Reduced number and function of endothelial progenitor cells in patients with aortic valve stenosis: a novel concept for valvular endothelial cell repair

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Aims
Endothelial destruction and calcification primarily occur at the aortic side of the calcified aortic valves (AVs). This study investigated whether degenerative AV stenosis (AS) is associated with the presence of valvular endothelial senescence and a reduction in the number and function of endothelial progenitor cells (EPCs).

Methods and results
Fifteen patients with severe AS and 18 age-matched control subjects were enrolled. Senescence-associated β-galactosidase activity was mostly localized on the valvular endothelial cells (ECs) of the explanted AVs and highly coincided with the calcified lesion at the aortic side. The number (9.3 ± 8.3 vs. 20.5 ± 9.0 cells per 10⁶ mononuclear cells; P < 0.01) and the migratory capacity (107.8 ± 54.6 vs. 185.0 ± 68.8 cells per 1000 cells; P < 0.01) of EPCs evaluated by FACS analysis or migration assay were significantly reduced in AS when compared with control. As possible mechanisms of alterations in EPCs, caspase-3 activity was significantly increased, whereas telomere-repeating factor-2 expression quantified by western blot was significantly reduced in EPCs from AS when compared with control.

Conclusion
Reduced regenerative capacity of valvular ECs due to senescence and decreased levels of EPCs might be, at least in part, a pathological link for the destruction of valvular ECs, resulting in progression of degenerative AS.

Keywords
Aortic valve stenosis • Apoptosis • Endothelial progenitor cells • Senescence • Ageing

Introduction
Degenerative aortic valve (AV) stenosis (AS) is the most common valvular disease and increases in prevalence with age.1,2 However, no established therapy to prevent development and progression of AS is currently available despite recent promising results with statins or angiotensin-converting enzyme-inhibitors (ACE-I).3–6 The pathogenesis of AS shares a number of similarities with atherosclerosis, such as endothelial dysfunction, increased leucocyte adhesion/infiltration, and calcification.7–9 The surfaces of valve leaflets are covered with endothelial cells (ECs), which are critical in maintaining a non-thrombogenic surface and for the transduction of mechanical and biochemical signals.10 Notably, the EC layer of calcified AV appears to be damaged especially on the aortic side.11

Mature ECs possess limited regenerative capacity.12 Thus, there is growing interest in circulating endothelial progenitor cells (EPCs), especially in their role for the maintenance of endothelial integrity and function.13,14 Insufficient numbers of EPCs are related to endothelial dysfunction15 and adverse clinical events,16 suggesting that endothelial injury in the absence of sufficient repair by circulating EPCs promotes the progression of vascular disease or valve disorder. It is well documented that the number and function of circulating EPCs are reduced in several atherosclerotic vascular diseases such

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as stable coronary artery disease (CAD), stroke, and peripheral occlusive vascular disease. In addition, EC senescence appears to be an important factor contributing to the pathogenesis of atherosclerosis.

However, the presence of senescent valvular ECs and the role of circulating EPCs remain unknown in the pathogenesis of valvular heart disease, particularly in patients with AS. Therefore, the aim of this study was (i) to evaluate the presence of senescent valvular ECs in explanted calcified AVs, (ii) to elucidate the amount and function of EPCs in patients with AS, and (iii) to investigate possible molecular mechanisms responsible for alterations in EPCs.

**Methods**

**Study population**

All the patients were recruited in our outpatient clinic between September and December 2007. Thirty-three individuals <75 years were enrolled in this study after assessment by Doppler echocardiography as well as cardiac catheterization and classified into two groups: (i) patients with severe AS (n = 15) from 65 screened patients scheduled for AV replacement; patients with congenital bicuspid AV, severe mitral valve disease, CAD, and chronic dialysis were excluded; (ii) control subjects (n = 18) from 40 screened patients with angina-like symptom; valvular heart disease including AS and CAD were excluded (Figure 1). The absence of CAD was defined as lack of or minimal coronary atherosclerosis by angiography. AS was excluded by direct measurement of the AV pressure gradient during catheterization.

