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Activated factor XI and tissue factor in aortic stenosis: Links with thrombin generation

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Abstract

Introduction—In our previous studies we showed that a significant proportion of patients with various cardiovascular diseases have active tissue factor (TF) and factor (F)XIa in their plasma.

Objective—To evaluate these two proteins in plasma from patients with aortic stenosis (AS) and established their relationship with the severity of the disease.

Methods—Fifty-four consecutive patients with AS, including 38 (70.4%) severe AS patients, were studied. Plasma FXIa and TF activity were determined in clotting assays by measuring the response to inhibitory monoclonal antibodies.

Results—TF activity was detectable in plasma from 14 of 54 patients (25.9%), including 13 of 38 with severe AS (34.2%) and 1 of 16 (6.25%) with moderate AS ($p=0.052$). FXIa activity was found in 12 (22.2%) patients, mostly in individuals with severe AS (11 of 38, 28.9%, $p=0.067$). All 12 patients with circulating FXIa had active TF in their plasma as well. Severe AS patients with detectable TF had higher maximal (111 ± 20 vs 97 ± 16 mm Hg, $p=0.02$) and mean (61 ± 12 vs 53 ± 8 mm Hg, $p=0.02$) transvalvular gradient, compared with those without such activity in plasma. In severe AS patients with detectable active TF, prothrombin fragment 1.2, a thrombin generation marker, was higher than in patients without TF (375 ± 122 vs. 207 ± 64 pM, $p<0.001$).

Conclusions—Detectable FXIa and TF activity was observed for the first time in AS patients, primarily in severe ones. This activity correlates with thrombin generation in those patients.

Keywords

Aortic stenosis; factor XIa; tissue factor; prothrombin fragment 1.2

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Conflict of interest statement

None declared.

Introduction

Aortic stenosis (AS) is nowadays the most common native valve disease occurring primarily among elderly patients [1]. It has been shown that moderate-to-severe AS is associated with disturbances in blood coagulation, both bleeding, which is mostly caused by acquired von Willebrand disease [2], and less frequently, thrombosis [3,4]. Data about a hypercoagulable state in AS are sparse. It has been demonstrated that patients with obstructed left ventricular outflow tract due to hypertrophic cardiomyopathy and AS, platelet activation and thrombin generation markers are higher and are positively correlated with transvalvular pressure gradient [5,6].

Tissue factor (TF) is a key initiator of blood coagulation *in vivo*. TF is continuously expressed on subendothelial cells and upon vascular injury forms a complex with circulating factor (F)VIIa initiating blood coagulation [7]. There have been reports that strongly indicate the importance of TF in the pathogenesis of atherosclerosis and its relation with cardiovascular risk factors. Increased TF levels in circulating blood have been observed in patients with diabetes and hypertension [8]. In early stages of atherogenesis, increased expression of TF is detectable on monocytes, in foam and smooth muscle and endothelial cells [8].

FXIa is a serine protease which activates FIX to FIXa leading to the intrinsic FXase (FIXa/FVIIIa/Ca⁺⁺/membrane) formation and to ultimate amplification of thrombin generation. Under physiologic conditions, a key activator of FXI is thrombin [9]. A similar mechanism of FXI activation was suggested for patients suffering from coronary artery disease (CAD) and chronic obstructive pulmonary disease [10–12].

In 2008 we reported that in plasma samples from patients with acute coronary syndromes (ACS) and less commonly in those with stable CAD, FXIa and TF can be detected, while in plasma of healthy individuals, active TF and FXIa are not observed [11]. There are many similarities between the pathogenesis of aortic sclerosis/stenosis and atherosclerosis [13,14]. It has been established that in AS patients there are atherosclerosis-like lesions in the aortic leaflets, including macrophage accumulation [14]. Additionally, risk factors for AS and atherosclerosis are similar [14–20].

The objective of this study was to test the hypothesis that active TF and FXIa can be detected in a fraction of patients with AS and that both factors, together with thrombin generation markers, are linked with the severity of the heart defect.

Patients and Methods

Patients

Fifty-four consecutive adult patients with calcific AS were enrolled. The Jagiellonian University Ethical Committee approved the study, and patients provided written, informed consent. The inclusion criterion was a mean transvalvular pressure gradient higher than 20 mm Hg measured in transthoracic echocardiography. Patients with atrial fibrillation or taking oral anticoagulants, thienopyridines and heparins were excluded. Other exclusion criteria were: impaired left ventricular (LV) systolic function (LV ejection fraction <50%), more than mild concomitant valvular disease, rheumatic AS, history of endocarditis, aortic aneurysm, acute cardiovascular event within the previous 6 months, acute illness (symptoms and/or C-reactive protein >10 mg/l), more than stage 2 of chronic kidney disease, hepatic insufficiency, severe pulmonary disease and cancer. Clinical characteristics of the study population are presented in Table 1.

