INTRODUCTION

Accumulating evidence supports the hypothesis that calcific aortic stenosis, conventionally viewed as a “degenerative” process might be based on an active inflammatory process (1, 2, 3). The sclerotic process in aortic stenosis presents many similarities with atherosclerosis, including the presence of activated leukocytes in stenotic aortic valves (3). Moreover, an association between elevated inflammatory markers and adverse cardiovascular outcomes has been demonstrated in patients with aortic sclerosis, suggesting that inflammation could be a common pathophysiologic mechanism in both aortic sclerosis and atherosclerosis (4).

For a better definition of “vulnerable patient”, several markers of systemic inflammation were proposed as parameters that could predict the risk of future cardiovascular events (5).

In the current study, we applied this approach to patients with aortic sclerosis (AS). We examined the serum levels of several inflammatory mediators - pro- and anti-inflammatory cytokines, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase-1 (TIMP-1) and soluble intercellular adhesion molecule-1 (sICAM) in patients with aortic sclerosis. The aim of the study was to see if elevated concentrations of systemic inflammatory markers might be relevant in relation with the progression of aortic valve calcification and also with the occurrence of acute coronary events.

MATERIALS AND METHODS

Study population

Fifty-one patients with aortic sclerosis (AS) were consecutively registered and recruited at the Department of Cardiology, the Institute of Cardiovascular Diseases „C.C. Iliescu”, Bucharest for the present study. The baseline characteristics are presented in Table 1.

ABSTRACT

The aim of the study was to evaluate several mediators of inflammation in patients with aortic sclerosis in relation to severity of cardiovascular disease. Serum level of cytokines, soluble intracellular adhesion molecule 1, matrix metalloproteinase (MMP) 2 and 9 and their tissue inhibitor TIMP-1, were measured by ELISA and MMPs activity by zymography in 51 aortic sclerosis patients. The increase in MMPs expression positively correlated with their gelatinase activity; also there was a positive correlation between MMP-9 and TIMP-1 serum levels. Moreover, IL-6 concentration positively correlated with both serum level and activity of MMP-9. The level of IL-6 and IL-1Ra were higher in patients with a great burden of atherosclerosis. Noteworthy, statistically significant higher levels of IL-6 were noticed for patients with coronary artery disease. There was a significant increase in IL-6 serum level as well as a significant decrease in IL-1Ra for patients with a history of myocardial infarction. A trend toward higher concentration of inflammatory mediators was noticed in relation to the increase in severity of the aortic valve disease. Our results support the hypothesis of an “inflammatory pattern” associated with AS pathology and suggest the persistence of a chronic inflammation in patients who experienced acute coronary events.

Key words: aortic sclerosis; inflammation; cytokine; matrix metalloproteinase
Twenty-one healthy subjects formed the control group (6 men and 15 women, mean age 28.6 ± 8.2 years). All the patients gave informed consent and the study was conducted according to the guidelines approved by the ethics committee of the Institute of Cardiovascular Diseases “C.C. Iliescu”.

Exclusion criteria consisted of: presence of bicuspid aortic valves (which could be a separate contributing factor for thickening of aortic cusps), infectious, inflammatory or neoplastic known co-morbidities (which would be a confounding factor for the inflammatory markers profile).

**Clinical and echocardiography examination**

Standard clinical examination was performed for all patients during the first 48 hours of hospitalization. Each patient underwent an echocardiographical assessment (HP Sonos 5500) for aortic valve morphology (thickness, calcifications) and also Doppler parameters (measurement of velocities and calculation of mean and peak aortic gradient according to standard recommendations). Aortic sclerosis was defined as an increased echogenicity, thickening or calcification of the valve leaflets with a transaortic velocity below 2 m/sec. A bilateral 2D carotid ultrasound examination was also performed in every patient for the assessment of carotid intima-media thickness (IMT).

The patients were also assessed for the presence of the metabolic syndrome, according to the NCEP ATP III criteria: 1) abdominal obesity (waist circumference >88 cm in women and >102 cm in men), 2) hypertriglyceridemia (≥150 mg/dl), 3) low high density lipoprotein (HDL) cholesterol (< 40 mg/dl in men and <50 mg/dl in women), 4) high blood pressure (≥130/85 mmHg), and 5) high fasting glucose (≥110 mg/dl).

