The Role of Endothelial Cell Reactive Antibodies in Peripheral Arterial Disease

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Objectives. It is hypothesised that endothelial cell reactive antibodies (ECRA) play a role in the progression of PAD through activation of endothelial cells and the release of inflammatory cytokines. We aimed to test this hypothesis by assessing levels of ECRA, E-selectin and IL-6 in patients with PAD of varying severity in a case controlled study.

Design, materials, methods. Patients were assessed clinically and with ankle–brachial pressure indices. Patients with critical ischaemia (CI, \( n = 30 \)), stable claudicants (SC, \( n = 30 \)), and age-matched controls (AMC, \( n = 20 \)) were studied. Antibody, E-selectin and IL-6 levels were measured using ELISA.

Results. ECRA levels were significantly raised in the CI group over AMC. IL-6 levels were significantly elevated in both SC and CI over the control group and in CI over SC. There were no significant differences in E-selectin levels between the AMC, SC and CI.

Conclusion. Our findings support the hypothesis that autoantibodies play a role in promoting PAD by elevating IL-6. The absence of an elevation in E-selectin in this study may be due to its short half-life, and casts doubt on its value as a marker of inflammation in atherosclerosis.

Keywords: Peripheral arterial disease; Endothelial cell reactive antibodies; Anticardiolipin; Anti-\( \beta \)-2-glycoprotein I; Interlukin-6.

Introduction

Peripheral arterial disease (PAD) is a common and disabling condition, which affects 4.6% of the population aged between 55 and 74.1 Meta-analysis of studies on the outcome of patients with PAD reveals, that over a 5-year period, as little as five percent of patients deteriorate to a degree that necessitates surgery and only two percent require an amputation.2 Furthermore, of those patients requiring amputation, less than half had symptoms of intermittent claudication 6 months previously,3 suggesting that a small cohort of individuals have an accelerated form of the disease. Several traditional risk factors are recognised to promote the development of PAD, however, these do not fully explain the relatively benign progression in the majority or the aggressive nature of the disease in the few.4

The formation of atheroma in the arterial wall is increasingly recognised as an inflammatory process.5 Greater understanding of this process, which includes the accumulation of macrophages and T lymphocytes, suggests that an autoimmune response directed against the vascular endothelium may play a role in the initiation and progression of atherogenesis.5 Endothelial cell reactive antibodies (ECRA) are a heterogeneous group of autoantibodies directed against antigens in the membrane of endothelial cells (EC). Originally described in 1971,6 these antibodies have been demonstrated in patients, with a number of autoimmune diseases, in whom accelerated atherosclerosis is well recognised.7,8 Anticardiolipin (anti-CL) antibodies and, their major subpopulation, anti-\( \beta \)-2-glycoprotein I (anti-\( \beta \)2GPI) antibodies are two ECRA which have been identified in these patients. Animal and in vitro studies have demonstrated a potential pathogenic role for these antibodies by the up-regulation of adhesion molecule expression and cytokine and chemokine production.
production by EC, resulting in a proinflammatory and prothrombotic state.\textsuperscript{9,10} Anti-CL antibodies have been shown in several studies to be correlated with angina, myocardial infarction and cardiac death.\textsuperscript{11,12} Anti-\(\beta\)2GPI antibodies have been found to be abundant in atherosclerotic plaques and elevated levels have been associated with unstable angina and ischaemic stroke.\textsuperscript{13–15} However, the significance of these antibodies in peripheral arterial disease has not been elucidated to date.

This study aims to investigate whether levels of anti-CL and anti-\(\beta\)2GPI antibodies are elevated in patients with peripheral arterial disease and whether they are a marker of increased disease severity. In addition the levels of interleukin-6 and E-selectin, two markers of endothelial activation will be examined. This will enable the testing of the hypothesis that elevated autoantibody levels may be implicated in the development and progression of PAD.

