Increase in tissue endothelin-1 and ETA receptor levels in human aortic valve stenosis

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Aims: Aortic valve stenosis (AS) is an actively regulated process like atherosclerosis, which is accompanied by changes e.g. in endothelin-related genes. However, the role of endothelin peptides in AS is unknown.

Methods and results: We characterized the expression of the endothelin system in aortic valves of patients with normal valves (n = 12), regurgitation, and fibrosis (n = 6) and AS (n = 18) by reverse-transcriptase–polymerase chain reaction and immunohistochemistry. The number of endothelin-1 (ET-1) positive cells was higher in AS than in control valves, while levels of ET-1 mRNA did not differ between groups. Endothelin receptor-Α (ETA) mRNA levels were upregulated in stenotic valves (4.3-fold, P = 0.032) associated with a remarkable increase in number of ETA-immunopositive cells. ETA-receptor mRNA levels did not change during disease progression. Endothelin-converting enzyme-1 (ECE-1) mRNA levels were 42% lower (P = 0.007) in stenotic valves. Finally, because ET-1 and ECE-1 have binding site for activator protein-1 (AP-1), we measured AP-1 DNA binding by gel shift assays, which showed significantly lower (76%, P = 0.003) activity in AS.

Conclusion: AS is characterized by distinct upregulation of ET-1 and its target receptor ETA, promoting growth, inflammation, and fibrosis. These findings suggest therapeutic potential for ETA-receptor antagonists in aortic valve calcification.

Keywords: Aortic valve stenosis • Endothelin-1 • Receptors • Endothelin-converting enzyme • Activator protein-1 • Nitric oxide synthase

Introduction

Calcific aortic valve disease is identified by thickening and calcification of the aortic valve leaflets in the absence of rheumatic heart disease. Aortic valve stenosis (AS) is present in 2–5% of elderly patients, is the second most common indication for cardiac surgery, and carries an 80% 5-year risk of progression to heart failure, valve replacement, or death. Calcified aortic valve disease represents a spectrum of disease spanning from aortic valve sclerosis in which the leaflets do not obstruct left ventricular outflow, to severe aortic stenosis in which obstruction to left ventricular outflow is present. The calcified aortic valve lesion contains many features which are typical of an active pathobiological process: lipid accumulation, lipoprotein deposition, and other hallmarks of atherosclerosis, as well as matrix metalloproteinase activation, active calcification and ossification, renin–angiotensin system activation, and the development of injury and inflammation. Moreover, downregulation of antifibrotic C-type natriuretic peptide (CNP) system as well as upregulation of neutral endopeptidase (NEP). CNP-degrading enzyme, has recently been reported in AS.

Endothelins (ET-1, ET-2, and ET-3) are potent 21 amino acid vasoconstrictor isopeptides produced in different vascular tissues, including vascular endothelium. ET-1 is the main endothelin generated by the endothelium in humans and is recognized as the major isoform of relevance in cardiovascular physiology and pathophysiology. ET-1 is initially synthesized as preproET-1, which is processed to an inactive intermediate big ET-1. Endothelin-converting enzyme-1 (ECE-1) is a neutral endopeptidase that degrades big ET-1 into active ET-1. In addition, ECE-1 is involved in the activation of other vasoconstrictor peptides, including angiotensin II (Ang II) and big ET-1. The role of ECE-1 in the pathogenesis of AS remains unclear.

In AS, active ET-1 production is associated with increased apoptosis and cell proliferation. ET-1 stimulates cell proliferation through the ETA receptor, while ETA antagonists inhibit cell proliferation.

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Endothelin and its receptors in stenotic valves

Methods

Patients

The present study involved 36 consecutive patients (26 male and 10 female, mean age 58.6 ± 16.9 years) referred for aortic valve or aortic root surgery during August 2006 and January 2008 at Oulu University Hospital (Table 1). The study protocol was approved by the Research Ethics Committee of Oulu University Hospital and it complies with the Declaration of Helsinki. Some of the patients of this study have been enclosed earlier to our other study examining the pathophysiology of AS.5 All operations were made following normal surgical procedures. Aortic valve cusps were immersed immediately after removal on liquid nitrogen and stored at −70°C until analysed. Part of the samples was placed on 10% buffered formalin solution for histological analysis.