Eighteen patients underwent coronary angiography for evaluation of coronary artery disease presence and extension. Four patients had single-vessel coronary artery disease, eight patients had multi-vessel disease, and six had non-significant coronary stenosis.

**Blood samples**

Blood samples were obtained by venipuncture from all the patients within 72h from the hospital admittance. For testing the inflammation markers, serum was separated and stored at -20°C until measuring.

**Cytokines and other measurements**

Serum concentration of cytokines and other mediators was measured by enzyme-linked immunosorbent assay (ELISA) technique, using commercially available kits as follows: IL-6 by high sensitivity ELISA (R&D Systems Europe Ltd, the lower limit of detection being of 0.039 pg/ml); IL-1Ra, MMP-9, TIMP-1 and ICAM-1 by Quantikine kits (R&D Systems, Abingdon, UK, the lower limit of detection of 3.9 pg/ml for IL-1Ra, 0.156 ng/ml for MMP-9, 0.08 ng/ml for TIMP-1 and 0.35 ng/ml for ICAM-1); IL-12 (p70) by BD OptEIA kits (Becton-Dickinson, San Diego, USA, the lower limit of detection of 7.8 pg/ml); IL-18 by MBL ELISA kit (Medical & Biological Laboratories, Japan, the lower limit of detection of 12.5 pg/ml).

**Detection of MMPs activity by gel zymography**

MMPs activity was tested by gelatin zymography. Serum samples were diluted 1/100 in sample buffer [50mM Tris-HCl (pH ~6.8), 10% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS), 0.5% (w/v) bromophenol blue] and separated in 8% SDS-PAGE gel polymerized with 1% (w/v) gelatin. Gels were removed from glass plates and soaked for 30 min. in 2.5% Triton X on a shaker followed by two brief washes in water. Thereafter gels were incubated overnight at 37°C in the reaction buffer [50 mM Tris-HCl (pH 7.5), 0.2M NaCl, 5 mM CaCl₂]. Gelatinolytic activity was visualized by staining the gels with 0.25% Coomassie

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**Table 1. Baseline characteristics of the study patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (mean ± SD)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>62.9 ± 10.6</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>60.8</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>43.1</td>
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<tr>
<td>Diabetes, %</td>
<td>25.5</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>68.4</td>
</tr>
<tr>
<td>Obesity, %</td>
<td>35.3</td>
</tr>
<tr>
<td>Body mass index</td>
<td>27.15 ± 5.3</td>
</tr>
<tr>
<td>Metabolic syndrome, %</td>
<td>32</td>
</tr>
<tr>
<td>Aortic valve calcification, %</td>
<td>68</td>
</tr>
</tbody>
</table>
Brilliant Blue R-250 blue for 1 h on a shaker at room temperature. Destaining step was performed in glacial acetic acid: methanol: distilled water (1:3:6, v/v) solution. Gels were scanned using a HP3690 scanner and MMP activity was quantified by densitometry. A sample of fetal calf serum (FCS) was used as positive control and 100% reference to determine relative bands intensity.

Statistical analysis
Results were expressed as mean ± standard deviation (SD). Continuous variables were compared by means of a two-sided Student’s t-test or non-parametric tests. Spearman’s correlation coefficient was used to assess the association between inflammatory markers levels and clinical and echographic continuous variables. Age correction was performed using a full factorial univariate linear model. A value of P < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS Version 9.0 software.

RESULTS

Serum levels of inflammatory mediators in AS patients vs. controls
When comparing the serum levels of inflammatory markers in patients and controls we noticed significant higher levels of IL-6, MMP-9, TIMP-1 and sICAM-1 in AS patients as compared to healthy subjects (data not shown). To investigate the involvement of matrix metalloproteinases, we assessed also the gelatinolytic activity of MMP-2 and MMP-9 in patients and controls (Figure 1).

The two bands representing the latent form of MMP-9 (92 kDa, upper band) and the latent form of MMP-2 (72 kDa, lower band) were detected by gel zymography in the sera of all subjects, but the serum MMP-9 activities of aortic sclerosis patients were higher than those of healthy controls. Densitometric analysis in sera of 51 patients and 21 healthy controls indicated that the mean MMP-2 was comparable in the two groups while the MMP-9 activity for patients was statistically significant higher than controls. Furthermore, a good linear correlation was found between the densitometry units measured by zymogram and the respective concentrations of MMP-9 measured by immunoassay in the sera of patients ($r = 0.727$, $P < 0.001$) and controls ($r = 0.572$, $P < 0.01$). A positive correlation was also noticed between measured TIMP-1 concentrations and MMP-9 concentrations ($r = 0.420$, $P < 0.001$) and MMP-9 activity ($r = 0.373$, $P < 0.05$) in studied patients whilst no similar correlation was found in control group for these mediators.

