Relationship between white blood cell count and circulating anti-endothelial cell antibodies detected in patients with peripheral arterial disease

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ABSTRACT

Background and objectives: Circulating anti-endothelial cell antibodies (AECAs) are elevated in peripheral arterial disease (PAD) patients. In this context, these autoantibodies have been associated with the endothelial dysfunction and the pro-inflammatory status that surrounds atherosclerosis. On the other hand, white blood cell (WBC) count and inflammatory markers have been consistently associated with cardiovascular events and PAD. Our aim is to investigate the relationship between circulating AECAs and WBC count in patients with PAD.

Methods: An observational translational study was conducted including 34 male patients with intermittent claudication and no previous autoimmune disease. WBC count was measured in all patients. Highly sensitive C-reactive protein levels (hsCRP) were investigated as a surrogate of inflammation. Circulating AECAs titer was detected using indirect immunofluorescence.

Results: We found higher hsCRP levels in patients with circulating AECAs (7.60 [4.25-10.20] vs. 4.90 [3.20-7.00] mg/L p=0.02). We also observed a higher monocyte count (0.72 [0.60-1.01] vs. 0.55 [0.45-0.79] X103/µL p=0.03) and eosinophil count (0.28 [0.22-0.36] vs. 0.25 [0.07-0.29] X103/µL p=0.05) in patients with these autoantibodies.

Conclusions: Circulating AECAs of PAD patients could be associated with the systemic inflammatory status that surrounds the disease and with high monocyte and eosinophil blood cell count.

INTRODUCTION

Atherosclerosis is a chronic inflammatory vascular disease based on the immune system activity. Therefore, complex interactions between genetic and environmental factors of the immune system and the vascular wall could act as modulators of the pathogenesis and development of peripheral arterial disease (PAD).

Indeed, white blood cell count (WBC) and inflammatory markers elevation have been consistently associated with cardiovascular events and PAD (1-5). Moreover, there are several data pointing to a possible autoimmune origin for atherosclerosis (6-11). In this context, circulating anti-endothelial cell antibodies (AECAs) are a heterogeneous group of circulating antibodies targeting endothelial cells, detected in different vasculitic, inflammatory or autoimmune situations, whose common denominator is endothelial damage (12).

AECAs have various physiopathological effects of scarcely known mechanisms, such as induction to apoptosis (13,14), cytotoxicity (15), increase in leukocyte adhesion and cytokine secretion (16,17), activation of coagulation and thrombosis. In a recent study it has been found an association between these autoantibodies and PAD in patients with no previous autoimmune disease (18). Our aim is to investigate the relationship between circulating AECAs and WBC count in patients with PAD in order to provide new insights in the field of the etiological pathophysiology of atherosclerosis.
METHODS

An observational translational study was conducted including male patients with intermittent claudication due to PAD after haemodynamic confirmation of the disease by Doppler and treadmill exercise testing. None of the patients included had been previously revascularised or presented tissue lesions of the lower limbs. All patients signed an informed consent form, according to the principles of the Helsinki Declaration and the study protocol was approved by our Hospital Ethics Committee.

All those patients with documented diagnosis of atopic, autoimmune or rheumatologic disorders, transplanted or immunodepressed, and those treated with immunosuppressors or systemic and/or inhaled corticosteroids were excluded. We also excluded all those patients with an acute inflammatory status. Remaining subjects underwent a serological screening for autoimmune, rheumatologic and neoplastic disease markers. Antibodies ANA, Anti-DNA, Anti-LKM, Anti-mitochondria and Anti-smooth muscle were analysed by means of indirect immunofluorescence and antibodies Anti-SM, Anti-Jo, Anti-LA, Anti-Scl 70, Anti basement membrane, Anti-RO and Anti-RNP were measured using the enzyme-linked Immunosorbent assay (ELISA) test. All patients seropositive to any marker were excluded from the analysis.

During two months, 248 patients were screened at our outpatient clinic, but only 34 met the inclusion criteria and were finally recruited for the research. The basal ankle-brachial-index higher value and the absolute claudication distance of the included subjects were 0.60 [0.57-0.70] and 175 [126-236] metres, respectively. Cardiovascular risk factors and medical treatment of the patients were recorded.

