Evidence for tissue factor expression in aortic valves in patients with aortic stenosis

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KEY WORDS
aortic stenosis, tissue factor, transvalvular gradient

ABSTRACT

INTRODUCTION The role of blood coagulation in the pathogenesis of aortic stenosis (AS) is unknown. Recently, tissue factor (TF) expression in stenotic aortic valves has been reported in animal model.

OBJECTIVES The aim of the study was to investigate TF expression in valve leaflets obtained from AS patients and to determine its associations with circulating coagulation markers and echocardiographic variables.

PATIENTS AND METHODS We studied 20 patients (10 men, 10 women) with dominant AS (age 62.9 ± 9.6, years, mean gradient 43.62 ± 14.62 mmHg), and 20 well-matched patients with dominant aortic insufficiency (AI) undergoing elective aortic valve replacement. Immunofluorescence was measured on decalcified leaflets using antibodies against human TF and macrophages. Prothrombin fragment 1+2 (F1+2) and circulating TF were determined in plasma prior to surgery.

RESULTS AS valves were characterized by an increased (all, p <0.001) percentage of TF-positive (24.6%) and macrophage-containing (27.3%) areas detected mainly on the aortic side of the leaflets, compared with AI valves (6.3% and 7.4%, respectively). Patients with AS had elevated F1+2 (262.1 ± 27.8 pmol/l, p <0.001) and plasma TF (median 131.8, interquartile range [91.42–310.56] pg/ml, p = 0.018) compared with AI subjects (136.1 ± 11.9 pmol/l, 65.38 [49.51–87.81] pg/ml, respectively). Percentage of TF-positive areas correlated with plasma TF (r = 0.68, p <0.0001), but not with F1+2. Maximum transvalvular gradient >75 mmHg, but not the aortic valve area, showed associations with percentage of TF-positive areas (r = 0.88, p = 0.0039).

CONCLUSIONS This study is the first full-length report demonstrating the presence of TF associated with macrophage infiltration in human aortic valve leaflets in AS patients.

INTRODUCTION Although aortic stenosis (AS) is recognized as the most common valvular heart disease in the elderly, its underlying pathophysiology is still poorly understood. The mechanisms leading to the development of aortic valve lesions were traditionally believed to be associated with age-dependent degeneration with passive calcium accumulation.¹ However, recent studies have demonstrated that valvular degeneration is preceded by endothelial damage, with subsequent inflammatory cell infiltration, including T lymphocytes and macrophages.²⁻³ Mast cells⁴ and proinflammatory cytokines, including interleukin-1β⁵ and tumor necrosis factor-α⁶, have also been identified in both stenotic tricuspid and bicuspid aortic valves⁷. Growing evidence indicates that AS shares clinical and histological similarities with the active pathobiology of atherosclerosis.³⁻⁷ The concept of AS as an atherosclerosis-like process is supported by epidemiologic studies showing that the development of AS is associated with cardiovascular risk factors such as smoking, hypercholesterolemia⁸, male gender⁹, hypertension¹⁰, and age¹¹.

Blood coagulation is implicated in atherosclerosis at every stage of the disease. However, the role of blood coagulation in AS is unknown. Recently, it has been reported that thrombin generation...
The exclusion criteria were: diabetes, autoimmune disorders, acute infection, Valsalva sinus aneurysm, aortic aneurysm, coronary artery stenosis requiring revascularization, cancer, endocarditis, previous cardiac surgery, a history of myocardial infarction, stroke, venous thromboembolism, or bleeding. Patients with tricuspid (n = 35) and bicuspid (n = 5) aortic valves were included. Patients who required additional surgical intervention or had other heart defects were ineligible.

Five aortic valves from age-matched healthy subjects, obtained at autopsy, without morphological AS and AI or other valvular disorders served as negative controls.