Echocardiography

Transthoracic echocardiographic examinations were performed with the use of the Toshiba Aplio SSA-770A echocardiograph (Netherlands). Images were stored digitally and analyzed offline by an experienced echocardiographer. Measurements were made in conventional parasternal, four- and five-chamber apical views using M-mode two-dimensional Color Doppler, pulsed-wave Doppler and continuous-wave Doppler according to the European Society of Echocardiography guidelines. The transvalvular gradient and peak velocity were measured by Doppler echocardiography with the use of the modified Bernoulli equation. Aortic valve opening area (AVA) was calculated using the standard continuity equation [21]. LV volume and ejection fraction were measured by the modified Simpson method [22]. LV mass, left atrial volume and AVA were indexed to body surface area. AS was considered as moderate if the mean transvalvular pressure gradient was 20–40 mm Hg and severe if the mean transvalvular pressure gradient was higher than 40 mm Hg [23].

Laboratory methods

Blood samples were obtained from patients with AS between 8–10 a.m. on the day of echocardiographic examination after an overnight fasting, with minimal stasis applied during blood draw. Blood cell counts, biochemical analysis, including lipid profile, creatinine, glucose, were assessed by standard automated laboratory methods. Venous blood samples for clotting assays were taken into 0.13 M trisodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged for 20 minutes within 10 min of collection at 24 °C and 2500g. Platelet-poor plasma was immediately frozen and stored at –80 °C. Fibrinogen and C-reactive protein were assayed by immunonephelometry (Dade Behring, Marburg, Germany). To assess thrombin generation in circulating blood, prothrombin fragment 1.2 (F1.2) in citrate plasma was measured using an immunoenzymatic assay (Dade Behring). All intra-assay and inter-assay coefficients of variation were <7%.

Plasma clotting assays

Plasma clotting assays were performed as described previously [11]. Briefly, plasma was thawed at 37 °C in the presence of corn trypsin inhibitor (prepared as previously described [24]; blocks the contact pathway of blood coagulation by inhibiting FXI activation by FXIIa) and either buffer or inhibitory monoclonal anti-FXI (α FXI-2) or anti-TF (α TF-5) antibody (both produced in house) at a final 0.1mg/ml concentration. CaCl_2 at a final 15 mM concentration was added and clotting was initiated by the addition of 2 μ M phospholipid vesicles (prepared as previously described [24]) composed of 25% dioleoyl-*sn*-glycero-3-phospho-L-serine and 75% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (both from Avanti Polar Lipids, Inc; Alabaster, AL). Clotting times were determined using the ST8 clotting instrument (Diagnostica Stago, Parsippany, NJ). FXIa and TF concentration in plasma was calculated from calibration curves built by sequential dilutions of human FXIa (a gift from Dr. R. Jenny, Haematologic Technologies, Inc., Essex Junction, VT) or relipidated TF_{1–242} (a gift from Dr. R. Lundblad, Baxter Healthcare Corp., Duarte, CA) in pooled 10-donor plasma. Laboratory personnel were “blinded” to the status of samples. Age- and sex-matched healthy control individuals (n=12) recruited from the hospital staff showed no detectable TF or FXIa. Quantification limits of FXIa and TF activity assays were 10 and 0.4 pM, respectively.

Statistical analysis

Data are presented as means $\pm$ standard deviation (SD) for continuous variables or number and percentage for categorical variables. The Kolmogorov-Smirnov test was used to determine normal distribution. Student *t* test, Mann-Whitney U test, and χ^2 test were used to

compare differences between groups. Linear regression analysis was performed to correlate the variables. A value of p below 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 54 patients (37 men and 17 women, aged 43–83 years) were studied. Sixteen had moderate AS and 38 had severe AS. The groups did not differ significantly with regard to age, gender, body mass index, the frequency of CAD, hypertension, diabetes and smoking habit (Table 1). Lipid profile, creatinine, and platelet count were comparable between the moderate and severe AS groups. The maximal and mean transvalvular pressure gradients ranged from 39 to 147 mm Hg and from 20 to 84 mm Hg, respectively. All patients had preserved LV function with a mean LV ejection fraction of 62.4%. Six patients in the moderate AS group were diagnosed with LV hypertrophy, defined as LV mass index $>110\text{g}/\text{m}^2$ for female or $>134\text{g}/\text{m}^2$ for male. Sixteen patients (42%) in the severe AS group had this condition. The remaining echocardiographic parameters were similar for both groups (Table 2).

