Degenerative Aortic Valve Stenosis, but not Coronary Disease, Is Associated With Shorter Telomere Length in the Elderly

David J. Kurz, Barbara Kloechener-Gruissem, Alexander Akhmedov, Franz R. Eberli, Ines Bühler, Wolfgang Berger, Osmund Bertel, Thomas F. Lüscher

Objective—The mechanisms responsible for the age-related increase in the incidence of calcific aortic valve stenosis (CAS) are unclear but may include telomere-driven cellular senescence. Because telomere length varies widely among individuals of the same age, we hypothesized that patients with shorter telomeres would be prone to develop CAS late in life.

Methods and Results—Mean telomere length was measured in leukocytes from a cohort of 193 patients ≥70 years of age with and without CAS. Pilot experiments performed in 30 patients with CAS and controls pair-matched for age, sex, and presence or absence of coronary disease demonstrated significantly shorter telomeres in the CAS group both by Southern blot hybridization (5.75±0.55 kbp versus 6.27±0.7 kbp, P=0.0023) and by a quantitative polymerase chain reaction-based technique (relative telomere length 0.88±0.19 versus 1.0±0.19, P=0.01). This finding was then confirmed in the whole cohort (CAS n=64, controls n=129, relative telomere length =0.86±0.16 versus 0.94±0.12, P=0.0003). Both groups were comparable for potential confounding characteristics. Subgroup analysis according to the presence or absence of coronary disease demonstrated no association of this disorder with telomere length.

Conclusions—In the elderly, calcific aortic stenosis, but not coronary disease, is associated with shorter leukocyte telomere length. (Arterioscler Thromb Vasc Biol. 2006;26:e114-e117.)

Key Words: aging ▪ aortic stenosis ▪ coronary disease ▪ risk factors ▪ telomere

Degenerative calcific aortic valve stenosis (CAS) is a strictly age-related disorder which can be distinguished from other forms of aortic stenosis such as post-rheumatic valve disease or congenital abnormalities (eg, bicuspid valve). CAS has a prevalence of ≈4% among those older than 85 years1 and is currently the most frequent reason for performing valve replacement surgery. However, the mechanisms leading to CAS and its tight association with advancing age remain largely unknown.

At the cellular level, aging leads to a permanent nondividing state known as replicative senescence, which ensues in somatic cells after a predetermined number of cell divisions and induces characteristic changes in gene expression, morphology, and cellular function.2 Entry into the senescent state can be triggered by the loss of telomere integrity.3 Telomeres build the physical caps of chromosomes and, in humans, consist of several thousand repeats of the sequence TTAGGG associated with telomere binding proteins. Telomeres shorten during replication of somatic cells, ultimately leading to senescence with progressing biological age.4 In the human vasculature, telomere length (TL) was found to be shorter at sites with increased hemodynamic stress.5,6 Because the cusps of the aortic valve are exposed life-long to high levels of mechanical and shear stress, they might also be prone to the accumulation of senescent cells, which in turn could contribute to the development of age-related CAS. Because TL varies widely among individuals of the same age,7–10 mainly because of hereditary factors,7,9,10 patients with heritably shorter telomeres might have a predisposition toward CAS. We therefore investigated whether the presence of age-related CAS was associated with shorter telomeres in peripheral blood leukocytes. These have been shown to represent a legitimate surrogate of tissue TL.8,11
Methods

Patients
The study population (n=193) was recruited prospectively from patients ≥70 years of age undergoing diagnostic coronary angiography. Case patients (n=64) all had critical CAS scheduled for valve replacement surgery and underwent elective angiography to assess for concomitant coronary artery disease (CAD). Absence of CAD was defined as lack of or minimal coronary atheromatosis as visualized by angiography. Patients with type I diabetes mellitus, total cholesterol >8 mmol/L, serum creatinine >200 μmol/L, or recent immunosuppressant/antineoplastic chemotherapy were excluded. Echocardiography was performed in case patients to assess the mean systolic pressure gradient over the aortic valve, aortic valve morphology, and left ventricular ejection fraction. In controls (n=129), aortic stenosis was excluded by direct measurement of the aortic valve pressure gradient during left ventricular catheterization. These 2 methods of quantifying transvalvular pressure gradients have been repeatedly cross-validated and are accepted to be of equivalent sensitivity and specificity. Written informed consent was obtained from all patients, and the protocol was approved by the institutional ethics committee.

TL Analysis
TL analysis was performed by a team blinded to patient characteristics. Blood samples were taken after an overnight fast. Whole EDTA blood was frozen immediately and stored at −80°C until further processing.