Methods Echocardiography Transthoracic echocardiography was performed in each patient prior to surgery using a MargotMac 5000 ultrasound machine. Measurements were performed using conventional techniques in accordance with the European Society of Cardiology guidelines. The aortic valve area (AVA) was calculated using the standard continuity equation. The transvalvular gradient was measured by Doppler echocardiography using the modified Bernoulli equation. Laboratory tests Fasting venous blood was drawn from patients 24 h before surgery. Citrated plasma samples (9:1 of 0.129 M citrate) were centrifuged at 3000 rpm at 20°C for 10 min and stored in aliquots at ~80 °C until analysis. Routine laboratory tests, including lipid profile, glucose, and creatinine, were assayed in serum by routine laboratory techniques. Fibrinogen was measured by the von Clauss method (Dade Behring, Marburg, Germany). High-sensitive C-reactive protein (hsCRP) in serum was determined using an immunoturbidimetric assay (Dade Behring, Marburg, Germany).

Prothrombin F1+2 and circulating TF were determined in citrated plasma using commercially available ELISA assays according to the manufacturer’s instructions (Dade Behring, Marburg, Germany for prothrombin F1+2 and American Diagnostica Inc., Stamford USA for TF).

Tissue sampling and immunohistochemical analysis Diseased aortic valves were collected during surgery for valve replacement. To decalcify the incised aortic valves, they were incubated in 15% EDTA (Sigma Co, St. Louis, MO, USA) at 4°C for 10 days. Decalcification was confirmed by calcium determination in 6 M HCl. After treatment valves were rinsed with phosphate-buffered saline (PBS), embedded in Tissue Tec-OCT compound (methylmethacrylate) (Sakura, Torrance, CA, USA) for tissue cryopreservation and cryosectioned vertically (8–10 µm thick) onto SuperFrost slides (Menzel-Glaser, Germany) by the Leica Jung CM 3000 cryostat. Slides were stored at ~20°C until morphological and immunohistochemical staining.

To ascertain the morphology and the global architecture of the leaflets, in each experiment one hematoxylin-eosin section, taken from a group of sections obtained from each leaflet, was observed under microscope before the other cryosections were subjected to immunocytochemical procedures.

Double-label immunofluorescence was performed using primary, rabbit monoclonal antibody against human TF (1:100) or primary, mouse monoclonal antibody against human macrophage (Mac-3 antigen) (1:100) (all antibodies obtained from Santa Cruz Biotechnology, Inc., CA, USA). After endogenous peroxidase activity blocking with 3% H2O2 in methanol (15 min) at room temperature (RT) and blocking of unspecific background with 3% bovine albumin (Sigma Co, St. Louis, MO, USA) for 30 min at RT, the sections were incubated overnight at 4°C with specific antibodies diluted in blocking serum at appropriate concentrations. After overnight incubation, the sections were washed with PBS and exposed to the corresponding secondary antibodies conjugated with fluorochromes, fluoroisothiocyanate (FITC, green) or phycoerythrin (PE, red) at 1:500 dilution (Santa Cruz Biotechnology, Inc., CA, USA) at RT for 1 h. A negative control (without primary antibody incubation) was routinely
TABLE 1  Patient characteristics

|                        | AS (n = 20) | AI (n = 20) | p   |
|------------------------|------------|------------|-----|
| male, n (%)            | 50/50      | 65/35      | NS  |
| age, years             | 62.9 ± 9.6 | 60.1 ± 7.4 | NS  |
| body mass index (kg/m²)| 28.8 ± 5.97| 31.89 ± 9.8| NS  |
| risk factors           |            |            |     |
| hypertension, n (%)    | 13 (65)    | 9 (45)     | 0.042|
| hypercholesterolemia, n (%) | 7 (35) | 6 (30) | NS  |
| active smoker, n (%)   | 3 (15)     | 3 (15)     | NS  |
| treatment              |            |            |     |
| β-blockers, n (%)      | 14 (70)    | 12 (60)    | NS  |
| acetylsalicylic acid, n (%) | 12 (60) | 4 (20) | 0.007|
| ACEI, n (%)            | 3 (15)     | 4 (20)     | NS  |
| statins, n (%)         | 7 (35)     | 3 (15)     | 0.039|
| echocardiography       |            |            |     |
| maximum gradient, mmHg| 75.9 ± 10.1| 11.22 ± 6.89| 0.001|
| mean gradient, mmHg    | 43.62 ± 14.62 | 5.36 ± 3.79 | 0.001|
| LVEF, %                | 59 ± 9.38  | 45.4 ± 8.13| 0.027|
| calcification of valve, n (%) | 20 (100) | 3 (15) | 0.001|
| calcified area/total area of valve, % | 31.7 ± 13 | 4.5 ± 2 | 0.001|
| aortic bulb diameter, cm | 3.64 ± 0.56 | 4.68 ± 0.78 | NS  |
| ascending aorta diameter, cm | 4.04 ± 0.68 | 4.61 ± 1.09 | NS  |
| aortic bulb diameter/ascending aorta diameter, ratio | 0.91 ± 0.12 | 1.02 ± 0.38 | NS  |
| AVA, cm²               | 0.95 ± 0.4 | –          |     |
| aortal regurgitation    | –          | III/IV     |     |