Figure 1. Gelatinase activity of MMP-2 (72 kDa) and MMP-9 (92 kDa) in AS patients and controls. Sera from 51 AS patients and 21 healthy controls were analyzed for their MMP-2 and MMP-9 activities by gel zymography. A sample of fetal calf serum (FCS) was used as positive control (lane 1). The figure shows representative results for serum samples from the two groups (lanes 2-5: controls, lanes 5-8: AS patients).

However, it was demonstrated that advanced age is associated with a hyperinflammatory state, referred to as 'inflamm-aging' (6). As there was a significant difference in the age of control and patients groups, the measured levels were age-adjusted (Table 2). As can be seen, after age correction, the values were still higher for IL-6, MMP-9 (level and activity), TIMP-1 and IL-18 and lower for IL-1Ra, in the patients group compared to controls although the differences were not statistically significant (with the exception of sICAM-1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean value ± SD</th>
<th>Statistical significance</th>
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<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>3.5 ± 0.6</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>IL-1 RA (pg/ml)</td>
<td>434.8 ± 43.5</td>
<td>454.9 ± 84.7</td>
</tr>
<tr>
<td>IL-18 (pg/ml)</td>
<td>490.7 ± 37.2</td>
<td>355 ± 73.3</td>
</tr>
<tr>
<td>MMP-9 (ng/ml)</td>
<td>628.3 ± 65.8</td>
<td>497.9 ± 133.2</td>
</tr>
<tr>
<td>MMP-9 activity (AU)</td>
<td>177.3 ± 39.8</td>
<td>155.6 ± 78.5</td>
</tr>
<tr>
<td>TIMP-1 (ng/ml)</td>
<td>184.8 ± 7.8</td>
<td>167 ± 13.3</td>
</tr>
<tr>
<td>sICAM-1 (pg/ml)</td>
<td>272.7 ± 12.3</td>
<td>200.2 ± 24.2</td>
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Table 2. Age-adjusted values of serum levels of inflammatory markers in AS patients and controls
Systemic inflammation and aortic sclerosis

Relation of systemic inflammatory markers to echocardiographic and angiographic measures of atherosclerosis

We wanted to see if there was any association between the increased values noticed for several inflammation mediators and the severity of atherosclerosis burden evaluated by carotid intima-media thickness (IMT). Indeed, we found higher age-adjusted values, although not statistically significant, for patients having a "high" value of IMT (defined as an IMT ≥0.9 mm) as compared to patients with IMT <0.9 mm for IL-6 (6 ± 1.4 vs. 2.2 ± 1.7 pg/ml, P= 0.122), IL-1Ra (492.3 ± 57.4 vs. 327 ± 67.5 pg/ml, P=0.083). Moreover, in the case of IL-1Ra, a positive correlation was found with the measured values of IMT (r = 0.477, P < 0.05).

Next, we analyzed the serum concentrations of inflammatory mediators in relation with the degree of coronary atherosclerosis evaluated by angiography. The noticed differences were at the level of IL-6 concentration and MMP-9 activity, which were higher in the subgroup of patients with multi-vessel disease compared to patients with less than two vessels affected (data not shown).

Relation of systemic inflammatory markers to cardiovascular risk factors, coronary artery disease and history of cardiovascular events

Generally, the age-adjusted values for systemic inflammatory markers did not differ significantly between patients with different numbers of cardiovascular risk factors. However, a slight increase of the serum levels was noticed for patients with more than three risk factors as compared to patients with less than three risk factors for IL-6 (4.2 ± 0.6 pg/ml vs. 2.7 ± 1 pg/ml) and IL-1Ra (531.5 ± 44 pg/ml vs. 397.7 ± 67.3 pg/ml).

Diabetic patients had significantly higher levels of IL-6 (Figure 2) and a higher MMP-9 activity (285.8 ± 70 ng/ml in diabetic patients vs. 179.85 ± 40.9 ng/ml in non-diabetic patients). Also, patients with metabolic syndrome had higher values of MMP-2 and MMP-9 activities as well as higher values of IL-6 and IL-1 RA serum concentrations as compared to the other patients (data not shown).