\section*{Materials and Methods}

\textbf{Patients}

This study was approved by the Ethical Committee of the Leeds Teaching Hospital University Trust and written consent was obtained from all those that took part. All patients attending the Vascular Unit were considered for entry into the study. Patients were selected on the basis of a history of claudication\textsuperscript{16} or critical ischemia\textsuperscript{17} and their ankle brachial pressure index. Two groups were studied: stable claudicants (SC) had symptoms of intermittent claudication with no subjective alteration in their exercise tolerance over the prior 6 months, plus an ankle brachial pressure index of less than 0.9 (diabetic toe pressure <70 mmHg); critical ischaemia (CI) these patients had persistent ischemic rest pain requiring analgesia for >2 weeks or ulceration/gangrene of the foot or toes, plus an ankle systolic pressure of less than 50 mmHg (diabetic toe pressure <40 mmHg).

Patients were excluded if they had suffered a myocardial infarction or stroke in the past 3 months as this might have altered their antibody profile, if they had presented with a proven thrombotic event or if they had ever been given a diagnosis of autoimmune disease, vasculitis or malignancy. In addition a control group was recruited: age matched controls (AMC), this group had no symptoms or clinical signs of vascular disease and had an ankle brachial pressure index of greater than 0.9. Thirty subjects were recruited into each of the study groups, and 20 patients into the control group. This was done over a period of 3 months.

\textbf{Clinical assessment}

A full medical history was taken from each subject entered into the trial, including a detailed history of cardiac, cerebral and peripheral vascular symptoms, risk factors and previous events. Current medication was recorded, as well as a detailed smoking history. An ECG and serial blood pressure measurements were taken. An uncuffed 50 ml venous blood sample was collected and sent for analysis of full blood count, APTT, glucose, lipids, U&E and LFT’s. The remaining blood was collected in serum tubes (Vacuette\textsuperscript{®}, Greiner International, Austria), centrifuged at 3000 rpm for 15 min, aliquoted into 0.5 ml units and stored at –80 \(^\circ\)C until required.

\textbf{Elisa assay}

Antibodies to anticardiolipin and anti-\(\beta\)2-glycoprotein I were measured using a commercially available enzyme-linked immunosorbent assay (ELISA), (Bindazyme MK040/MK041, UK). Interleukin-6 was measured using a commercially available ELISA (R&D Systems D6050, USA). E selectin levels were assessed using an in-house ELISA. In brief, microtitre plates (Costar, Corning Life Sciences, The Netherlands) were coated overnight at 4 \(^\circ\)C with a monoclonal antibody against human E-selectin (BBA16, R&D Systems, USA) at a concentration of 2 \(\mu\)g/ml. After washing with 0.1 M PBS/0.05% Tween-20, the plates were blocked with 2% BSA for 1 h at room temperature (RT). Subsequently patient serum samples (1:1000 dilution in PBS) were added in triplicate. Recombinant E-selectin (ADP1, R&D Systems, USA) was used as calibration with eights standards on each plate (range 1 \(\mu\)g/ml–7.8 ng/ml). The results from these eight calibration standards plus a zero standard were used to plot a calibration curve. Wells were washed and biotinylated polyclonal antibody against human E-selectin (BAF575, R&D Systems, USA) was used as calibration with eights standards on each plate (range 1 \(\mu\)g/ml–7.8 ng/ml). The results from these eight calibration standards plus a zero standard were used to plot a calibration curve. Wells were washed and biotinylated polyclonal antibody against human E-selectin (BAF575, R&D Systems, USA) was added at a concentration of 0.2 \(\mu\)g/ml for 1 h at RT. E-selectin was detected by the addition of streptavidin-peroxidase (1 mg/ml) (S-2438, Sigma-Aldrich, UK) diluted of 1:200 for 1 h at RT. Finally the plates were washed and the colour reaction was initiated by the addition of substrate (tetrathiomethylene benzidine and \(H_2O_2\)). After
20 min the colour reaction was stopped by adding 200 μl of 0.1 M H2SO4. The plates were read at 490 nm. The results were calculated from the standard curve. The accuracy of this ELISA was validated by using a range of positive and negative standards on each plate and also by performing the assay in duplicate.