DNA Isolation
Genomic DNA was extracted using the PureGene DNA extraction kit (Gentra Systems, Minneapolis, Minn) or magnetic bead technology (Chemagen, Baesweiler, Germany). Control experiments confirmed that TL analysis of the same sample extracted by both techniques delivered virtually identical results.

Terminal Restriction Fragment Determination by Southern Blot
Terminal restriction fragment (TRF) length analysis was performed using standard techniques. For details, see the online data supplement available at http://atvb.ahajournals.org. Each case sample was run with its pair-matched control on adjacent lanes. Results are the mean of 2 independent assessments for each sample. The interassay variability was 7.8% ± 7%.

TL Determination by Quantitative Polymerase Chain Reaction
In a parallel approach, relative TL was determined using a previously described and validated polymerase chain reaction-based method. For details, see the online data supplement available at http://atvb.ahajournals.org.

Results
Pilot experiments were performed on a set of 30 cases and 30 controls, selected from the cohort to be pair-matched for age, sex, and the presence or absence of CAD, factors that have previously been associated with differences in leukocyte TL. Clinical, risk factor and hemodynamic characteristics of these patients were comparable and are summarized in Table I (see online data supplement available at http://atvb.ahajournals.org). The 2 groups were comparable with regard to cardiovascular risk factor profiles, plasma levels of lipoproteins and C-reactive protein, body mass index, and statin use.

TRF length determined by Southern blot analysis demonstrated that the CAS patients had on average significantly shorter telomeres than the controls, with the difference between the means of the 2 groups equaling 0.52 kbp (5.75±0.55 versus 6.27±0.7 kbp, P=0.002) (Figure 1). The average annual decline in TRF length as calculated by linear regression was 35.2±21.8 base pairs/year and was not significantly different between groups (ANCOVA, data not shown). These results were confirmed using a quantitative polymerase chain reaction-based method of TL analysis. Although less sensitive, this approach has recently become more widely used because of its suitability for high-throughput analysis. The mean relative TL of the CAS patients and controls included in the pilot experiments was 0.88±0.19 and 1.0±0.19, respectively (P=0.01). Linear regression analysis for the correlation between these 2 very different methods of measuring TL delivered a correlation coefficient of 0.262 for the relationship between relative TL and TRF length (P=0.0001, data not shown).

We next tested whether the association between shorter TL and CAS found in the pilot study could be reproduced in the entire cohort of 193 patients, made up of 64 CAS patients and 129 controls. Baseline characteristics were comparable and hardly differed from the pilot study pair-matched groups (Table). The mean relative TL in the CAS group was

Statistical Analysis
Student t tests were used to compare continuous variables. The distribution of categorical variables was compared with the χ2 test. Values of P<0.05 were considered significant.
The leukocyte TL of these elderly patients with CAS was independent of smoking status and statin use. Similarly, relative TL showed no confounding effect of these on our result. We found no significant association between relative TL and serum levels of lipid fractions or C-reactive protein. Similarly, relative TL showed no difference in relative TL (young aortic stenosis: $1.02 \pm 0.16$ versus controls $1.00 \pm 0.11$, $P=0.6$).

**Discussion**

Calcific aortic valve stenosis is an archetypal age-related degenerative disease. Approximately half of all octogenarians have some form of aortic valve sclerosis when assessed by echocardiography, and $\approx 10\%$ of these will develop severe aortic stenosis. It is unknown by which mechanism the aging process contributes to the development of CAS. In this study, we followed the hypothesis that the limited regenerative capacity resulting from the telomere-based cellular senescence program may be involved in the development of CAS. The aortic valve represents a predilection site for degenerative disease caused by its chronic exposure to high levels of mechanical stress, a factor known to result in increased endothelial turnover. The emergence of senescent cells on the valve might then contribute to the progression of valve sclerosis not only by the loss of endothelial integrity but also by their altered function and gene expression profile.

During recent years, a number of groups have reported the presence of senescent endothelial cells in vascular pathology. The indirect approach of using leukocyte TL as a surrogate for vascular tissue, as used here, is based on 2 reports demonstrating a strong intra-individual concordance between TLs of leukocytes and skin or synovial tissue from the same donor, indicating that the genetic determination of TL is tissue-independent. Using this strategy, others have reported an association of shorter leukocyte TL with CAD and increased cardiovascular mortality.