**Basic laboratory, WBC and highly sensitive C-reactive protein measurements**

Patients who met the inclusion criteria were called to attend our outpatient clinics after fasting for 12 hours. We obtained a general sample for basic laboratory determinations (glycaemia, electrolytes and renal function), lipid profile and WBC count. Using the same puncture, we also obtained an independent blood sample for plasma concentration of Highly sensitive C-reactive protein (hsCRP) and circulating AECAs titer measurements. Plasma concentration of hsCRP was measured using a highly sensitive, automated immunnoassay (Roche Diagnosis, Basel, Switzerland). This test provides a low detection limit of 0.2 mg/l and a variation coefficient of 4.2% in 4 mg/l and 6.3% in 1 mg/l. Each sample was performed in triplicate, and the mean of the three values was used for the analysis.

**Circulating AECAs detection**

The autoantibody titer was detected by indirect immunofluorescence using a diagnosis reagent kit from EUROIMMUN (Medizinische Labordiagnostika AG, Luebeck, Germany) with a TITER-PLANE technique. This technique provides 100% sensitivity and specificity for AECAs detection. Cultivated umbilical vein endothelial cells covered the reaction areas of a BIOCHIP. Slides were incubated with patient’s diluted serum samples. Positive reactions were marked by granular staining in cytoplasm of human umbilical vein endothelial cells with fluorescein-labeled antibodies and were visualised by fluorescence microscopy. (Figure 1). According to the manufacturers, titers of <1:10 represent the reference range and the lower detection limit of the test.

**Statistical analysis**

Data was processed using the SPSS 15.0 statistical software package (Microsoft). Differences between groups were considered statistically significant for a P<0.05 in two-tailed test. The normality of continuous variables was analysed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The association between categorical variables was studied using the Chi-square test and the Fisher’s Exact test when required. The association between continuous variables was analysed using the Mann-Whitney U test and Spearman’s correlation test. Categorical variables were expressed as a percentage and continuous variables as the median (interquartile range [p25-p75]). All the extreme values and outliers were identified and double-checked.

**RESULTS**

Circulating AECAs titer ≥1:10 was detected in the serum sample of 20 (59%) patients. All the autoantibodies were IgG isotype and all the serum samples with AECAs titer ≥1:10 reacted against beta2-glycoprotein I, proteinase 3 and collagenase antigens. The baseline characteristics of the included patients are described in Table 2.

**Relationship between circulating AECAs and WBC**

There were no statistical differences in lipid profile or WBC count between the analysed groups (Table 2). However, patients with AECAs titer ≥1:10 showed a higher blood monocyte count (0.72 [0.60-1.01] vs. 0.55 [0.45-0.79] X10³/µL p=0.03). Eosinophils count was also higher in patients with AECAs titer ≥1:10 (0.28 [0.22-0.36] vs. 0.25 [0.07-0.29] X10³/µL p=0.05) (Table 2 and figure 2).
Table 1: Baseline characteristics of the included patients

<table>
<thead>
<tr>
<th></th>
<th>AECAs titer ≥1:10 % (n=20)</th>
<th>AECAs titer &lt;1:10 % (n=14)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63 [60-68]</td>
<td>67 [57-74]</td>
<td>0.98</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5 (25%)</td>
<td>3 (21%)</td>
<td>1</td>
</tr>
<tr>
<td>History of Smoking</td>
<td>10 (50%)</td>
<td>6 (43%)</td>
<td>0.73</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>4 (20%)</td>
<td>4 (28%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Hypertension</td>
<td>11 (55%)</td>
<td>7 (50%)</td>
<td>1</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
<td>0.50</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>2 (10%)</td>
<td>1 (7%)</td>
<td>1</td>
</tr>
<tr>
<td>Chronic pulmonary disease</td>
<td>1 (5%)</td>
<td>2 (14%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Medical treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-platelet</td>
<td>15 (75%)</td>
<td>11 (78%)</td>
<td>1</td>
</tr>
<tr>
<td>Statins</td>
<td>6 (30%)</td>
<td>4 (28%)</td>
<td>0.61</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>8 (40%)</td>
<td>5 (36%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Nitrates</td>
<td>1 (5%)</td>
<td>2 (14%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>2 (10%)</td>
<td>2 (14%)</td>
<td>1</td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>5 (25%)</td>
<td>4 (28%)</td>
<td>0.56</td>
</tr>
<tr>
<td>Beta-agonists</td>
<td>2 (10%)</td>
<td>2 (14%)</td>
<td>1</td>
</tr>
<tr>
<td>Oral anticoagulants</td>
<td>0 (0%)</td>
<td>2 (14%)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Figure 2: Relationship between white blood cell count (WBC) and anti-endothelial cell antibodies (AECAs). Monocyte count (a) and eosinophil count (b) were higher in patients with circulating AECAs titer ≥1:10. *Blood count units: X10³/µL.
Table 2: Lipid profile and white blood cell count of the included patients