**Statistical analysis**

Antibody levels for each group are presented as median ± inter-quartile range. The differences between antibody levels for each group were assessed using an ANOVA with post hoc analysis. Differences in group characteristics were assessed using the independent samples t-test.

**Results**

The groups were well matched for sex and age (Table 1). Levels of cigarette smoking were similar in all groups. Mean ABPI was in keeping with the selection criteria, as is the past medical history of each group. As would be expected cardio/cerebro-vascular events were more common in patients with critical ischaemia (CI) although only cerebrovascular events reached statistical significance. This fact is reflected in the level of antiplatelet use. It is interesting to note that less than half of the patients with PAD were taking a statin at the time of recruitment (2001). With new guidelines, lowering the threshold for statin use in patients with vascular disease it is likely that statin use will be much higher in this population now.

The stable claudicants showed some elevation of both anti-CL levels and anti-β2GPI although this did not reach significance. The patients with critical ischaemia had significantly elevated mean levels of both anti-CL and anti-β2GPI antibodies when compared with SC. No significant difference in the incidence of hypertension, antiplatelet or statin use was observed between the SC and CI groups.

**Table 1. Patients and control characteristics**

<table>
<thead>
<tr>
<th></th>
<th>AMC</th>
<th>SC</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean age</td>
<td>67</td>
<td>66</td>
<td>69</td>
</tr>
<tr>
<td>Range</td>
<td>56–78</td>
<td>52–80</td>
<td>35–84</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>9/11</td>
<td>13/17</td>
<td>17/13</td>
</tr>
<tr>
<td>Smoking</td>
<td>10 (50)</td>
<td>17 (57)</td>
<td>17 (57)</td>
</tr>
<tr>
<td>Mean ABPI</td>
<td>1.0</td>
<td>0.71</td>
<td>0.31</td>
</tr>
<tr>
<td>Ambulation</td>
<td>0</td>
<td>0</td>
<td>8 (27)</td>
</tr>
<tr>
<td>Isch ulcer</td>
<td>0</td>
<td>0</td>
<td>12 (40)</td>
</tr>
<tr>
<td>PMHx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Art surgery</td>
<td>0</td>
<td>2 (7)</td>
<td>13 (43)</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>10 (34)</td>
<td>16 (53)</td>
</tr>
<tr>
<td>CVA/TIA</td>
<td>0</td>
<td>1 (3)</td>
<td>12 (40)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>21 (70)</td>
<td>19 (63)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>6 (20)</td>
<td>11 (37)</td>
</tr>
<tr>
<td>Anticoag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>0</td>
<td>23 (77)</td>
<td>12 (40)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>0</td>
<td>0</td>
<td>8 (27)</td>
</tr>
<tr>
<td>Warfarin</td>
<td>0</td>
<td>0</td>
<td>10 (33)</td>
</tr>
<tr>
<td>None</td>
<td>100%</td>
<td>7 (23)</td>
<td>0</td>
</tr>
<tr>
<td>Statin</td>
<td>0</td>
<td>12 (40)</td>
<td>13 (43)</td>
</tr>
</tbody>
</table>

Values are number of patients (%). Corresponding *(No) indicates significance p<0.05 between the two variables. There was no significant difference in the ages or smoking habits of the AMC, SC and CI groups. There was a significant increase in the incidence of diabetes, myocardial infarction, arterial surgery and CVA/TIA in CI when compared with SC. No significant difference in the incidence of hypertension, antiplatelet or statin use was observed between the SC and CI groups.