Patients were divided into three groups: control group consists of 12 patients with normal, non-calcified, smooth, and pliable aortic valve cusps. Fibrosis group consists of six patients, who were identified to have macroscopic thickenings of aortic valve cusps, which were macroscopically identified mainly as fibrotic and mild sclerotic lesions and did not contain calcified lesions. Patients in these groups were operated due to aortic root pathology or pathology of ascending aorta and did not have significant transvalvular gradient (as patients with AS). AS group consists of 18 patients, who had non-rheumatic, severe aortic valve sclerosis. The peak transvalvular gradient, assessed in Doppler echocardiography, was 84 ± 19 mmHg (range 40–120 mmHg) in AS group. The patient (male, age 65 years) with low peak gradient (40 mmHg) also had low ejection fraction (35%).

The patients’ demographics are listed in Table 1. Distribution of bicuspid aortic valves was similar in all groups. There were no significant differences in comorbidities or drugs used between the groups. However, there were more female in the AS group and patients in AS group were significantly older than patients in control group.

Expression of the endothelin system in aortic valves was characterized by the reverse-transcription–polymerase chain reaction (RT–PCR) analysis with TaqMan chemistry on an ABI PRISM 7700 Sequence Detection System as described previously.5 For histological analysis, the aortic valve samples were fixed in buffered formalin solution and embedded in paraffin. Five micrometre thick sections were cut and stained with haematoxylin and eosin for the calculation of aortic valve cusp area and quantification of calcified area.5 Localization of
ET-1, ETA, and ECE-1 in aortic valve cusps was made by using immunohistochemical staining. Supplementary data, including more detailed description of methods, extraction of RNA, RT–PCR, histological analysis, and gel mobility shift assay are online-only content (http://eurheartj.oxfordjournals.org).

**Statistical analysis**

Results are expressed as mean and standard deviation (SD). Demographic data between groups were compared by the analysis of variance (continuous variables) or by Fisher’s exact test (categorical variables). Because groups differed by age and sex (Table 1), these variables were set as covariates into analysis of covariance (ANCOVA) to control their influence on measurements. If ANCOVA showed a significant group effect ($P_g$), the ANCOVA model based means between groups were compared by Sidak’s adjustment with three replications was used to control multiple comparison problem and adjusted $P$-values ($P_a$) were reported. The logarithmic or square-root transformation was used if the dependent variable was heavily skewed to the right. Analyses were performed by SPSS for windows (version 14.0, SPSS inc., Chicago, IL, USA). Two-tailed $P$-values were reported. However, the $P$-values should be interpreted with caution, given the large number of statistical tests performed.

**Results**

**ET-1 in human aortic valve cusps**

As measured by RT–PCR, ET-1 was found to be expressed in all stages of calcified disease in human aortic valves (Figure 1A). While levels of ET-1 mRNA did not differ significantly between groups ($P_g = 0.45$, Figure 1A), the number of ET-1 positive cells in aortic valves was higher in stenotic than in control valves (Figure 1B–E; Supplementary material online, Table B). ET-1-positive staining was found in endothelial cells and myofibroblasts, adjacent to blood vessels, in stenotic aortic valves (Figure 1D–F). Regarding the cell types involved, we performed additional staining with alpha-smooth muscle actin (Figure 1G) to identify myofibroblasts and myocytes as well as CD 68 staining to identify macrophages (Figure 1H). Few macrophages, if none, could be detected (Figures 1F and H). ET-1-positive staining did not differ between the bicuspid and trileaflet valves or between patients with left ven-

ducal ejection fraction of 30–50% ($n = 3$) and $> 50%$ ($n = 15$) in AS group. There was no statistically significant correlation between peak gradient and tissue ET-1 levels (measured by immunohistochemistry, $n = 10$) in stenotic valves (data not shown).