For histological analysis, the non-calcified AVs to serve as control for β-galactosidase (β-gal) staining were collected from four surgical patients affected by AV insufficiency (AI) due to aneurysms of the ascending aorta, with a diagnosis based on pre-operative Doppler echocardiography and cardiac catheterization. Materials from four patients with AI were used only for the validation of the histological analysis but not for the measurement of EPCs (Figure 1).

The Ethics Committee of the University Leipzig approved the study protocol, and written informed consent was obtained from all patients before enrolment.

**Detection of senescence-associated β-galactosidase activity and immunohistochemical staining**

Senescence-associated β-gal activity was examined in the explanted AV tissue as described previously. Briefly, the samples were incubated for 24 h at 37°C (no CO2) in freshly prepared β-gal staining solutions containing 40 mmol/L citric acid/sodium phosphate, 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, 2 mmol/L MgCl2 adjusted to pH 6.0. After the stained tissue samples were photographed, they were fixed with 1% glutaraldehyde and embedded in paraffin. For immunohistochemical analysis, paraffin sections (3 μm) were prepared and the expression of CD31 and Krüppel-like factor-2 (KLF-2) was visualized with a polyclonal anti-CD31 antibody (Dako, Hamburg, Germany) or a polyclonal anti-KLF-2 antibody (Santa Cruz, Heidelberg, Germany) as described.

**Figure 1** Flow chart of the study. AS, AI, and CAD indicate aortic valve stenosis, aortic valve insufficiency, and coronary artery disease, respectively.
Quantification of circulating endothelial progenitor cells by flow cytometry

The peripheral blood was taken in the outpatient clinic. Especially for patients with AS, the blood was taken before surgery to exclude the effects of cardiopulmonary bypass and peri-operative procedures. To quantify the amount of circulating EPCs (defined as CD34pos/KDRpos cells) by flow cytometry, 7.5 x 10^6 mononuclear cells (MNCs) from circulating peripheral blood, isolated by density gradient centrifugation, were incubated at 4°C in the dark for 30 min with phycoerythrin-conjugated mouse anti-human KDR (R&D Systems, Wiesbaden, Germany), FITC-conjugated mouse anti-human CD34 (Miltenyi, Bergisch Gladbach, Germany), and PerCP-conjugated mouse anti-human CD3 (Becton Dickinson, Heidelberg, Germany). After incubation, cells were washed with phosphate-buffered saline (PBS), fixed with 0.5% paraformaldehyde/PBS, and analysed by FACS (LSRII, Becton Dickinson). For clear analysis, at least 3 x 10^5 total events or 1000 CD34pos events were collected by flow cytometry. In a second step, gated CD34pos cells were then examined for the expression of KDR.

Endothelial progenitor cell culture assay

Approximately 1 x 10^7 isolated MNCs were plated on six-well culture dishes coated with gelatin and maintained in EGM II medium (Cambrex, Verviers, Belgium). After 4 days in culture, non-adherent cells were removed by a thorough washing with cell culture medium, and adherent cells underwent migration, western blot, and caspase-3 activity assays at day 7.

Migration assay

After 7 days in culture, cells were detached using Trypsin/EDTA, and the migratory capacity of 1 x 10^5 EPCs towards SDF-1α (Sigma, Taufkirchen, Germany) was evaluated using a modified Boyden chamber as described recently.27 For quantification, cell nuclei were stained with Hoechst 33342 (Sigma) and the migrated cells were counted manually in three random microscopic fields.