**Table 2. Anti-cardiolipin antibody, anti-β2-glycoprotein I antibody, E-selectin and interlukin-6 levels for each of the four groups**

<table>
<thead>
<tr>
<th></th>
<th>AMC</th>
<th>SC</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Cardiolin (U/ml)</td>
<td>1.97±1 (1.67–2.42)</td>
<td>2.51±1 (1.96–3.43)</td>
<td>2.93±1 (2.27–5.45)</td>
</tr>
<tr>
<td>Anti-β2-Glycoprotein I (U/ml)</td>
<td>16.07±1 (15.09–17.68)</td>
<td>16.35±1 (14.07–18.58)</td>
<td>18.64±1 (16.33–22.00)</td>
</tr>
<tr>
<td>E-selectin ng/ml</td>
<td>9.47±1 (9.00–12.03)</td>
<td>9.92±1 (9.53–12.75)</td>
<td>9.92±1 (9.50–13.55)</td>
</tr>
<tr>
<td>Interlukin-6 (pg/ml)</td>
<td>6.05±1 (5.39–6.83)</td>
<td>8.36±1 (7.20–10.02)</td>
<td>22.35±1 (12.88–67.82)</td>
</tr>
</tbody>
</table>

Values are expressed as median (interquartile range). Corresponding *(No) indicates significance p<0.05 between the two variables. Both anti-CL and anti-β2GPI antibody levels were significantly raised in the CI group over SC and AMC. Anti-CL: CI 2.77 U/ml vs AMC 1.97 U/ml, p = 0.012; anti-β2GPI: CI 18.72 U/ml vs AMC 16.07 U/ml, p = 0.001. Anti-CL: CI 2.77 U/ml vs SC 2.51 U/ml, p = 0.054; anti-β2GPI: CI 18.72 U/ml vs SC 16.35 U/ml, p = 0.037. There were no significant differences in E-selectin levels between the AMC, SC and CI IL-6 levels were significantly elevated in both SC and CI over the control group. IL-6: AMC 6.03 pg/ml vs SC 8.36 pg/ml, p<0.0001; AMC 6.03 pg/ml vs CI 24.88 pg/ml, p<0.0001. There was also a significant increase between SC and CI. IL-6: SC 8.36 pg/ml vs CI 24.88 pg/ml, p<0.02.
compared with controls and stable claudicants (Figs. 2 and 3). Seven patients had pathological levels of anti-beta2GPI antibodies, 6 CI and 1 SC (anti-beta2GPI > 22 U/ml). Seven patients had pathological levels of anti-CL antibodies, 6 CI and 1 SC (anti-CL > 11 GPL/ml). Three of the patients with critical ischaemia had pathological levels of both anti-CL and anti-beta2GPI antibodies (Fig. 1). There was a significant elevation of IL-6 in patients with stable claudication when compared with the control group and a much larger elevation in IL-6 was seen in patients with critical ischaemia compared with controls (Fig. 4). The level of IL-6 seen in patients with critical ischaemia was significantly raised compared with patients with stable claudication.

There was no significant elevation in E-selectin levels in the patients with PAD compared with controls. Stable claudicants and patients with critical ischaemia had similar levels of E-selectin (Table 2).

Each group was independently examined for differences in antibody level, IL-6 and E-selectin level related to sex, smoking, diabetes, MI, CVA, hypertension, previous arterial surgery and previous amputation. No significant differences were found. In addition, as the groups were well matched for sex, age and smoking, these factors were examined using the data as a whole, independent of group to see if they significantly altered antibody, IL-6 and E-selectin levels. No significant difference was found.

Discussion

This study set out to examine the levels of anticardiolipin and anti-beta2-glycoprotein antibodies in patients with PAD and assess their potential pathogenicity in its development and progression. The groups of patients were chosen to represent cohorts with varying severities of atherosclerosis. The patients with critical ischaemia represent a cohort with more severe disease, which may have resulted from an accelerated atherosclerosis. However, there was no significant difference in risk factors such as smoking, hypertension, and diabetes between the two groups. The observation that traditional risk factors do not accurately predict the progression to critical ischaemia is supported by epidemiological data. The prior identification of this cohort would be of great value in clinical practice, allowing aggressive therapy for...
the minority and reassurance for the majority. The studies reported here show that the mean levels of both anti-CL and anti-β2GPI antibody levels were significantly elevated in the patients with critical ischaemia when compared with the stable claudicants and age-matched controls. Furthermore nine of the critically ischaemic patients showed pathological levels of antibodies compared with two stable claudicants and no controls. In addition we have shown that patients with stable claudication and critical ischaemia have progressively higher levels of interleukin-6. This would support our hypothesis that these autoantibodies are elevated in a proportion of patients with more severe PAD and that they may play a role in the accelerated progression of PAD seen in some patients.