<table>
<thead>
<tr>
<th></th>
<th>AECAs titer ≥1:10 (n=20)</th>
<th>AECAs titer &lt;1:10 (n=14)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>211 [171-251]</td>
<td>203 [151-248]</td>
<td>0.43</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>148 [132-188]</td>
<td>139 [107-167]</td>
<td>0.53</td>
</tr>
<tr>
<td>High-density lipoprotein (mg/dl)</td>
<td>45 [38-50]</td>
<td>49 [42-58]</td>
<td>0.53</td>
</tr>
<tr>
<td>Low-density lipoprotein (mg/dl)</td>
<td>116 [106-166]</td>
<td>116 [73-173]</td>
<td>0.48</td>
</tr>
<tr>
<td>Very low-density lipoprotein (mg/dl)</td>
<td>29 [26-38]</td>
<td>28 [21-33]</td>
<td>0.53</td>
</tr>
<tr>
<td><strong>White blood cell count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell count (X103/µL)</td>
<td>8.26 [5.89-12.32]</td>
<td>7.48 [5.83-10.15]</td>
<td>0.39</td>
</tr>
<tr>
<td>Neutrophils (X103/µL)</td>
<td>4.40 [3.63-7.21]</td>
<td>3.75 [2.25-5.49]</td>
<td>0.12</td>
</tr>
<tr>
<td>Lymphocytes (X103/µL)</td>
<td>2.62 [1.98-3.61]</td>
<td>1.67 [1.59-3.53]</td>
<td>0.09</td>
</tr>
<tr>
<td>Monocytes (X103/µL)</td>
<td>0.72 [0.60-1.01]</td>
<td>0.55 [0.45-0.79]</td>
<td>0.03</td>
</tr>
<tr>
<td>Eosinophils (X103/µL)</td>
<td>0.28 [0.22-0.36]</td>
<td>0.25 [0.07-0.29]</td>
<td>0.05</td>
</tr>
<tr>
<td>Basophils (X103/µL)</td>
<td>0.06 [0.05-0.08]</td>
<td>0.05 [0.04-0.06]</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Relationship between circulating AECAs and hsCRP

We found higher hsCRP levels in patients with circulating AECAs titer ≥1:10 (7.60 [4.25-10.20] vs. 4.90 [3.20-7.00] mg/L p=0.02) (Figure 3).

Relationship between hsCRP and blood cell count

We did not find any significant statistical correlation between WBC and hsCRP in our sample.

DISCUSSION

According to the findings of this study, the presence of circulating AECAs in patients with PAD could be associated with the systemic inflammatory status that surrounds the disease and with high monocyte and eosinophil blood cell count. Circulating AECAS have been related to PAD. Subjects seropositive to these autoantibodies presented worse endothelial dysfunction parameters (Flow-mediated arterial dilatation), higher levels of inflammation markers (hsCRP) and higher Intiman-media thickness measurements (18). This data suggest a possible role of circulating AECAs on the atherogenic process.