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**Table 1. Patient Characteristics of the Entire Study Cohort**

<table>
<thead>
<tr>
<th>Aortic Valve Stenosis</th>
<th>Controls</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>77.2±5.2</td>
<td>75.9±4.3</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>28 (44%)</td>
<td>73 (56%)</td>
</tr>
<tr>
<td>Presence of CAD, n (%)</td>
<td>35 (55%)</td>
<td>75 (58%)</td>
</tr>
</tbody>
</table>

**Cardiovascular Risk Factors**

- Current or ex-smoker, n (%) 24 (38%) 42 (32%) 0.47
- Hypertension, n (%) 42 (66%) 95 (73%) 0.28
- Hypercholesterolemia, n (%) 31 (48%) 70 (54%) 0.47
- Type 2 diabetes mellitus, n (%) 14 (22%) 26 (20%) 0.76
- Family history of CAD, n (%) 4 (6%) 19 (15%) 0.09

**Body mass index, kg/m²**

- 26.4±4.0 26.1±4.6 0.71

**Systolic blood pressure, mm Hg**

- 136±20 138±23 0.53

**Pulse pressure, mm Hg**

- 59±20 63±20 0.17

**Left ventricular ejection fraction, %**

- 55±14.6 61±13.5 0.04

**C-reactive protein, mg/L**

- 7.4±17.4 10.6±18.7 0.27

**LDL cholesterol, mmol/L**

- 3.3±1.1 3.0±1.1 0.17

**Statin therapy, n (%)**

- 29 (45%) 57 (44%) 0.85

Values represent means±standard deviation or number of patients and percent. *Measured by echocardiography in CAS, and by left ventricular angiography in controls.

CAD indicates coronary artery disease; LDL, low-density lipoprotein.
The main finding of this study is that patients with severe CAS have on average shorter telomeres than appropriately matched controls. A second important finding was the lack of association between shorter TL and the presence of angiographically verified CAD in this elderly population. This finding was consistently present in both the CAS and control groups (Figure 2B). This result contrasts with other publications reporting shorter leukocyte TL in patients with CAD. However, of these 1 study investigated only patients who had sustained an acute myocardial infarction before the age of 50, whereas in the other study reported by the same group only 1 patient and 2 controls were older than age 70 years. One might speculate that shorter TL in patients with premature CAD might be related to a more inflammatory nature of the disease compared with CAD in the elderly.

Although our findings show a clear association between shorter TL and CAS, they do not demonstrate a causal relationship. Additional factors, such as chronic inflammation, hypercholesterolemia, or increased oxidative stress, might contribute to the development of CAS and independently thereof accelerate leukocyte telomere erosion. Nonetheless, in our population no such confounding associations were identified. Alternatively, one could speculate that the hemodynamic shear forces active over the stenotic aortic valve might increase leukocyte turnover and would thus lead to shorter telomeres. Our findings from a smaller group of young patients with severe aortic stenosis attributable to other causes (posttraumatic disease, bicuspid valve) and age- and sex-matched controls showed that this was not the case, supporting a role for shorter telomere length selectively in the development of age-related degenerative CAS.

Evolutionary aging theories suggest that genes or processes that were selected to benefit young organisms can have unselected deleterious effects that become manifest in older organisms and thereby contribute to the aging phenotype. The process of telomere-based replicative senescence fits this concept in humans by protecting against the development of cancer during the years of reproductive activity, but resulting in degenerative disorders at advanced ages. The association we report here between age-related CAS and shorter TL is compatible with the concept that the telomere-dependent exhaustion of regenerative capacity may participate in the development of this disorder.

Acknowledgments
We acknowledge technical assistance of S. Pancera and Y. Gruissem in extracting DNA.

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Disclosure(s)
None.

References
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Online data supplement

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Methods

Terminal restriction fragment determination by Southern blot

Terminal restriction fragment (TRF) length analysis was performed using standard techniques. Aliquots (1.5 μg) of genomic DNA were digested with 20 units each of the restriction enzymes HinfI and RsaI during 3 hours at 37°C (final concentration 1 U/μl). Set-up experiments verified complete DNA digestion under these conditions. Digests were separated on a 0.7% agarose gel and transferred to a positively labeled nylon membrane (Hybond N+, Amersham Biosciences) under high-salt conditions. The blots were hybridized with a 32P-labeled oligonucleotide telomere probe (CCCTAACCTAACCCTAA) and exposed to a phospho-imager after standard high-stringency washes. Hybridization signals were quantified by computer-assisted scanning densitometry using the public domain NIH Image software. The mean TRF lengths were calculated by integrating the signal intensity over the entire TRF distribution as a function of TRF length using the formula TRF length = Σ(OD_i) / Σ(OD_i/L_i), where OD_i and L_i are the signal intensity and TRF length respectively at position i on the autoradiogram. Identical molecular weight standards run on either side and in the middle of the gel confirmed homogeneous migration conditions. In order to minimize
problems resulting from inter-gel variability, each case sample was run with its pair-matched control on adjacent lanes of the same gel.