Data are given as mean ± standard deviation, median, or number (percentage).

Abbreviations: ACEI – angiotensin-converting enzyme inhibitor, AI – aortic insufficiency, AS – aortic stenosis, AVA – aortic valve area, LVEF – left ventricular ejection fraction, NS – nonsignificant

performed. Sections were viewed in a fluorescence microscope (Zeiss, Berlin, Germany). Photomicrographs were taken using a Canon A640 camera. Three images were acquired from a randomly selected location in 9 slides per valve.

Specific stained areas/cells were evaluated using image analysis software, CellProfiler. The percentage of positive stained tissue area/cells (within each layer) was classified as

\[ \frac{\Sigma \text{of immunopositive-stained areas/cells}}{\text{total tissue area/total number of cells}} \]

Statistical analysis  Data were analyzed using Statsoft 7.1 PL package (StatSoft, Inc., 2005). Values are expressed as mean ± standard deviation or median or otherwise stated. Kolmogorov-Smirnov test was used to assess conformity with a normal distribution. Pair-wise comparisons were made using the Tukey’s test for continuous variables and the χ² test for proportions. The Mann-Whitney U test was used to compare non-normally distributed variables between two groups. Spearman correlation coefficient was calculated to evaluate associations between the values. p <0.05 was considered statistically significant.

RESULTS  Patient groups  AS and AI patients did not differ with regard to age, sex, and body mass index. Clinical and echocardiographic data for patients are summarized in Table 1. Valvular stenosis in AS patients was predominantly moderate-to-severe with maximal transvalvular gradient of 75.9 ±10.1 mmHg and mean gradient of 43.62 ±14.62 mmHg. Patients with AS exhibited higher incidence of arterial hypertension (p = 0.042) and received acetylsalicylic acid (p = 0.075) and statins (p = 0.04) more frequently than those from the AI group. There were no differences in the frequency of hypercholesterolemia between the AS and AI groups (Table 1).

Laboratory parameters  Patients from the AI and AS groups did not differ with regard to laboratory parameters such as glucose, creatinine, lipid profile, and fibrinogen, while hsCRP levels were higher in patients with AS than in those with AI (p <0.0001) (Table 2). Elevated plasma F1+2 (p = 0.004) and TF (p = 0.001) were observed in AS patients compared with AI subjects (Table 2).

There were no correlations of these parameters with any of the examined echocardiographic variables (data not shown).