Clotting variables

In severe AS patients, F1.2 was elevated compared with moderate AS ($p=0.006$). There was a positive correlation between F1.2 and both maximal ($r=0.71$; $p<0.001$, Figure 1a) and mean ($r=0.68$; $p<0.001$, Figure 1b) transvalvular gradients, but not AVA ($r=-0.17$; $p=0.23$, Figure 1c) or AVA index ($r=-0.16$, $p=0.26$, Figure 1d).

Active TF was detectable in plasma from 14 patients (25.9%), i.e. in one of 16 patients with moderate AS (6.25%) and in 13 of 38 patients (34.2%) with severe AS. In most (9 of 14 patients; 64.3%) TF activity was low, below 0.4 pM. Five patients, all from the severe AS group, had higher active TF levels up to 3.4 pM.

When the severe AS patients were divided in two subgroups based on the presence or absence of TF activity, in the TF(+) subgroup the mean F1.2 concentration was significantly higher (375 ± 122 vs 207 ± 64 pM, $p<0.001$) (Figure 2). Severe patients with detectable TF had higher maximal (111 ± 20 vs 97 ± 16 mm Hg, $p=0.02$) and mean (61 ± 12 vs 53 ± 8 mm Hg, $p=0.02$) transvalvular gradient, compared with those without such activity in plasma. Interestingly, the TF(+) subgroup was younger than the TF(−) subgroup (58 ± 10 vs 66 ± 11 , $p=0.03$).

Circulating FXIa was detected in 12 patients with AS (21.8%), including 11 patients with severe AS (28.9%). Concentrations reached as high as 105 pM. Importantly, all 12 of the patients with FXIa had active TF as well. The patients with FXIa present in their plasma had higher transvalvular gradient that corresponded with the severity of AS, however there was no correlation between the plasma level of FXIa and maximal transvalvular gradient ($r=0.32$, $p=0.34$), mean transvalvular gradient ($r=0.12$, $p=0.72$), AVA ($r=-0.23$, $p=0.49$), or AVA index ($r=-0.21$, $p=0.53$).

Discussion

This study is the first to demonstrate quantifiable active FXIa, not inhibited by plasma serpins, circulating in AS patient blood and accompanied by procoagulant TF activity. Previously, circulating FXIa was detected in 96% of patients with acute myocardial infarction (MI) and 76% of subjects with a history of previous MI. TF activity was observed in 38% of acute MI patients and only 7% of stable CAD patients with previous MI [11]. In the current study, the activity of circulating TF in the AS patients was similar to that

observed in acute coronary ischemia, whereas FXIa levels were only slightly lower compared to those observed in stable subjects following AMI. The presence of FXIa and TF activity in both advanced AS and CAD provides additional evidence for the similarities in the pathogenesis of both common diseases.

The current study corroborates the previous observation showing enhanced thrombin generation reflected by increased plasma F1.2 in AS patients [5]. We have also observed that in AS patient population F1.2 is positively correlated with transvalvular pressure gradient. The lack of significant correlations between AVA and thrombin generation markers (despite their higher levels in patients with TF and XIa) is unexpected. It appears that an increase in thrombin generation observed in AS like in hypertrophic cardiomyopathy [6] is a gradient related phenomenon alone. The association between the levels of the thrombin marker and FXIa suggests that most likely thrombin accounts for FXI activation in severe AS [11]. Moreover, higher concentrations of F1.2 in patients with detectable activity of TF in plasma might indicate that TF is associated with enhanced thrombin generation occurring in AS.

The significance of circulating active TF in plasma of AS patients remains obscure. Marechaux et al. in the experimental study on animals found that lesions on aortic valve leaflets display TF expression [25]. Natorska et al. [26] confirmed the presence of TF in aortic valves, predominately on the aortic side, examined after aortic valve replacement in patients with severe AS. The percentage of the area showing TF expression correlated with plasma TF measured using a commercially available immunoenzymatic assay. Very recently, the presence of TF in human stenotic valve leaflets close to calcification regions has also been shown by Breyne et al. [27]. In the present study, we observed that in AS patients with high transvalvular pressure gradient, there is an increased probability of procoagulant activity of TF in circulating blood. It remains to be established whether this activity is associated with TF expression within the aortic valves.