**Decreased ECE-1 gene expression in stenotic valves**

ECE-1, cleaving big ET-1 to mature biologically active ET-1, was found to be expressed in aortic valves (Figure 2A). The mRNA levels of ECE-1 were 42% lower (95% CI 13–71, $P_g = 0.012$, $P = 0.007$) in stenotic valves when compared with control valves (Figure 2A). To evaluate the localization of ECE-1 in aortic valves, we used immunohistochemistry. ECE-1 positive staining was found in valvular endothelial cells (Figure 2B–D).

**Expression of endothelin receptors ETA and ETB in aortic valve cusps**

The levels of ETA, mediating contractility and growth-promoting effects of ET-1, were significantly upregulated in stenotic aortic valves (4.3-fold, 95% CI 2.4–6.1, $P_g = 0.016$, $P = 0.022$) between control and AS groups and 2.9-fold (95% CI 1.1–4.7, $P_g = 0.016$, $P = 0.044$) between fibrosis and AS groups (Figure 3A). The results of the RT–PCR analysis were supported by immunostaining, which showed a remarkable increase in the number of ETA-immunopositive cells in stenotic compared with control valves (Figure 3C and D). The mRNA levels of ETB receptor did not differ between groups ($P_g = 0.88$, Figure 3B). Blue ink was used for visualizing the aortic side of control valve to show the orientation (Figure 3D). There was no statistically significant correlation between ETA or ETB mRNA levels and degree of calcification of aortic valve cusps in AS group (data not shown).

**Binding activity of AP-1 during progression of calcified aortic valve disease**

Because mRNA levels of ECE-1 were significantly downregulated and the number of ET-positive cells was increased, and the promoter regions of both ECE-1 and ET-1 have a binding site of activator protein-1 (AP-1), we next measured AP-1 DNA binding activity from aortic valves by gel mobility-shift assay using a oligonucleotide probe containing the AP-1 site of the B-type natriuretic peptide (BNP) promoter. Interestingly, the binding activity of AP-1 was significantly lowered by 66% (95% CI 47–84) in AS group when compared with control group ($P_g = 0.003$, $P = 0.003$, Figure 4A and B).

**Downregulation of eNOS gene expression in stenotic valves**

The disruption of the balance between ET-1 and vasodilating and anti-proliferative endothelium-derived factors, such as NO is associated with various vascular pathologies. Therefore, we further analysed the endothelial dysfunction in stenotic aortic valves by measuring mRNA levels of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS). The mRNA levels of both eNOS and iNOS were detectable in all patient groups. The expression of eNOS gene was 75% (95% CI 47–100, $P_g < 0.001$, $P < 0.001$) lower in AS group than in the control group (Figure 5A). Furthermore, in stenotic valves, the mRNA levels of eNOS were 61% lower (95% CI 9–100, $P_g < 0.001$, $P = 0.032$) compared with fibroelastic group. Levels of iNOS mRNA, on the other hand, did not differ statistically significantly between groups ($P_g = 0.20$, Figure 5B).

**Discussion**

Aortic valve sclerosis is a common clinical problem in developed countries with prevalence of about 25% in people at age of more than 65 years. At present, there is no approved pharmacologic treatment for AS and the unavoidable operation as curative therapy of the disease is valve replacement surgery. Targeted drug
therapy to prevent the progression of calcific aortic valve disease should ideally be based on the knowledge of the molecular pathogenesis of the disease. Here we show that ET-1 and ET-1-associated genes are expressed in human aortic valve cusps. Remarkably, calcified aortic valve disease was characterized by increased tissue levels of mature biologically active ET-1. Furthermore, there was disequilibrium between gene expression of ETA- and ETB-receptors. In addition, we documented a significant down-regulation of ECE-1, cleaving big ET to mature ET-1, as well as lower levels of eNOS mRNA in stenotic valves.