Endothelial dysfunction acts as a primary pathogenic event for atherosclerosis, as it occurs before structural changes are evident on angiogram or ultrasound scan and it is not correlated with the disease’s severity (19). The loss of endothelial regulation has been attributed to a reduction in nitric oxide (NO) bioactivity, and to an increased oxygen-free radical formation in the context of the pro-inflammatory status found in the disease (20,21).
In this context, circulating AECAs may cause endothelial damage through several mechanisms. Polyclonal IgG fractions of these autoantibodies can mediate endothelial cell lysis when incubated in vitro with the complement or in the presence of mononuclear cells to induce an antibody-dependent cellular cytotoxicity (15). Circulating AECAs can also induce endothelial apoptosis by themselves or together with natural killer cells. Signaling for apoptosis could be initiated through the interaction of these autoantibodies with the Fas receptor or with the integrin α3β1 and α6β1-NAG-2 protein complex (12). Furthermore, some published data have suggested that circulating AECAs might cause endothelial cell apoptosis through the NO pathway (22,23).

On the other hand, inflammation has a key role in every aspect of the atherosclerotic process. Recent investigations reported that the presence of high-risk carotid plaques is associated with increased levels of inflammatory markers, such as hsCRP and leukocyte count (24-26). Another study found that the prevalence of unstable hypoechoic plaques was higher in PAD than in coronary artery disease patients, probably because, consistent with previous studies, the PAD group has more pronounced inflammatory profile (27). Neutrophil count was associated with a more severe clinical presentation of atherosclerosis independently of sex, age and classic risk factors (27).

Relationship between circulating AECAs, monocyte blood cell count and hsCRP levels

In the present research we have observed an association between AECAs titer ≥1:10 and high monocyte blood cell count in patients with PAD. These findings are of great interest, considering that monocytes contribute actively to atherosclerotic lesion development. The infiltration of circulating monocytes into the arterial wall is one of the earliest events in the development of the atherosclerotic lesion. These white blood cells are believed to play a critical role in the atherosclerosis initiation, plaque instability and artery remodeling stages (28-30). Blood monocytes have been also independently associated with subclinical PAD after adjustment for other inflammatory markers (4).

In this context, circulating AECAs could activate endothelial cells through a NF-κB dependent mechanism, leading to the expression of leukocyte adhesion molecules (intercellular adhesion molecule 1 [ICAM-1], vascular cell adhesion molecule 1 [VCAM-1], E-selectin) and increasing the release of pro-inflammatory cytokines (interleukin 1 [IL-1], interleukin 6 [IL-6]) in vitro (16,17). These observations are consistent with the finding of raised hsCRP levels in patients with PAD and circulating AECAs. The relationship found between these autoantibodies and hsCRP levels suggest that autoimmunity could play an important role in the genesis of the chronic inflammation observed in patients with PAD. On the other hand, the hypothetical endothelial inflammatory chemotaxis enhancement related to circulating AECAs could justify higher monocyte count levels when the antibody is present.

Relationship between circulating AECAs and eosinophil blood cell count

We also found higher eosinophils count in patients with circulating AECAs. Recent observations suggest that eosinophils may play a role in coronary atherosclerosis (31). Eosinophil count has been associated with an increased risk for future cardiovascular events (32,33). When activated, eosinophils secrete several proteins, among which eosinophil cationic protein (ECP) stands up (34). This molecule interacts with other immune cells and plasma proteins such as coagulation factors and the complement system (35). ECP also up-regulates endothelial ICAM-1 expression (36) allowing monocytes adhesion on endothelium. Therefore, eosinophils might cooperate with, or amplify, circulating AECAs immune mechanisms in mediating endothelial damage.

Our findings do not establish a causal relationship. However, the observed association of circulating AECAs with monocytes and eosinophils count in patients with PAD supports the hypothesis of an alleged autoimmune origin of atherosclerosis, given the clear relationship of these blood cells with autoimmune diseases and circulating AECAs pathogenic mechanisms. Although the data obtained from this study does not have a direct clinical application, its potential benefits lie in the improvement of the etiopathogenic insights of the atherogenic process.

CONCLUSION

The presence of circulating AECAs in patients with PAD could be associated with the systemic inflammatory status that surrounds the disease and with high monocyte and eosinophil blood cell count. However, further prospective studies are required to establish a causal relationship.

REFERENCES

REFERENCES