The biological effect of increased thrombogenicity in AS patients is not clear. Despite enhanced activity of TF and FXIa and elevated platelet activation and thrombin generation markers, clinically evident thromboembolic events are infrequent. The explanation for this apparent discrepancy could be related to the compensation of increased thrombogenicity by impaired platelet function caused by the loss of largest multimers of the von Willebrand factor, which can lead to bleeding tendency observed in some patients with moderate-to-severe AS [28]. However, it might be speculated that a subset of AS patients positive for circulating TF and/or FXIa are at an increased risk of thrombotic manifestations. A larger study with a long follow-up would be needed to address this issue.

It should be emphasized that in stable AS patients detectable amounts of active FXIa are permanently present in plasma despite the abundance of numerous inhibitors for serine proteases because none of the physiologic inhibitors can effectively inhibit FXIa [29]. As a consequence, 80 to 90% of FXIa can survive in blood for the 30 minutes or more required for citrate plasma preparation [11]. The presence of FXIa cannot be attributed to *de novo* FXIa generation observed in tubes containing citrate plasma at room temperature over a period of 75 min either in the presence or absence of contact pathway inhibition [11]. These data indicate that plasma FXIa in the AS patients does indeed reflect the *in vivo* levels of this enzyme.

According to European Society of Cardiology guidelines, aortic valve replacement is recommended in severe AS either when symptoms occur (spontaneously or in exercise test) or when there is impairment of left ventricular systolic function (ejection fraction <50%) [30]. Management of asymptomatic AS patients with preserved left ventricular (LV)

function requires weighing the benefits and the risk associated with surgery. There are factors that indicate early surgical treatment, such as excessive LV hypertrophy, fast peak velocity progression and massive valve calcification [30]. It remains to be established in a larger prospective cohort study whether FXIa and TF activities and their association with thrombin generation could be useful for the prognosis of AS and might represent a new indication for earlier surgery.

The study has several limitations. First, the number of patients studied was limited. Second, our analysis was based on the determination of each variable at a single time point. It is likely that with time, TF and FXIa would change (most likely increase) in AS patients. Third, the impact of asymptomatic CAD cannot be excluded. However, in the current study a small subset of AS patients who had symptomatic advanced CAD did not differ from patients without it with respect to FXIa and TF activity. Fourth, patients with mild AS were not included since our previous observations indicate that such subjects had TAT and F1.2 levels within the normal range (data not shown). Finally, our hypothesis-generating study needs to be validated because the clinical significance of our findings is unclear. It might be speculated that TF and/or FXIa increase the risk of thromboembolic events and contributes to the lesion growth within aortic valve leaflets.

In conclusion, FXIa and TF activity associated with thrombin generation was observed in a fraction of patients with AS, almost exceptionally in those with a severe manifestation of the disease. This observation suggests that quantitation of these two proteins could have certain potential for the stratification of severe and moderate AS patients. However, further prospective studies using a larger patient population are needed to evaluate clinical outcomes relevant to the presence or absence of these factors and related thrombin formation.

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1a. Correlations between maximal transvalvular pressure gradient and F(1.2) concentration ($r=0.72$, $p<0.001$)

1b. Correlations between mean transvalvular pressure gradient and F(1.2) concentration ($r=0.68$, $p<0.001$)

1c. Correlations between AVA and F(1.2) concentration ($r=-0.17$, $p=0.23$)

1d. Correlations between AVA index and F(1.2) concentration ($r=-0.16$, $p=0.26$)

Fig. 1. Correlations between transvalvular pressure gradient and F1.2 concentration. A) Maximal pressure; B) Mean pressure; C) Aortic valve opening area (AVA); D) AVA index.

Fig. 2.
Box plot of F1.2 concentrations in the severe AS group segregated by the presence or absence of TF activity.

Table 1

Patient characteristics

Parameter	All (n=54)	Moderate AS (n=16)	Severe AS (n = 38)	p
Age, years	64.6 ± 11.3	67.7 ± 10.3	63.3 ± 11.5	ns
Male, n(%)	37 (68%)	13 (88%)	24 (63%)	ns
BMI, kg/m ²	27.2 ± 3.9	26.3 ± 3.6	27.6 ± 4.0	ns
Hypertension, n(%)	34 (63%)	12 (75%)	22 (58%)	ns
Diabetes, n(%)	11 (20%)	5 (31%)	6 (16%)	ns
CAD, n(%)	15 (28%)	5 (31%)	10 (26%)	ns
Current smoking, n(%)	25 (46%)	5 (31%)	20 (53%)	ns
Glucose, mM	5.9 ± 2.2	6.4 ± 3.5	5.7 ± 1.4	ns
Total cholesterol, mM	4.9 ± 1.0	4.6 ± 1.0	5.0 ± 1.0	ns
HDL cholesterol, mM	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	ns
LDL cholesterol, mM	3.0 ± 1.0	3.0 ± 1.1	3.0 ± 1.0	ns
Triglycerides, mM	1.5 ± 0.7	1.6 ± 0.9	1.4 ± 0.6	ns
Creatinine, μM	88.8 ± 21.8	90.1 ± 17.9	88.2 ± 22.4	ns
Platelets, 10 ³ /μl	197.5 ± 49.2	209.4 ± 34.8	192.0 ± 54.1	ns
C-reactive protein, mg/l	4.5 ± 5.8	4.1 ± 3.7	4.7 ± 6.5	ns
F1.2, pM	237.8 ± 113.5	174.3 ± 69.6	264.6 ± 118.3	<0.001

