells in the interstitium are positive for TGF-beta. (B) In hypertrophied myocardium, numerous structures contain TGF-beta as fine granules. Row III = immunofluorescence for fibronectin (green, nuclei are red). (A) Normal myocardium shows fine layers of fibronectin between the myocytes, corresponding to labeling for collagen I. (B) In hypertrophied myocardium, the amount of fibronectin is greatly increased. In three-dimensional reconstruction in the confocal microscope, the myocytes are blue. Row IV (bottom) = immunofluorescence for ACE (green, nuclei are red). (A) Only a few small capillaries are positive for ACE in normal myocardium. (B) In hypertrophied myocardium, labeling for ACE is more prominent.
with ACE inhibitors had more unfavorable hemodynamic characteristics as compared with patients not treated with ACE inhibitors (LVEF 42.4 ± 6.0% vs. 52.5 ± 4.3%; LVEDP 22.5 ± 4.0 vs. 13.5 ± 1.9 mm Hg; mean pulmonary artery pressure 21.0 ± 0.8 vs. 13.5 ± 0.3 mm Hg; p < 0.05 for all). Therapy with ACE inhibitors was associated with reduced myocardial ACE activity (patients with AS treated with ACE inhibitors: 23.3 ± 4.6 mmol/min/g wet weight; patients with AS not treated with ACE inhibitors: 47.9 ± 21.4 mmol/min/g wet weight; p = 0.009) and a tendency toward a reduced ACE mRNA content, but no other significant changes in gene expression.

**DISCUSSION**

We report an increase in the expression of ACE and TGF-beta_1_ in human hearts with AS and AR. The increases in ACE and TGF-beta_1_ mRNA correlate with each other and are accompanied by an increase in ACE activity and TGF-beta_1_ protein. The increased expression of the RAS components was accompanied by upregulation of collagen I and III and fibronectin at the mRNA level. Together with our in vitro studies of human hearts (32), the data suggest that activation of the cardiac RAS occurs early in human aortic valve disease and contributes to the development of myocardial fibrosis. Significant correlations were found between the expression of collagen I and III and fibronectin mRNA and hemodynamic variables in patients with AS, which points to a clinical relevance of the observed changes.

**Activation of the cardiac RAS in human aortic disease.**

Activation of the cardiac renin-angiotensin-aldosterone system contributes to cardiac fibrosis in a number of experimental conditions, and ACE inhibitors prolong survival and delay the transition to heart failure in rats with AS (5–7,10,33–35). Our data show, for the first time, to our knowledge, that the cardiac RAS and its mediator TGF-beta_1_ are also activated in human hearts with AS and AR, and that the increase in ACE and TGF-beta_1_ occurs in hearts with normal systolic function. This supports, at a molecular level, the clinical observation that angiotensin II is involved in the pathogenesis of cardiac dysfunction in mechanically overloaded human hearts (36).

Angiotensin II activates the synthesis and secretion of TGF-beta_1_ in vivo and in vitro (9,11–14). We found a significant upregulation of TGF-beta_1_ mRNA in human AS with normal and impaired systolic function, as well as in AR, and it was correlated to the upregulation in ACE mRNA. Because TGF-beta_1_ mRNA content is not regulated only by the activation of the RAS, but also by direct mechanical load and tissue catecholamines (37), and because the number of individuals studied was small and they had a broad range of clinical conditions, a better correlation than that observed may not be expected. In our previous in vitro studies in isolated human cardiac fibroblasts and human atrial tissue samples, TGF-beta_1_ upregulated collagen I and III mRNA (32).

The observed downregulation of hHC mRNA in AS is in agreement with previous large animal experiments where right ventricular hypertrophy was associated with significant decreases of right ventricular chymase expression and increases in ACE expression (38). We found, in as yet unpublished studies, that the expression of chymase was linked to the number of mast cells and expression levels of cytokines in the human heart, and both seem to be reduced in AS (unpublished data by Fielitz et al., 2000).

**Collagen and fibronectin gene regulation in myocardial hypertrophy.**

The upregulation of myocardial collagen mRNA also occurred in patients with AS with normal systolic function (i.e., in the compensated state of mechanical loading) and was increased in patients with more severely compromised cardiac function. This suggests a contribution to the transition from pure hypertrophy to cardiac failure. Similar observations have been made in animal models (3,39). The significant positive correlation between collagen I and III and fibronectin and LVEDP confirmed the available evidence that a rise in collagen concentration is associated with abnormal diastolic stiffness and diastolic dysfunction (2,40). A significant negative association existed between collagen and fibronectin mRNA and LVEF in AS and supports the notion that an increased myocardial collagen content is also associated with systolic dysfunction, as well as diastolic dysfunction.

The upregulations of collagen I and III and fibronectin mRNAs correlate closely with each other in aortic valve disease. This is in contrast with findings in human cardiomyopathy, where significant alterations in the relative amounts of collagen I and III mRNAs and isoforms, and thus different regulation of both genes, are reported (21,22).

**Therapy with ACE inhibitors.**

Medical therapy with ACE inhibitors was used in some patients to treat hypertension or heart failure. Inclusion of the ACE inhibitor-treated patients in the analysis was regarded as justified, because they did not differ from the rest of the group in terms of the expression of any gene except ACE itself, which represents a known effect of ACE inhibitor therapy on ACE gene expression (41). Because ACE inhibitors were not administered in a controlled manner, but were more frequently used in patients with more severe hemodynamic impairment, we cannot judge their specific effects on myocardial gene expression from our patient sample.

**Study limitations.**

This study started from the large body of published data that testifies to the increase in fibrous tissue in collagen and fibronectin protein in human aortic valve disease (2,15,16,18,19). The material was limited and preferentially used for mRNA quantitation. Because of the shortage of material, measurements of ACE activity and TGF-beta_1_ protein content and immunohistochemistry could only be performed in subgroups.

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