Anti-phospholipid antibodies, such as anti-CL antibodies, were initially identified in patients who suffered from increased venous and arterial thrombosis. It has been shown that β2-glycoprotein I is a key co-factor for the recognition of cardiolipin. The antibodies have been found in patients with autoimmune diseases, such as SLE and the anti-phospholipid syndrome, and have been linked to increased cardiovascular disease in these patients. Whilst these antibodies can increase the likelihood of thrombosis, patients with either SLE or APS also show an accelerated atherosclerosis, raising the possibility that these antibodies play a role in the pathogenesis of atherosclerotic disease. High levels of anti-CL have been shown to be an independent risk factor for myocardial infarction and cardiac death in middle aged men. Anti-β2GPI antibodies have been shown to be elevated in patients with ischaemic heart disease compared with controls. In addition, whilst 45% of patients with unstable angina had elevated levels of anti-β2GPI antibody, only 11.8% of patients with stable, effort induced angina showed elevated levels of the antibody, suggesting a role in the progression of the disease. Several studies have shown an association between elevated levels of anti-β2GPI antibodies and ischaemic stroke. Whilst this clinical evidence demonstrates an association between raised anti-CL and anti-β2GPI antibody levels and atherosclerosis, it cannot determine whether these antibodies play a role in the pathogenesis of atherosclerosis.

The pathogenesis of atherosclerosis is a complex process. No single factor can be identified as playing a pivotal role in its development or progression; rather it relies on a complex jigsaw of diverse processes. Anti-CL and anti-β2GPI are just two of a large heterogeneous group of antibodies, which react with the vascular endothelium. These endothelial cell reactive antibodies (ECRA) were first described in 1971. Anti-phospholipid, anti-DNA, ANCA and anti-tubulin antibodies have all been identified as endothelial cell reactive antibodies that can up-regulate pro-inflammatory markers in endothelial cells. The heterogeneity of endothelial autoantigens suggests that reactivity with cardiolipin or β2GPI may only identify a sub-group of autoantibodies that can accelerate atherosclerosis.

Anti-CL and anti-β2GPI antibodies have been shown to induce an endothelial pro-atherogenic phenotype mediated by the expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 and increased secretion of inflammatory cytokines IL-1β and IL-6 in in vitro studies. These studies show that patients with stable claudication had elevated IL-6 levels when compared with controls and that patients with critical ischaemia had significantly higher levels of IL-6 still. This supports the in vitro work and lends weight to our hypothesis that these autoantibodies are contributing to the accelerated progression of disease seen in the small cohort of patients who go on to develop critical ischaemia. The absence of an increase in E-selectin levels was surprising given the strong in vitro evidence that anti-CL and anti-β2GPI antibodies cause up regulation of E-selectin. However, the short term and relatively transient expression of E-selectin may mean that it is not the most suitable marker of chronic inflammation in these patients. In conclusion this study has shown for the first time that significantly elevated levels of anti-CL and anti-β2GPI antibodies are present in PAD patients with the most advanced disease, implicating these antibodies in the progression of PAD. The lack of a direct correlation between patients with elevated anti-CL and anti-β2GPI antibodies and IL-6 demonstrates that further work needs to be done to examine the full panel of ECRA, each of which in vitro, have been shown to cause pro-inflammatory effects on EC and, therefore, have the potential to be pro-atherogenic.

References

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