Data are given as mean ± SD or n (%).

Abbreviations: BMI, body mass index, CAD, coronary artery disease.

Table 2

Echocardiographic parameters

	All (n=54)	Moderate AS (n=16)	Severe AS (n=38)	
Gradient max., mm Hg	89.9 ± 25.0	61.5 ± 11.1	101.9 ± 22.2	<0.001
Gradient mean, mm Hg	48.7 ± 14.5	31.5 ± 5.7	56.0 ± 7.8	<0.001
LVEDV, ml	155.4 ± 50.2	156.5 ± 59	155.0 ± 47.2	ns
LVESV, ml	62.4 ± 38.3	65.6 ± 37.2	61.1 ± 39.1	ns
LAV, ml	58.0 ± 30.4	60.6 ± 28.7	57.0 ± 31.4	ns
LAVI, ml/m ²	31.2 ± 15.7	32.2 ± 13.9	30.8 ± 16.5	ns
LVMI, g/m ²	119.4 ± 25.2	110.9 ± 21.7	123.0 ± 25.9	ns
EF, %	62.6 ± 11.1	62.3 ± 9.4	62.3 ± 11.9	ns
Aorta diameter, mm	37.4 ± 4.9	39.2 ± 5.9	36.7 ± 4.3	ns

Abbreviations: LVEDV- left ventricle end-diastolic volume, LVESV- left ventricle end-systolic volume, LAV- left atrial volume, LAVI- left atrial volume index, LVMI- left ventricle mass index, EF - ejection fraction.

Table 3

Characteristics of the severe AS patients divided in two subgroups based on the presence of [TF(+)] or absence [TF(-)] of active TF

	TF(-) (n=25)	TF(+) (n=13)	p
Age, years	66.1 ± 11.4	57.9 ± 10.1	0.03
Gender- males, n(%)	16 (64%)	8 (62%)	ns
BMI, kg/m ²	27.7 ± 4.2	27.5 ± 3.8	ns
Hypertension, n(%)	16 (64%)	6 (46%)	ns
CAD, n(%)	8 (32%)	3 (23%)	ns
Diabetes, n(%)	5 (20%)	1 (8%)	ns
Current smoking, n(%)	12 (48%)	8 (62%)	ns
Glucose, mM	5.7 ± 1.6	5.6 ± 1.0	ns
Creatinine, mM	93.7 ± 23.4	77.8 ± 20.4	ns
Platelets, 10 ³ /μl	210 ± 52	182 ± 54	ns
Total cholesterol, mM	4.9 ± 1.0	5.2 ± 1.1	ns
HDL cholesterol, mM	1.4 ± 0.3	1.4 ± 0.4	ns
LDL cholesterol, mM	2.8 ± 0.9	3.5 ± 1.2	ns
Triglycerides, mM	1.4 ± 0.6	1.6 ± 0.6	ns
C-reactive protein, mg/l	5.2 ± 7.7	3.8 ± 3.0	ns
Gradient max., mm Hg	97 ± 16	111 ± 20	0.02
Gradient mean, mm Hg	53 ± 8	61 ± 12	0.02
LAV, ml	63.0 ± 35.5	45.5 ± 17.2	ns
LAVI, ml/m ²	33.9 ± 18.7	24.7 ± 9.1	ns
LVMI/m ²	119.5 ± 24.3	129.7 ± 28.6	ns
FXIa, pM	---	47.5 ± 33.8	
FXIa, n(%)	0 (0%)	11 (85%)	
EF, %	60 ± 12	69 ± 9	ns
Aorta diameter, mm	35.3 ± 2.9	39.4 ± 5.4	ns
F1.2, pM	207 ± 64	375 ± 122	<0.001

Abbreviations: see table 1 and 2.