Dysfunction of the vascular endothelium is an early finding in the development of cardiovascular disease and is closely related to atherosclerosis. Under physiological condition, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an
autocrine/paracrine factor. Under pathophysiological conditions, the production is stimulated in the large number of different cell types, including endothelial cells. The biological effects of ET-1 are transduced by two receptor subtypes ETA and ETB. In the vasculature, ETA-receptor mediates vasoconstriction, whereas ETB-receptor, when located endothelially, will cause vasodilatation via the release of NO and prostacyclin. ET-1 in subnanomolar concentrations has been demonstrated to activate macrophages resulting in release of pro-inflammatory and chemotactic mediators which are of importance in the atherosclerotic process, and ET-1 itself also has growth-promoting effects and pro-inflammatory effects.

A key finding of this study was that AS is characterized by the increased amount of ET-1 peptide. Thus, many features which are typical to human calcified aortic valve disease such as uptake of oxidized LDL, atherosclerosis lesion formation, calcification, inflammation (via actions and secretion of inflammatory cytokines and chemotactic mediators, respectively), and local renin–angiotensin system activation may be consequences of increased paracrine/endocrine production of ET-1 in aortic valve. The pathophysiological significance of increased ET-1 peptide levels in AS may be further enhanced by the concomitant up-regulation of ETA-receptors. The molecular mechanisms that mediate up-regulation of ET-1 in AS remain to be studied, but one possibility would be the changes in the degradation of ET-1. This seems, however, unlikely because an increase in NEP activity in stenotic aortic valves has been described recently. Increased amount of ET-1 in stenotic valves may also be related to uptake of circulating ET-1 via ETB-receptors. Previously, plasma levels of ET-1 have been shown to be increased in patients with AS and ETB-receptors are involved in elimination and uptake. Yet, the levels of ETB-receptors were not different between control and stenotic valves in the present study.

Since continued stimulation of cells with agonists generally results in a down-regulation of receptors, one would expect either ETA or ETB or both receptors to be down-regulated due to higher ET-1 peptide levels in stenotic valves. However, in human calcified aortic valves, several recent studies have reported...
higher mediator activity in parallel with increased receptor levels or decreased ligand levels in combination with down-regulation of target receptors. For example, angiotensin II-forming enzymes and AT1 receptors are all upregulated,1,4 and NEP activity increased in parallel with increased expression of profibrotic BK1 receptors in stenotic valves.6 Moreover, a significant down-regulation of both CNP levels and its target receptors has been reported in calcific stenotic valves.5 Thus, although unanticipated, our results showing increased amount of ET-1 and its target receptor ETA agree with these observations.

The molecular mechanisms that mediate the down-regulation of ECE-1 in stenotic valves could be explained at least partially with decreased binding activity of AP-1, having a binding site in the promoter area in the position of −232 to −222 bp upstream in ECE-1. AP-1 site is located in the promoter area of ET-1 as well,19 suggesting that the decrease of AP-1 binding activity in diseased valves may account for the unchanged ET-1 mRNA levels. Another reason for unchanged mRNA levels of ET-1 may be the fact that mRNA levels of ET-1 have been shown to decrease by high shear stress,11 associated to severe AS.12 Moreover, increased ET-1 peptide levels with unchanged ET-1 mRNA levels may reflect possible differences in the efficiency of ET-1 RNA message being converted in the big ET-1 protein, increased production of ET-1 from big ET-1, or uptake of ET-1 from circulation and decreased ET-1 degradation, as discussed above. Moreover, the stenotic valve contains many cell types in addition to endothelial cells and myofibroblasts,1 diluting the proportion of ET-1 gene expressing cells to total mass of tissue analysed, and thus ET-1 mRNA levels normalized to 18S.

Figure 3 (A) ETA receptor and (B) ETB receptor mRNA levels in human aortic valves. Results (mean ± SD) are expressed as ratio of ETA or ETB mRNA to 18S as determined by RT–PCR analysis and are relative to control group. P-values reported are between groups in concern. C, control (n = 12); Fibrosis, fibrosis (n = 6); AS, aortic stenosis (n = 18). (C and D) Representative light photomicrographs showing ETA-receptor immunostaining in aortic valves. Sections were stained with a specific ETA-antibody. (C) Graph of a stenotic valve showing ETA-receptor positivity in endothelial cells in several vessels (arrows). Additionally, stromal myofibroblasts adjacent to the vessels show ETA-receptor positivity. (D) A control valve stained with ETA-receptor antibody. No positive staining was detected in the valve. Blue ink (arrows) indicates the aortic side of the valve.