Western blot analysis

After 7 days in culture, adherent cells were harvested, lysed in lysis buffer, and after centrifugation (10 min at 10 000 g), an aliquot of the supernatant was separated on 10% polyacrylamid gel and transferred to PVDF membrane.27 To explore the expression of telomere-repeating factor-2 (TRF-2), a TRF-2-specific antibody (Santa Cruz, CA) was used. After 7 days in culture, adherent cells were harvested, lysed in lysis buffer, and after centrifugation (10 min at 10 000 g), an aliquot of the supernatant was separated on 10% polyacrylamid gel and transferred to PVDF membrane.27 To explore the expression of telomere-repeating factor-2 (TRF-2), a TRF-2-specific antibody (Santa Cruz, CA) was used. Western blot analysis was performed using the standard formula as implemented in an online software algorithm developed by David A. Schoenfeld at Harvard school of public health (http://hedwig.mgh.harvard.edu/sample_size.html).28

Results

Characteristics of study population

Thirty-three individuals (15 patients with AS and 18 controls) were included into this study. The two groups did not differ significantly with regard to baseline parameters including age, cardiovascular risk factors, and medication (Table 1).

Endothelial cell senescence in human stenotic calcified aortic valves

Applying the β-gal-staining technique, macroscopic β-gal activity (blue colour) was remarkably visible at the aortic side of the explanted valve (Figure 2A). The specificity of the staining was verified using a piece of left internal mammary arteries (LIMAs) from several patients undergoing bypass surgery. Using the same staining conditions as for the calcified AV, no β-gal activity was observed in the AV without calcification from patients with aneurysms of ascending aorta and LIMA. To further investigate the β-gal-positive cell, a microscopic picture was taken from the β-gal-positive area. As shown in Figure 2B, β-gal-positive cells were mainly located on the surface of the leaflet at the aortic side. In order to analyse whether the EC layer is intact or disrupted, immunohistochemical staining for CD31 was performed. As shown in Figure 2C, a clear

| Table 1: Clinical characteristics of included individuals |
|-------------|-------------|-------------|
|             | Control (n=18) | AS patients (n=15) | P-value |
| Age, years  | 62 ± 7       | 62 ± 8       | 0.79     |
| Male, n (%) | 9 (50)       | 8 (53)       | 0.85     |
| Cardiovascular risk factors, n (%) | | | |
| Hypertension | 14 (77.7) | 13 (86.6) | 0.66 |
| Diabetes    | 4 (22.2) | 4 (26.6) | 1.00 |
| Hypercholesteralma | 6 (33.3) | 5 (33.3) | 1.00 |
| Smoking     | 3 (16.6) | 1 (6.7) | 0.61 |
| Medication, n (%) | | | |
| β-Blocker  | 12 (66.6) | 7 (46.6) | 0.25 |
| ACE-IVARB  | 8 (44.4) | 11 (73.3) | 0.16 |
| Statins    | 6 (33.3) | 5 (33.3) | 1.00 |
| Calcium antagonists | 3 (16.6) | 2 (13.3) | 1.00 |
CD31-positive staining showing a nearly intact EC layer was obvious at the ventricular side of the valve, whereas the EC layer at the aortic side was interrupted.

**Immunohistochemical staining for Krüppel-like factor-2**

In order to examine whether different flow conditions on the aortic and ventricular sides could explain the destruction of the EC layer at the aortic side, immunohistochemical staining for KLF-2 was performed. KLF-2, a protein that is downregulated by turbulent shear stress, was less expressed at the aortic side when compared with the ventricular side (Figure 2D).

**Amount of circulating CD34pos cells and endothelial progenitor cell**

Since EPCs are rare events in the circulation, at least \(3 \times 10^6\) MNCs were evaluated for the expression of CD34 and KDR by flow cytometry. An example of such an analysis is shown in Figure 3A. Analysing the expression of CD34pos cells in the peripheral blood of patients with AS and controls, no significant difference could be detected (control: \(406 \pm 148\) vs. AS: \(429 \pm 254\) events per \(1 \times 10^6\) MNC; \(P = 0.75\)) (Figure 3B). In contrast, a 2.1-fold lower amount of EPCs was detected in patients with AS when compared with the control group (control: \(20.5 \pm 9.0\) vs. AS: \(9.3 \pm 8.3\) events per \(1 \times 10^6\) MNC; \(P < 0.01\)) (Figure 3C).