Aortic valve tissue samples  AS cusps showed marked thickening and massive calcific deposits (Figure 1A). AI valves demonstrated soft and thin cusps with no calcifications (Figure 1B). By hematoxylin-eosin staining, control and AI valves were characterized with a preserved layered structure and homogenous extracellular matrix (data not shown). In the leaflets from control aortic valves no TF and macrophages were observed (data not shown). TF and macrophages were found predominately on the aortic side of AS valve leaflets, mainly in the lesion area, involving both subendothelial region and the adjacent fibrosa. Representative images of AS and AI cusps are shown in Figure 1C-F. Specifically, compared with AI, AS valves were characterized by an increased (all, p < 0.001) percentage of TF-positive (24.6 ± 6.93%) areas of the leaflet and macrophages infiltrating the aortic valve tissue (27.3 ± 7.56%). TF was expressed mainly, but not exclusively, at the sites of macrophage infiltrations (>50% TF-positive areas) (Figure 1C, 1E). Macrophages were noted in 100% of the valve leaflets with AS. The pattern of macrophage accumulation varied from aggregates of a few to a substantial number of cells. They were located near the surface of the lesion (data not shown). In contrast, areas positive for TF (6.3 ± 1.17%) and macrophages (7.4 ± 2.85%) were observed in 65% of the valves from all patients. All antigens could be detected on both aortic and ventricular sides, mainly in the outer layer (Figure 1D, 1F). Quantitative immunohistochemical data for these antigens are presented in Figure 2.
In AS valves we found a positive correlation between TF-positive areas and the percentage of area infiltrated with macrophages within the AS valve leaflets ($r = 0.59$, $p = 0.0014$). No such correlation was observed for AI valves ($r = 0.62$, $p = 0.28$).

There were no age-, gender-, or smoking-related differences in immunohistochemical data.

None of immunohistochemical parameters were affected by statin therapy.

Interestingly, in patients with AS, but not those with AI, increased percentage of TF-positive areas correlated with plasma TF ($r = 0.68$, $p = 0.0001$) and low-density lipoprotein (LDL) cholesterol ($r = 0.62$, $p = 0.0034$) levels, but not with prothrombin $F_1+2$ ($r = 0.07$, $p = 0.88$). There were no associations between TF-positive areas in AS leaflets and circulating CRP levels. However, in AS patients with hsCRP $>3$ mg/l ($n = 12$) we found positive correlations of CRP with TF-positive areas ($r = 0.52$, $p = 0.016$) and plasma TF ($r = 0.59$, $p = 0.007$). Moreover, AS patients with hsCRP $>3$ mg/l had an elevated percentage of TF-positive areas within the valve, $F_1+2$ and TF compared with the remainder (all, $p < 0.05$) (TABLE 3).

In the AS group, there were no associations between mean transvalvular gradient and valvular TF expression as well as circulating TF levels (data not shown). However, in AS patients with maximal transvalvular gradient $>75$ mmHg ($n = 11$), there were significant associations between percentage of TF-positive areas and both maximal ($r = 0.88$, $p = 0.0039$) and mean gradients ($r = 0.71$, $p = 0.0064$) (FIGURE 3), but not with ejection fraction ($r = 0.18$, $p = 0.17$) or aortic valve area ($r = 0.16$, $p = 0.65$). In AI patients TF-positive areas were not associated with any of the examined echocardiographic parameters or circulating coagulation markers (data not shown).

**DISCUSSION**

To our knowledge, the current study is the first full-length report demonstrating that TF associated with macrophages is present
We found significant associations between percentage of TF-positive areas with maximal and mean gradients, which indicates that hemodynamics of this valvular defects predisposes to TF expression within the diseased leaflets.

It has been also reported that thrombin generation, circulating TF, and platelet activation are enhanced in patients with large thoracic ascending aortic aneurysm (with maximal aortic diameter >45 mm) compared with patients with limited dilatation and controls.

Therefore, we excluded patients with aortic aneurysm and analyzed patients with a similar aortic bulb diameter, which abolished a potential confounding impact of dilated ascending aorta.

Importantly, we hypothesized that TF expression is partly associated with hemodynamic stress, reflected by increased transvalvular gradient in AS valves of patients, which is consistent with recent findings in animal models and supports the concept of similar pathogenesis of AS and atherosclerosis.

Stenotic lesions develop at the basal aortic side of the leaflet, a region exposed to disturbed flow and low shear stress that increase permeability to macromolecules such as LDL, accumulating in subendothelial matrix, thereby leading to recruitment of monocytes into the leaflet, which represents the initial step of the inflammatory response. Inflammation, with an accumulation of monocytes/macrophages, may also contribute to thrombosis by stimulating TF expression. In our study, there were positive correlations between TF-positive areas and LDL cholesterol levels, which supports the hypothesis that lipid infiltration contributes to the development of AS.