NO is a gaseous lipophilic free radical cellular messenger modulating, among several other cellular responses, inflammation, cell growth, leukocyte adhesion, smooth muscle cell proliferation, and platelet aggregation.15,32 However, little information exists concerning changes of NOS enzymes in human aortic valves. It has been documented that eNOS and iNOS positivity exist in non-rheumatic AS cusps when measured immunohistochemically,33 while gene expression levels as well as comparison to normal cusps has not been reported. Here we show that eNOS and iNOS are expressed in human aortic valves in calcified aortic valve disease. Interestingly, there was no significant compensatory activation of NOS in diseased
valves, and in fact, eNOS gene was significantly downregulated in calcific aortic valve disease. Thus, the progressive, active process of aortic valve sclerosis and calcification involves alterations in both the ET-1 and NO signalling pathways in that ET-1 signalling appears to be potentiated by reduced bioavailability of protective NO, similar to endothelial dysfunction in atherosclerosis.11–14 The downregulated NOS activity may be one of the central factors common to cardiovascular disease, although it is unclear whether this is a cause of, or result of, e.g. human calcific AS.

Although AS is characterized by distinct upregulation of ET-1 peptide and its target receptor ETA, promoting growth, inflammation, and fibrosis, it remains to be determined whether ET receptor antagonists might have beneficial effects in slowing down the progression of disease or which effect ET receptor antagonists in general or selective ETA-receptor antagonists might have in patients with AS. Experimentally, ETA-receptor blockade prevents endothelial dysfunction and structural vascular changes in atherosclerosis as well as inhibits hypercholesterolaemia-induced atherosclerosis.35 On the other hand, the vasodilating and blood pressure lowering properties of ET receptor antagonists might present a clinical problem in AS. In patients with heart failure, ETA-receptor antagonists have controversial effects (e.g. darusentan36), although neurohormones and natriuretic peptides decrease favourably by treatment.37

In conclusion, this study shows that ET-1, its processing enzyme ECE-1, and receptors ETA and ETB are expressed in human aortic valves. A major finding of this study was that ET-1 signalling via ETA-receptors in calcific aortic valve disease appears to be potentiated by imbalance between ETA and ETB receptors and downregulated eNOS gene expression. Therefore, our findings raise the possibility that the process of AS, showing hallmarks of atherosclerosis, may be attenuated by therapeutic interventions aiming at blocking ET receptors before the late-stage pathology of the disease. In view of our present results, it would be of interest to investigate whether selective ETA-receptor antagonists, particularly, have therapeutic potential in AS.

Figure 4 (A) AP-1 binding activity in human aortic valves. Results are mean ± SD. P-values are reported vs. control group. C, control (n = 10); Fibrosis, fibrosis (n = 5); AS, aortic stenosis (n = 12). (B) Gel mobility shift assay of nuclear extracts from human aortic valves. Nuclear extracts were incubated with radiolabelled BNP AP-1 oligonucleotide probe. C, control; Fib, fibrosis; AS, aortic stenosis.

Figure 5 (A) eNOS and (B) iNOS mRNA levels in human aortic valves. Results (mean ± SD) are expressed as ratio of eNOS or iNOS mRNA to 18S as determined by the RT–PCR analysis and are relative to control group. For eNOS, P-values are reported vs. group in concern. C, control (n = 12); Fibrosis, fibrosis (n = 6); AS, aortic stenosis (n = 18).
Limitations of the study
The results should be interpreted with caution, however, because of the small number of patients with AS in the study. Moreover, there is no follow-up or experimental treatment of the patients with ET receptor antagonists; therefore, possible treatment impact in AS is unknown. Furthermore, there are no data on plasma levels of ET-1 or big ET-1 in these patients.

Supplementary material
Supplementary material is available at European Heart Journal online.

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