**Migratory capacity of isolated endothelial progenitor cells**

Migratory capacity of EPCs was significantly lower in patients with AS when compared with controls (AS: \(107.8 \pm 54.6\) vs. control: \(185.0 \pm 68.8\) migrating EPCs per 1000 cells, \(P < 0.01\)) (Figure 4).

**Telomere-repeating factor-2 expression and caspase-3 activity of cultured endothelial progenitor cell**

To elucidate the impact of AS on EPC senescence, the expression of TRF-2, a telomere-binding protein with important roles in telomere protection and telomere-length regulation, was analysed in a
Figure 3  FACS analysis of CD34<sup>pos</sup> cells and endothelial progenitor cells in patients with aortic valve stenosis (AS) and controls. A representative example of a FACS analysis for the quantification of CD34<sup>pos</sup> cells and endothelial progenitor cells is shown (A). P1, cells positive for CD34; P2, cells positive for CD34 and KDR. For quantitative analysis (B), the amount of CD34<sup>pos</sup> cells or endothelial progenitor cells is calculated per 10<sup>6</sup> events measured. Results are expressed as mean ± SD.

Figure 4  Evaluation of the migratory capacity of endothelial progenitor cells from controls and patients with aortic valve stenosis (AS). Cultured endothelial progenitor cells were assayed in a modified Boyden chamber for the capacity to migrate along an SDF-1 gradient. Representative examples of a Hoechst 33342-stained membrane of a Boyden chamber are depicted. For quantitative analysis, the amount of migrated cells is calculated for 1000 cells added into the Boyden chamber. Results are expressed as mean ± SD.
subset of patients (AS: n = 11, control: n = 11). As shown in Figure 5A, a 2.9-fold lower expression of this important regulatory protein was evident in the EPCs from patients with AS when compared with controls (AS: 0.17 ± 0.13 vs. control: 0.51 ± 0.30 AU; P < 0.01).

Increased activity of caspase-3 is a hallmark for the activation of the apoptotic process. A 6.4-fold elevated caspase-3 activity was measured in EPCs from patients with AS when compared with controls (AS: 0.050 ± 0.029 vs. control: 0.007 ± 0.016 change in OD405/min mg; P < 0.001) (Figure 5B).

In order to evaluate whether reduced TRF-2 expression and the increased activity of caspase-3 may be involved in the reduced concentration of circulating EPCs in patients with AS, correlation analyses were performed. A positive correlation was observed between the expression of TRF-2 and the amount of EPCs (r = 0.69, P < 0.001, Figure 5C), and a negative correlation was seen between caspase-3 activity and the amount of EPCs (r = −0.63, P < 0.01, Figure 5D). Furthermore, a correlation was also detected between TRF-2 expression and the migratory capacity of EPCs (r = 0.53, P = 0.01).

**Discussion**

The destruction of the EC layer on the AV leaflet may be one important trigger for the progression of AV disease. Under normal physiological conditions, the EC layer is kept intact after injury either by division of mature ECs or by restoration with circulating EPCs. Three major findings emerge from this clinical study: (i) senescent ECs are present on the aortic side of degenerative AVs; (ii) the number and the migratory capacity of EPCs are significantly decreased in patients with AS when compared with the controls without AS; (iii) a molecular mechanism for the reduced levels of

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**Figure 5** Quantitative analysis of telomere-repeating factor-2 (TRF-2) expression (A) and caspase-3 activity (B) in endothelial progenitor cells (EPCs) isolated from controls and patients with aortic valve stenosis (AS). The values for telomere-repeating factor-2 expression are depicted as ratio over the expression of GAPDH. A representative western blot is shown on top of the quantitative evaluation. In addition, a correlation analysis between the amount of circulating endothelial progenitor cells and the expression of telomere-repeating factor-2 (C) and caspase-3 activity (D) are shown. Results are expressed as mean ± SD.
circulating EPCs may be the increased cell senescence as well as an enhanced apoptosis of EPCs from AS patients. These results suggest that in patients with AS, valvular EC regeneration is impaired not only by an increased senescence of valvular ECs but also by a reduced number and function of circulating EPCs.