Increased serum levels of inflammatory mediators have been reported to be correlated with the risk for acute coronary syndromes. In our study, 22 patients were diagnosed with coronary artery disease (CAD). For these patients, the age-adjusted values of serum IL-6 were significantly higher than for the other patients (Figure 3).

Among these CAD patients, 15 patients had a history of myocardial infarction (MI). For this subgroup, the serum concentrations were significantly higher for IL-6 and lower for IL-1Ra as compared with no history of infarction (Figure 4 A,B).

For IL-6, serum level significantly correlated with the severity of myocardial dysfunction as assessed by the determination of ventricular ejection fraction (r = -0.376, P < 0.05). No correlation was noticed in the case of IL-1Ra.

Relation of systemic inflammatory markers to aortic valve disease

As the main objective of this study was to investigate the relevance of inflammatory markers in aortic sclerosis, we looked for any possible correlation between the serum levels of different mediators and the severity of aortic valve disease, estimated by the degree of calcification, maximum aortic jet velocity and the presence of aortic regurgitation.

We did not find a direct correlation between the degree of calcification and any of the determined inflammatory markers. However, a trend toward higher values was noticed for IL-6 concentration with increase in the severity of aortic sclerosis (data not shown). Also patients with moderate and severe aortic
stenosis (maximum aortic jet velocity $\geq 2$ m/s, $n=10$) had higher values of serum IL-6 than the others ($5.1 \pm 1.4$ pg/ml vs. $3.1 \pm 0.9$ pg/ml) although the difference was not statistically significant. Patients with aortic regurgitation ($n=23$) had significantly higher values of total MMP activity (95.6 ± 54.9 ng/ml vs. 44.9 ± 12.4 ng/ml, $P < 0.05$) than patients without aortic regurgitation ($n=14$).

![Graph A](image)

![Graph B](image)

Figure 4. Serum concentration of IL-6 (A) and IL-1Ra (B) in AS patients in relation to the presence of a previous MI. Data represented as mean ± SD

DISCUSSION

Recently, it has been suggested that, similar to atherosclerosis, chronic inflammation may play a role in the pathogenesis of aortic sclerosis (1, 2, 7, 8). As inflammatory markers have been also considered a predictor of future cardiovascular events such as acute coronary syndromes, in this study we investigated a panel of inflammatory mediators in patients with aortic sclerosis.

In a first attempt to measure the inflammatory markers in patients vs. controls, we noticed statistical-ly significant increased values for IL-6, MMP-9, TIMP-1 and s-ICAM serum concentration in AS patients. However, a major limitation of this study was the significant differences in the mean age of the two groups (62.9 ± 10.6 years in patients vs. 28.6 ± 8.2 years in control group) due to difficulty to find age-matched subjects without cardiovascular diseases. Therefore, we applied an age correction to the measured values. In these conditions, the differences between the two groups were no longer significant, although higher values were noticed for patients as compared to healthy donors for several parameters (Table 2). In particular, the level of IL-6 was greater in AS patients. IL-6 is an important local and circulating marker of inflammation in cardiovascular tissues and increased levels were found in patients with acute myocardial infarction and unstable angina (9, 10). In our study, the concentration of IL-6 was significantly higher for patients with CAD and also for patients with diabetes mellitus or metabolic syndrome. This is considered an important finding, as elevated levels of IL-6 were associated in literature with unfavorable short- and long-term prognoses in patients with coronary artery disease (9).

Besides inflammatory cytokines, elevated levels of circulating soluble adhesion molecules have been reported in patients with acute coronary syndromes (11, 12) and non-rheumatic aortic stenosis (13). In our study we looked for serum levels of soluble intercellular adhesion molecule 1 (sICAM-1) and found statistically significant differences between patients and controls. Higher levels of circulating sICAM-1 in AS patients might be explained by activation of endothelium by cytokines that mediate the inflammatory response.

