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Concentrations of selected proteins and homocysteine in patients with atherosclerosis

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Short title: Pathogenesis of atherosclerosis

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INTRODUCTION

The development of atherosclerosis is not only associated with endothelial dysfunction (due to inflammation or injury) and subendothelial lesions, but also with coagulation disorders and systemic metabolic abnormalities. However, we are often confronted with inconsistent results in the studies in this area.

Therefore, we decided to study some selected autoimmune markers, proteins taking part in coagulation and homocysteine in atherosclerotic patients aiming to identify some biomarkers indicative of the risk and progression of atherosclerosis.

METHODS

Seventy-eight men and women aged from 54 to 77 years diagnosed with atherosclerosis based on clinical manifestations and angiography were divided into three groups: 33 patients with coronary artery disease (CAD), 15 with peripheral artery disease (PAD) and 30 with both coronary and peripheral artery disease (CPAD). A wide range of peripheral blood tests was performed using a µ-Quant ELISA reader. The following parameters were determined: 1/ coagulation system – protein C, free protein S, annexin V, 2/ autoimmune markers – antineutrophil cytoplasmic antibodies (pANCA, cANCA), antinuclear antibodies (ANA), anti-double stranded DNA antibodies (anti-dsDNA), antiendothelial cells antibodies (AECAs), anticardiolipin antibodies-aCLa (IgG, IgM) and 3/ homocysteine levels to represent metabolic disorders.

Blood samples were collected in between 07:00 and 08:00 hours; serum/plasma samples were immediately coded and stored at -80°C until assay. The study was approved by the Bioethics Committee; all patients gave their written consent to blood sampling.

The exclusion criteria were as follows: concomitant chronic liver / kidney, diseases collagenoses, thyroid disease, immunosuppressive therapy (during the past 12 months), a history
of neoplastic disease, acute or chronic inflammatory disease (exclusion was based on medical history, medical examination and determination of CRP levels in serum, which in the examined groups of patients were in normal range for this parameter, i.e.<5mg/L). The exclusion criteria concerned also psoriatic patients [1]. Candidates with atherosclerosis and dyslipidemia, diabetes, arterial hypertension were included.

The study protocol included: 1/ comparative analysis of laboratory results between the CAD, PAD and CPAD groups and 2/ evaluation of relationships between humoral parameters in the CAD, PAD and CPAD groups.

Statistical analysis

The statistical analysis included the Shapiro-Wilk test for normality of distribution followed by calculation of the arithmetic mean (X) and standard deviation (SD). The following tests were performed: Mann-Whitney U test, Spearman’s correlation coefficient (r), Kruskal-Wallis test, Student’s t test, and one-way ANOVA test. Differences with p-value ≤0.05 were considered statistically significant. In relation to a few determined parameters differences between X and median were revealed; nevertheless in comparative analysis X and SD values were calculated because in the most of examined parameters the values of X and median were comparable.

RESULTS and DISCUSSION

The Kruskal-Wallis test revealed that differences between the mean levels of annexin V (p = 0.057) in CAD, PAD and CPAD participants approached statistical significance. One-way ANOVA showed statistically significant differences in protein C levels between the CAD, PAD and CPAD groups (p=0.023, Table S1- included in the supplementary material).

Table 1 presents comparative analysis of laboratory results between the CAD, PAD and CPAD groups.
The following facts should be considered: in the PAD group the mean protein C level and the value of AECA index exceeded the upper reference limit (relatively 6.5 µg/mL and 0.165) (Table 1). In all study group 10 participants had ANA levels exceeding the upper reference limit (25 AU/mL). It also concerned homocysteine levels (>15 µmol/L) in 17 participants.