Possible involvement of turbulent flow in the endothelial denudation on the aortic valve

It is well established that endothelial dysfunction is characterized by a progressive loss of ECs and thought to represent a crucial step in the initiation and progression of atherosclerotic vascular disease.\(^1\) The surface of the AV leaflets is also covered with ECs, which are important for proper function.\(^2\) During the development and progression of AS, EC layer is damaged followed by infiltration of inflammatory cells,\(^3\) which can induce a viscous cycle leading to progression of the disease.\(^2,3\) Importantly, the loss of endothelial integrity as well as calcification occurs primarily on the aortic side of the valve leaflets.\(^4\) This notion is supported by our observation that using CD31 immunohistochemical staining; an interrupted EC layer is observed at the aortic side but not at the ventricular side (Figure 2C). In line with our observation, a previous report using electron microscopy showed deteriorated ECs on the aortic side of the valve.\(^5\) Interestingly, asymmetric development of valve lesions in an area of abnormal flow rheology is similar to that of atherosclerotic arteries at branch points, where turbulent flow is involved.\(^6\) An indicator for a flow-specific pattern is the expression level of KFL-2 in ECs.\(^7\) Of note, we demonstrate for the first time that the expression of KFL-2 is markedly reduced on the aortic side of the leaflet when compared with the ventricular side (Figure 2D), suggesting an involvement of turbulent flow in the destruction of EC integrity. In addition to haemodynamic stress and its influence on ECs, other factors are important for the initiation and progression of AS, because degenerative AS is not an inevitable consequence of ageing.\(^8\)

Valvular endothelial cell senescence in human calcified aortic valves

Haemodynamic stress on the AV may result in an increased rate of EC turnover,\(^9\) telomere loss,\(^10\) and focal areas of EC senescence,\(^11\) contributing to impaired regeneration of valvular EC. Somatic cells reaching a certain age enter into a replicative senescence, a non-dividing state with short telomeres.\(^12\) The importance of cell senescence in patients with AS was supported by a recent report of Kurz et al. showing a shorter leucocyte telomere length in patients with AS when compared with an age-matched control group.\(^13\) Analysing cell senescence by using β-gal staining, we apparently observed an intensive β-gal activity in calcified AVs which was completely absent in control AVs and LIMA (Figure 2A). In particular, β-gal activity highly coincides with calcified lesions which are marked in the base and mid-portion of the cusp but not at the commissure with sparing of closing edge (Figure 2A). Combined with the evidence that ECs with senescence-associated phenotypes exist in human atherosclerotic lesions,\(^14\) it is conceivable that functional changes in senescent valvular ECs in vivo may, at least in part, play an important role in the pathophysiology of age-associated AV disease.

Impaired regenerative capacity of circulating endothelial progenitor cells in patients with aortic valve stenosis

Accumulating evidence suggests that EPCs contribute to endothelial recovery after injury and maintenance of endothelial function.\(^15,16\) Knowing that constant mechanical stress leads to a destruction of endothelial integrity and increased EC turnover, repair mechanisms are of considerable importance.\(^17,18\) Unfortunately, the role of circulating EPCs as a potential source of valve repair is largely unknown, since previous studies investigating the mechanisms of degenerative AVs have focused predominantly on the microenvironment within valvular tissues.\(^19\) In the present study, we found that the amount and the migratory capacity of circulating EPCs are significantly diminished in patients with AS when compared with controls without AS (Figures 3 and 4). These results are of great importance, because they may represent possible mechanisms for persistent systemic endothelial dysfunction\(^15,17\) or adverse cardiovascular events such as stroke in patients with AS,\(^16,18\) rapid progression of the disease,\(^19\) and the inability to efficiently repair the damaged EC layer on the aortic side (Figure 2C). In addition, CAD and AV sclerosis/AS are often present in the same patients,\(^19,40\) further strengthening the concept. On the basis of our results, atherosclerosis should be increased in patients with AS. This is supported by a recent report showing that >50% of patients with AS reveal CAD.\(^39\) On the other hand, patients with atherosclerosis should, to a certain amount, suffer from an altered AV and in particular from a dysfunction of the endothelial layer of the valves. This notion is also supported by a study of Soydinc et al. demonstrating that 50 out of 133 patients with CAD had AV sclerosis.\(^40\)