Matrix metalloproteinases (MMPs) represent a family of endopeptidases with proteolytic activity against extracellular matrix components involved in various pathological processes such as inflammation, tumor metastasis, respiratory diseases, myocardial injury, vascular aneurysms, and remodelling (14). It has been shown that MMPs are upregulated in calcific AS and might modulate matrix remodelling (15, 16). MMP-9 (gelatinase B or 92-kDa type IV collagenase) was found to be highly expressed in the vulnerable regions of atherosclerotic plaques, and it has been suggested to be causally involved in the remodelling processes associated with atherogenesis and plaque rupture (14). In our study, we found positively correlated increases in expression and activity of MMP-9 as well as in serum level of TIMP-1, a tissue inhibitor of MMPs in AS patients compared to controls, although the differences were not statistically significant after age correction. Moreover, the IL-6 concentration positively correlated to activity of MMP-9, suggesting an "inflammatory pattern" of the investigated patients. As in the case of IL-6 concentration, MMP-9 activity was significantly greater for patients with diabetes.
It has been reported that the balance between inflammatory response by T-helper related cytokines and anti-inflammatory response by T-helper-2 related cytokines is associated with coronary atherosclerosis (17). In our study, the age-adjusted serum levels of IL-6 and IL-1Ra were higher in patients with a great burden of atherosclerosis (defined by IMT ≥ 0.9 mm). Our results are in agreement with a previous report in which increased levels of IL-6 and IL-1Ra were found in patients with stable angina pectoris, suggesting that an increased inflammatory activity may play a role in chronic ischemic heart disease (18).

When the results were analyzed in relation with angiographic findings, we found higher serum levels of IL-6 concentration and MMP-9 activity for patients with multivessel disease, which suggest an "excessive inflammation" pattern in patients with severe atherosclerosis. However, our result does not support the hypothesis of a preferential involvement of Th-1 type response in AS patients as reported by others in patients with coronary artery disease (17, 19). A trend toward higher concentration of serum IL-6 in relation to increase in the severity of aortic sclerosis was noticed. As we reported previously, values of IL-6 might be predictive for the presence of aortic valve calcifications in patients with AS (20).

An interesting finding was the persistence of increased levels of IL-6 in patients who experienced an acute myocardial infarction. For these patients, age-adjusted serum concentration of IL-6 correlated significantly with the severity of myocardial dysfunction as assessed by the determination of ventricular ejection fraction. Recently, the role of interleukin-6 for LV remodelling and survival was re-examined in an experimental model using IL-6 knockout mice (21). The authors suggested that elevated levels of IL-6 in permanent ischemia post-MI might reflect a response to the extent of the underlying injury rather than being directly involved in remodeling or heart failure. Our results might be explained by this hypothesis.

Through its capacity to bind to IL-1 receptor, the naturally occurring antagonist IL-1 receptor antagonist (IL-1Ra) is considered to limit the potentially deleterious effects of IL-1. The IL-1/IL-1Ra balance may determine the severity of both acute and chronic inflammation, and the relative absence of IL-1Ra is suggested to play a role in the pathogenesis of some inflammatory disorders, including atherosclerosis (22, 23). In agreement with other studies, which report increased expression (23) or enhanced ex vivo synthesis of IL-1Ra (24) associated with cardiovascular events, we found increased levels of serum IL-1Ra in AS patients as compared to controls. However, we detected lower levels of IL-1Ra (statistically significant after age correction) in the group of patients with a history of MI, as compared to the other patients. In contrast to our results, early elevated levels of IL-1Ra have been reported in patients with acute myocardial infarction (25). The discrepancy between our results and the cited study might be explained by the potential protective role of IL-1Ra against deleterious effects of inflammatory cytokines. It may be presumed that in the case of post-MI patients, the level of IL-1Ra was insufficient to fully control inflammation resulting in its long-term persistence.

In conclusion, in this study we demonstrated an association of several markers of systemic inflammation with echocardiographic findings, suggesting an "inflammatory pattern" of aortic sclerosis patients. As our study was not a prospective one and did not accumulate data sequentially with time, we could not investigate the association between inflammation and adverse clinical outcomes in patients with aortic sclerosis as suggested or the predictive value of studied inflammation parameters (4). Despite this limitation and the relatively small number of subjects, we demonstrated that several inflammation mediators, such as IL-6, MMP-9, TIMP-1, IL-1Ra, IL-18 and sICAM-1 are present in higher concentration in sera of AS patients. Although the patients and control group were not age-matched, a relative increase in serum concentration of inflammatory mediators was still noticed after age correction in AS patients vs. healthy controls. In addition, our findings support the hypothesis of a chronic inflammatory "background" in patients with a history of acute coronary events.

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