The correlations between analysed agents, most frequently in the CPAD group were observed, i.e. negative and statistically significant or approaching statistical significance relationships between protein C/ cANCA (r = -0.513, p = 0.004), protein S/AECA (r = -0.358, p = 0.052), annexin V/homocysteine (r = -0.468, p = 0.010), anti-dsDNA/homocysteine (r = -0.382, p = 0.038), AECA/homocysteine (r = -0.512, p = 0.004) and positive ones between protein S/homocysteine (r = 0.350, p = 0.058), annexin V/AECA (r = 0.557, p = 0.002), pANCA/cANCA (r = 0.496, p = 0.006), pANCA/ANA (r = 0.417, p = 0.023), pANCA/IgG aCLa (r = 0.478, p = 0.008), cANCA/ANA (r = 0.365, p = 0.048), cANCA/IgG aCLa (r = 0.394, p = 0.032), ANA/anti-dsDNA (r = 0.551, p = 0.002), anti-dsDNA/AECA (r = 0.312, p = 0.093).

In the CAD group positive and statistically significant or approaching statistical significance correlations between cANCA/anti-dsDNA (r = 0.391, p = 0.025), ANA/anti-dsDNA (r = 0.388, p = 0.026), ANA/IgG aCLa (r = 0.341, p = 0.053), anti-dsDNA/AECA (r = 0.330, p = 0.061), homocysteine/ IgM aCLa (r = 0.394, p = 0.024), homocysteine/ IgG aCLa (r = 0.354, p = 0.044) and negative ones between AECA/homocysteine (r = -0.597, p = 0.000) were revealed.

In the PAD group positive and statistically significant relationships between pANCA/ANA (r = 0.536, p = 0.042), ANA/anti-dsDNA (r = 0.755, p = 0.002), ANA/IgG aCLa (r = 0.526, p = 0.047) and anti-dsDNA/ IgG aCLa (r = 0.603, p = 0.020) were observed.
The lowest concentrations of protein C in the peripheral blood of CAPD participants, might hypothetically contribute to the development and extent of angiopathy; however, the mean concentration of protein C in this group was within normal limits. The PAD group, on the other hand, had the lowest although still normal concentrations of free protein S and the lowest annexin V levels.

Considering the above coagulation components some correlations with autoimmunological parameters (cANCA, AECA) and homocysteine in the CPAD group were revealed. These results are difficult to interpret because the data on this subject are scanty. Numerous reports indicate some association between hypercoagulability and development / progression of atherosclerosis [2,3]. It is also interesting though that Vig et al. [4] found thrombophilia in a quarter of their PAD patients. It should also be mentioned that plasma annexin V is deemed to be inversely related to the severity of coronary stenosis [5] and that patients with acute phase of myocardial infarction had low plasma annexin V levels [6].

In the CPAD group there were also associations between ANCAs (both pANCA and cANCA) and IgG aCLa. These correlations as well as the relationships between ANAs and pANCAAs in the PAD and CPAD groups, between anti-dsDNA antibodies and cANCAs in the CAD group and between cANCAs and ANAs in the CPAD group seem to provide evidence for some relationships between ANCAs and prothrombotic factors as well as between ANAs and anti-dsDNA antibodies.

The interest in ANCAs mainly stems from their association with vasculitis. Vessel inflammation has been implicated as a causative agent in atherosclerosis; some authors believe the inflammatory process is the predominant initiating factor. It should be noted though that a negative ANCA test does not exclude vasculitis and a positive ANCA cannot be considered an unambiguous proof of the disease [7].
ANA levels of several CAD and CPAD participants exceeded the upper reference limits. Similar observations were made regarding AECAs in the PAD and CPAD groups. In addition a positive correlation between AECAs and anti-dsDNA antibodies was noted in the CAD and CPAD groups. These findings might speak in favor of some atherosclerosis-related processes with endothelial involvement, and are consistent with higher prevalence of AECAs in patients with ischemic heart disease [8].

Although upper reference limits of anti-dsDNA antibodies, IgG aCLa, pANCAs and cANCAs were not exceeded in our study population, the relationships between ANAs and these antibodies are noteworthy and warrant large cohort studies. It should be emphasized that the role of ANAs in atherogenesis remains unclear.