TF has several properties that might contribute to the development of AS. It plays a major role in monocyte migration, contributes to the thrombogenicity of vascular lesions, and may participate in the formation of microthrombi. Stein et al. have demonstrated organized microthrombi associated with fibrin deposits at the aortic side of calcified and stenotic valves. They hypothesized that thrombus formation may be associated with TF induction when the valve is exposed to additional stimuli, such as disturbed flow or low shear stress. As previously reported in animal models, TF expression can be predominantly detected at the aortic side of the leaflets. The percentage of TF-positive stained area in the animal model was similar to that observed in the current study (20 ±3% vs. 24.6 ±6.93%, respectively) and TF immunostaining was associated with myofibroblasts and with macrophages.

Our observation is consistent with previous reports that macrophage infiltration plays a critical role in TF synthesis. We have shown, using double-labeled immunofluorescence, that TF is expressed mainly at the sites of macrophage infiltrations. TF expression outside the areas rich in macrophages might be associated with myofibroblasts. Indeed, myofibroblast activation in AS has been observed in human and rabbit valves. Moreover, it has been shown that TF expression is associated with mineralized and calcified areas of the valve. In turn, valvular calcification and mineralization lead to increased transvalvular gradients. Importantly, in AS patients with maximal transvalvular gradient >75 mmHg we found significant associations between percentage of TF-positive areas with maximal and mean gradients, which indicates that hemodynamics of this valvular defects predisposes to TF expression within the diseased leaflets.

It has been also reported that thrombin generation, circulating TF, and platelet activation are enhanced in patients with large thoracic ascending aortic aneurysm (with maximal aortic diameter >45 mm) compared with patients with limited dilatation and controls. Therefore, we excluded patients with aortic aneurysm and analyzed patients with a similar aortic bulb diameter, which abolished a potential confounding impact of dilated ascending aorta.

Importantly, we hypothesized that TF expression is partly associated with hemodynamic stress, reflected by increased transvalvular gradient.

| TABLE 3 Immunohistochemical and coagulation markers in AS patients with hsCRP levels >3 or ≤3 mg/l |
|---------------------------------|------------------|------------------|------------------|------------------|
| hsCRP ≤3 (n = 8) | hsCRP >3 (n = 12) | p      |
| TF-positive area (%) | 20.28 ±1.95 | 25.73 ±3.93 | 0.041 |
| prothrombin fragment 1+2 (pmol/l) | 162.24 ±50.89 | 270.97 ±35.25 | 0.019 |
| plasma TF (pg/ml) | 110.67 ±19 | 200.04 ±37.3 | 0.012 |

Abbreviations: see TABLE 1 and TABLE 2

![FIGURE 2 Quantitative analysis of immunostained tissue factor (TF) and macrophages (MF) in patients with aortic insufficiency (AI) and aortic stenosis (AS). Each bar represents the mean ± standard deviation. * p <0.001.](image)

![FIGURE 3 Correlation between transvalvular maximal gradient (top) and mean gradient (bottom) and tissue factor (TF)-positive area (measured as percentage of positive areas within leaflets) within stenotic valves](image)
in AS patients. The association in subjects with the highest gradient supports this concept. TF abundant in AS valves should be probably perceived as a marker of advanced calcific AS. Further studies, based on a detailed evaluation of the valves with a range of disease severity, are needed to define cellular and molecular factors involved in valve degeneration and its associations with circulating coagulation markers.

Of note is also the fact that in the current study statin therapy did not affect TF expression in the valve tissue and plasma. It may be caused by a low number of patients receiving such therapy (n = 7). However, recent findings in animal models, which demonstrated that rosuvastatin decreased TF expression only in the first sections of the ascending aorta, but not in the sections from aortic valves, suggest negligible modulation of TF in AS valves.