Potential mechanisms regulating the number and function of endothelial progenitor cells

The amount of circulating EPCs can be regulated by two different mechanisms: (i) controlling the release of EPCs from the bone marrow and (ii) the survival and life span of EPCs. In the present study, we could provide evidence that mechanisms regulating life span and cell death might be altered in EPCs isolated from patients with AS. Increased caspase-3 activity, a hallmark for the activation of the apoptotic process, in EPCs could be one mechanism regulating the number of EPCs in patients with AS; a 6.4-fold elevated caspase-3 activity in EPCs isolated from patients with AS was observed (Figure 5B). Estimating apoptosis by the caspase-3 activity is adequate and commonly performed in the literature.\(^41\) An alternative possibility to evaluate apoptosis is measuring annexin-V-positive EPCs by FACS analysis. This measurement is demanding, since the number of CD34\(^+\)/KDR\(^+\)/annexin-V\(^+\) cells is very small, and therefore reliable results are difficult to obtain. The involvement of apoptotic cell death in regulating the amount of EPCs in patients with AS is further supported by the negative correlation between caspase-3 activity and cell number (Figure 5D). The mechanism that increased apoptosis regulates the number of
EPCs in other diseases such as diabetes or experimental models is well known for a long time.

Another mechanism to regulate the number and function of circulating EPCs is an increase in cell senescence. A critical factor controlling telomere length and function appears to be TRF-2. Interestingly, it could be shown that the removal of TRF-2 triggers apoptosis or cell senescence in different cell culture systems. Furthermore, the expression of TRF-2 can influence the migratory capacity of EPCs. This is supported by our data on the correlation between TRF-2 and the migratory capacity of EPCs as shown in the present study.

Taken together, it seems reasonable to assume that enhanced apoptosis as well as increased senescence induced by a lower expression of TRF-2 may lead to a reduction in circulating EPCs in patients with AS. These remarkable findings provide evidence that patients with AS reveal signs associated with ‘biological’ ageing. Owing to the presence of senescent valvular ECs and the reduced levels of EPCs, it may be no longer possible to keep the EC layer intact, thereby leading to the development of AS (Figure 6).

Clinical implications
In view of an increasing prevalence of AS with age and increasing life expectancy of the population, an effective treatment strategy to inhibit the progression of AS will have major clinical benefits. Potential treatment target is the disrupted EC layer of the valve leaflet. The present study suggests that various therapeutic attempts to preserve the number and function of EPCs at an earlier stage of disease and longer treatment may result in an effective protection for valvular ECs, and thus preventing the development of AS. Candidates include statins, ACE-I, and angiotensin-receptor blockers, all of which have the potency to suppress EPC senescence and increase the number or functional capacity of EPCs. It is still controversial whether statins or ACE-I influences the progression of disease in patients with AS.

Limitations
One of the limitations of this study is that it did not examine the level of circulating EPCs at an earlier stage of disease and an association between the number of EPCs and the disease progression. This correlation would have further strengthened our hypothesis. To address these issues, an animal study with a model developing AV sclerosis/AS is currently conducted.

Conclusions
This study reports for the first time the presence of senescent valvular ECs on calcified AVs and a reduction in circulating EPCs related to ‘biological’ ageing in patients with AS. These data may offer a novel pathophysiological concept for impaired valvular EC regeneration, leading to the progression of age-associated calcific AV disease.
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