Several participants in each of the groups met the criteria of hyperhomocysteinemia. The CAD group showed a positive and statistically significant correlation between IgG and IgM aCLa and homocysteine levels and a negative relation between homocysteine and anti-dsDNA in the CPAD patients. In addition in the PAD group a positive relationship between anti-dsDNA and IgG aCLa was observed. Interestingly some antiphospholipid antibodies were more prevalent in patients with acute phase of myocardial infarction [9], which seems to support the concept of immune mechanisms and coagulation abnormalities in pathogenesis of atherosclerosis. Homocysteine and antiphospholipid antibodies exert similar biological effects in homeostasis; however, their interactions remain unclear.

The CAD group showed a negative correlation between homocysteine levels and AECAs. This relationship was also revealed in the CPAD but not in the PAD group, which is noteworthy but difficult to account for. It is also difficult to analyse the role of the correlation in the pathogenesis of atherosclerosis and the more so because we have not come across any reports on this subject.
We conclude that the obtained results might point to the role of autoimmunity and homocysteine in the pathogenesis of certain cases of CAD, PAD and CPAD. The data obtained in this study suggest the necessity of conducting further investigations carried out on larger groups of patients to elucidate pathomechanisms of the relationships between analysed agents, observed most frequently in CPAD group.

REFERENCES


Table 1. Comparative analysis of the study proteins and homocysteine concentrations in patients with atherosclerosis

<table>
<thead>
<tr>
<th>Study parameter</th>
<th>CAD vs PAD</th>
<th>CAD vs CPAD</th>
<th>PAD vs CPAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=33  N=15</td>
<td>N=33  N=30</td>
<td>N=15  N=30</td>
</tr>
<tr>
<td></td>
<td>X (SD)</td>
<td>X (SD)</td>
<td>X (SD)</td>
</tr>
<tr>
<td>Protein C, µg/mL</td>
<td>6.09  (1.97)</td>
<td>6.75  (2.66)</td>
<td>6.09  (1.97)</td>
</tr>
<tr>
<td>Free protein S, %</td>
<td>129.11 (13.09)</td>
<td>121.42 (12.76) *</td>
<td>129.11 (13.09)</td>
</tr>
<tr>
<td>Annexin V, µg/mL</td>
<td>3.74  (2.03)</td>
<td>3.37  (1.94)</td>
<td>3.74  (2.03)</td>
</tr>
<tr>
<td>pANCA, AU/mL</td>
<td>2.81  (1.93)</td>
<td>3.54  (4.75)</td>
<td>2.81  (1.93)</td>
</tr>
<tr>
<td>cANCA, AU/mL</td>
<td>2.61  (2.31)</td>
<td>4.25  (2.77) *</td>
<td>2.61  (2.31)</td>
</tr>
<tr>
<td>ANA, AU/mL</td>
<td>17.59 (35.95)</td>
<td>8.33  (8.04)</td>
<td>17.59 (35.95)</td>
</tr>
<tr>
<td>Anti-dsDNA IgG, IU/mL</td>
<td>3.67  (1.35)</td>
<td>3.50  (1.67)</td>
<td>3.67  (1.35)</td>
</tr>
<tr>
<td>AECA, index</td>
<td>0.04  (0.03)</td>
<td>0.17  (0.27)</td>
<td>0.04  (0.03)</td>
</tr>
<tr>
<td>IgM aCLa, AU/mL</td>
<td>3.49  (0.85)</td>
<td>7.58  (16.53)</td>
<td>3.49  (0.85)</td>
</tr>
<tr>
<td>IgG aCLa, AU/mL</td>
<td>3.63  (0.66)</td>
<td>3.76  (0.76)</td>
<td>3.63  (0.66)</td>
</tr>
</tbody>
</table>

Data are presented as mean and standard deviation, * - statistically significant difference, o - difference approaching statistical significance,
aCLa - anticardiolipin antibodies, AECA - antiendothelial cells antibodies, ANA - antinuclear antibodies, ANCA - antineutrophil cytoplasmic antibodies, ANOVA - analysis of variance, anti-dsDNA - *anti-double stranded DNA* antibodies, CAD - coronary artery disease, CPAD - coronary and peripheral artery disease, CRP - C reactive protein, ELISA - enzyme-linked immunosorbent assay, N - group size, PAD - peripheral artery disease, SD - standard deviation, vs. - versus, X - arithmetic mean