**Telomere length determination by quantitative PCR**

In a parallel approach relative TL was determined using a previously described and validated PCR-based method. In a reaction volume of 10 µl, genomic DNA (6ng) was combined with a mastermix in 384-well plates as dispensed by robotic pipetting. Each plate contained sample DNA as well as five serial dilutions of reference DNA, which served as standards in calculating the relative TL. Each DNA sample was analyzed at least 4 times. A first mastermix contained telomere-specific primers (100nM of tel1b: CGGTTTGT TTGGGTGTTTTGTTTGGTTTGGTTTGGTT, and 900 nM of tel2b: GGTGGCCTACCCCTACCCCTACCCCTACCCCTACCCCT) in the presence of 1.7 mM MgCl₂ while a second mastermix, analyzed on a separate plate, contained primers specific for the single copy gene beta globin (hbgl) (300 nM of hbgl1: GCTTCTGACACA CACTGTGTTCACTAGC and 700 nM of hbgl2: CACCAACTTCATCCACGTTCACTAGC) in the presence of 2.5 mM MgCl₂. Amplification was performed with 0.8 U Hotstar polymerase and its corresponding 10x buffer and MgCl₂ stock (Quiagen) in the presence of 0.15x SYBR Green (Applied Biosystems, Rotkreuz, Switzerland) and 2.5 mM dithiothreitol. Amplification was performed with a Prism 7900 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). The temperature cycling regime was adapted from the preprogrammed cycling conditions as follows: after 15 minutes at 95°C, 25 cycles followed for the telomere probe at 56°C (30 seconds) and 72°C (30 seconds) while 35 cycles were applied for the hbgl probes with the annealing temperature of 58°C. ABI Prism 7900 SDS software version 2.2 was used
for analysis. Relative TL ($2^{-\Delta\Delta Ct}$) was calculated as described in the User Bulletin #2 (ABI PRISM 7700 Sequence detection System) from mean Ct values of 4 to 8 replicates for each sample. Patient and control TLs were calculated relative to those obtained for a standard DNA sample, present on all plates. The functionality and reproducibility of the assay was confirmed on a series of DNA extracts from serially passaged human endothelial cells with a known linear decrease in TL (data not shown).
Table I:
Characteristics of pair-matched patients in the pilot study with and without calcific aortic valve stenosis

<table>
<thead>
<tr>
<th>Feature</th>
<th>Aortic valve stenosis (n = 30)</th>
<th>Controls (n = 30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age* (y)</td>
<td>76.9 ± 4.0</td>
<td>76.9 ± 4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Male* (n, %)</td>
<td>14 (47%)</td>
<td>15 (50%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Presence of CAD* (n, %)</td>
<td>13 (43%)</td>
<td>14 (47%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Cardiovascular Risk Factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or ex-smoker (n, %)</td>
<td>14 (47%)</td>
<td>8 (27%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td>22 (73%)</td>
<td>24 (80%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Hypercholesterolemia (n, %)</td>
<td>17 (57%)</td>
<td>14 (47%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus (n, %)</td>
<td>8 (27%)</td>
<td>7 (23%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Family history of CAD (n, %)</td>
<td>2 (7%)</td>
<td>6 (20%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>26.1 ± 4.2</td>
<td>25.7 ± 3.0</td>
<td>0.72</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>137 ± 19</td>
<td>147 ± 27</td>
<td>0.11</td>
</tr>
<tr>
<td>Pulse pressure (mm Hg)</td>
<td>56 ± 22</td>
<td>69 ± 23</td>
<td>0.03</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%) †</td>
<td>56 ± 16</td>
<td>64 ± 15</td>
<td>0.07</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>4.64 ± 4.25</td>
<td>7.83 ± 11.92</td>
<td>0.19</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol/l)</td>
<td>3.37 ± 1.12</td>
<td>3.09 ± 0.81</td>
<td>0.32</td>
</tr>
<tr>
<td>Statin therapy (n, %)</td>
<td>12 (40%)</td>
<td>11 (37%)</td>
<td>0.79</td>
</tr>
<tr>
<td>Calcific aortic valve stenosis (n, %)</td>
<td>30 (100%)</td>
<td>0 (0%)</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean pressure gradient (mm Hg)</td>
<td>50 ± 15</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Calculated aortic valve area (cm²)</td>
<td>0.75 ± 0.17</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values represent means ± standard deviation or number of patients and percent. * features used to pair-match subjects. †measured by echocardiography in CAS, and by left ventricular angiography in controls. CAD, coronary artery disease; LDL, low density lipoprotein; ns, not significant; N/A, not applicable.
References