The study has several limitations. First, the number of the patients enrolled in this preliminary study was small. However, both groups were meticulously matched and subjects with likely confounders, e.g., diabetes mellitus, were ruled out. Second, expression of TF was determined from a semiquantitative analysis system, thus potentially making the estimation less precise. A laser scanning confocal microscope with a software able to determine the fluorescence density would be the best technique to evaluate the exact immunopositive areas. However, we believe that a large amount of images analyzed per one valve provide reliable data, convincingly documenting the presence of TF in stenotic aortic valves in humans. Third, the present study is a cross-sectional study of the valves obtained from patients scheduled for surgery. The lesions are likely to be representative of the same stage of the disease, i.e., advanced, and the extent of TF expression in the AS valves cannot be possibly extrapolated on subjects with less severe AS. Fourth, in our study we did not use RT-PCR analysis, because we have focused on native protein presence not on gene expression on the mRNA level. Moreover, immunohistochemistry analysis enables detection of two or more co-localized antigens.

In conclusion, to our knowledge, we are the first to have shown that TF expression associated with macrophages infiltration are present in human AS valves. These findings suggest that blood coagulation contributes to the development of AS in a similar manner to that observed in atherosclerotic plaques. The results suggest that a better understanding of the associations between valve biology and circulating factors may help to prevent or slow down the disease progress in individuals with early valve leaflet changes.

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ARTYKUŁ ORYGINALNY

Obecność czynnika tkankowego w płatkach zastawek aortalnych u pacjentów ze stenozą aortalną

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SŁOWA KLUCZOWE

czynnik tkankowy, gradient przezastawkowy, stenoza aortalna

STRESZCZENIE

WPROWADZENIE Rola układu krzepnięcia w patogenezie stenozy aortalnej (aortic stenosis – AS) jest niewyjaśniona. Ostatnio opisano na modelu zwierzęcym obecność czynnika tkankowego (tissue factor – TF) w zwężonych zastawkach aorty.

CELE Badanie ekspresji TF w płatkach zastawek aortalnych pobranych od pacjentów z AS oraz ustalenie jej związków z krążącymi markerami krzepnięcia oraz parametrami echokardiograficznymi.

PACJENCI I METODY Badaniu poddano 20 pacjentów (10 mężczyzn i 10 kobiet) z dominującą AS (wiek 62,9 ± 9,6 lata; średni gradient 43,62 ± 14,62 mm Hg) i 20 odpowiednio dobranych pacjentów z dominującą niedomykalnością zastawki aortalnej (aortic insufficiency – AI), poddanych planowanej operacji wymiany zastawki. Immunofluorescencję mierzono na odwapnionych skrawkach płatków używając ciał przeciw ludzkiemu TF i makrofagom. Fragment protrombiny 1+2 (F1+2) i krążący TF oznaczano w osoczu przed zabiegiem.

WYNIKI Zastawki pacjentów z AS charakteryzowały się istotnie statystycznie zwiększonym (dla wszystkich porównań, p < 0,001) odsetkiem obszarów TF- pozytywnych (24,6%) i zawierających makrofagi (27,3%), występujących głównie po aortalnej stronie płatków, w porównaniu z zastawkami pacjentów z AI (odpowiednio 6,3% i 7,4%). U pacjentów z AS obserwowano zwiększy poziom poziom F1+2 (262,1 ± 27,8 pmol/l; p < 0,001) i TF (mediana [przedział międzykwartylowy] 131,8 [91,42–310,56] pg/ml, p = 0,018) w porównaniu z pacjentami z AI (odpowiednio 136,1 ± 11,9 pmol/l i 65,38 [49,51–87,81] pg/ml). Odsetek obszarów TF-pozytywnych korelował z poziomem TF w osoczu (r = 0,68; p = 0,0001), ale nie ze stężeniem F1+2. Maksymalny gradient przezzastawkowy >75 mm Hg, lecz nie powierzchnia zastawki aortalnej, korelował z powierzchnią wykazującą ekspresję TF (r = 0,68; p = 0,0039) i r = 0,711; p = 0,0064).

WNIOSKI To badanie jest pierwszym doniesieniem naukowym przedstawiającym obecność TF związanego z naciekami makrofagów w płatkach ludzkich zastawek aorty u chorych z AS.

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