Platelet Monocyte Aggregates and Monocyte Chemoattractant Protein-1 are not Inhibited by Aspirin in Critical Limb Ischaemia

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Objectives. Platelet monocyte aggregates (PMA) and monocyte chemoattractant protein-1 (MCP-1) play a significant role in atherosclerotic disease but the effect of aspirin and their role in peripheral arterial disease (PAD) requires further investigation. We have compared p-selectin, PMA and MCP-1 in patients with PAD treated with aspirin (75mg daily), with age matched controls not treated with aspirin.

Materials and methods. Using flow cytometry and ELISA, p-selectin, PMA and MCP-1 were compared in 3 populations; healthy controls (n = 12), intermittent claudication (n = 19) and critical limb ischaemia (CLI), (n = 10).

Results. P-selectin was significantly higher in CLI patients (3.48% positive) compared to the claudicants (1.36% positive) and the controls (1.76% positive). PMA levels were significantly higher for CLI population (44.5% positive) compared to the claudicants (20.48% positive) and the controls (28.33% positive). MCP-1 levels expression was significantly higher for the CLI patients (175.4 pg/mL) compared to the claudicants (76.1 pg/mL) and the controls (117.0 pg/mL).

Conclusion. Despite aspirin treatment CLI patients have higher platelet activation and MCP-1 expression than controls and claudicants. With increasing severity of disease aspirin is unable to suppress markers of platelet activation and pro-atherosclerotic chemokine expression which may represent another form of aspirin resistance.

Keywords: Platelets; Platelet monocyte aggregates; Monocyte chemoattractant protein 1; P-selectin; Peripheral arterial disease; Aspirin.

Introduction

Platelet monocyte aggregates (PMA) occur with vascular inflammation and may represent a sensitive marker of platelet activation. Risk factors for cardiovascular disease have been associated with increased PMA expression. Smoking, diabetes and hyperlipidaemia have also been linked to elevated PMA levels.1–3 The PMA is not unique to thrombo-embolic conditions. Its role in sepsis has become increasingly apparent with elevated levels seen in Systemic Inflammatory Response Syndrome.4 In addition PMA’s have been associated with myeloproliferative disorders and chronic venous disease.5–7 Numerous studies have found associations between PMA’s and ischaemic heart disease.10–14 However, the role of the PMA in cerebral-vascular disease is not so well defined with a single study showing elevated levels following acute ischaemic stroke.15 The role of PMA’s in patients with lower limb peripheral arterial disease (PAD) also requires further investigation.

MCP-1 is an inflammatory chemokine released from multiple cell types including endothelial cells, smooth muscle cells and macrophages. MCP-1 recruits mononuclear cells to sites of inflammation, promoting endothelial adhesion and monocyte extravasation through the endothelium.16 This chemokine facilitates chronic inflammation, thrombosis, angiogenesis, oxidative stress and vascular smooth muscle cell proliferation and migration. Animal models knockout for MCP-1, and its receptor CCR2, show significant reduction in the atherosclerotic disease process.17,18 Other work has suggested that MCP-1 may have a role in ischaemic reperfusion injury.19,20 Reduced MCP-1 activity inhibits neointimal hyperplasia and atheroma in the animal model.21 In the presence of activated platelets, cultured human umbilical endothelial cells show increase MCP-1 expression.22 Similar effects have been seen in aortic smooth muscle cells when incubated with α-thrombin stimulated platelets.23 Despite the
recent growing interest in the role of MCP-1 in atherosclerosis, very little work has been published involving PAD.24

PMA’s and inflammatory cytokines may represent novel diagnostic tools and possible therapeutic targets in the treatment and prevention of atherosclerosis. The current study was performed to investigate the expression of PMA, p-selectin and MCP-1 in PAD patients treated with aspirin and to compare them to healthy controls not treated with aspirin.

**Materials and Methods**

**Patients**

The study received local ethical approval and was performed according to the declaration of Helsinki. Informed consent was obtained from all patients prior to their participation. Claudicants were recruited from vascular outpatients and patients with critical limb ischaemia (CLI) selected from the inpatient population. Controls were selected from patients attending hospital for day case hernia repair or minor local anaesthetic procedures for non-inflammatory and non-infective conditions.

**Inclusion and exclusion criteria**

Patients aged between 40 and 85 years were included. Subjects were included into one of three populations; proven PAD (intermittent claudication with ABPI < 0.9 and angiography or duplex proven lower limb disease), proven PAD with CLI (rest pain, ABPI ≤ 0.5, ulceration and angiography or duplex proven lower limb disease), age matched controls (patients attending for day case hernia repair or minor local anaesthetic procedures for non-inflammatory and non-infective conditions).

**Blood collection**

All blood sampling was completed before midday. Venous blood was taken from the antecubital fossa without tourniquet pressure using a 19G needle. The first 5mls was discarded before collection into vacutainers containing 1/10 volume of 3.8% sodium citrate for flow cytometry and MCP1 assays. Plasma was isolated from blood by centrifuge for 10 minutes at 1000 g, and stored at −80 °C until assayed.

**Flow cytometry**

A whole blood two-colour staining technique was used to quantify P-selectin as previously described.25,26 Anti-CD61 antibody, conjugated to a peridinin chlorophyll protein (BD Biosciences, Oxford, UK), was used to identify the platelet population. Anti-CD62P antibody, conjugated to R-phycocerythrin (BD Biosciences, Oxford, UK), was used to quantify platelet surface expression of P-selectin. Isotypic control samples were run in parallel (Y1, BD Biosciences, Oxford, UK). 15000 platelet events were collected and the results expressed as percentage cells positive for Anti-CD62P antibody. PMA estimation was performed as previously described.25,26 Anti-CD45 antibody conjugated to peridinin chlorophyll (BD Biosciences, Oxford, UK) was used as a pan-leukocytic marker. Anti-CD14 antibody conjugated to R-phycocerythrin (Dako, UK) was used to identify the monocyte population from other leukocyte subpopulations. Platelet monocyte aggregates were measured as the percentage of the monocyte population expressing the platelet specific marker GpIIb/IIIa, quantified with fluorescein isothiocyanate conjugated anti-CD61 antibody (Dako, UK). Isotypic control samples were run in parallel (IgG1, Dako, UK). 30,000 events were collected and the results expressed as the percentage cells (monocytes) positive for anti-CD61 antibody.

**Monocyte chemoattractant protein-1 ELISA**

MCP-1 was measured by ELISA using a commercially available kit (R&D systems (Oxon, UK). The assay procedure was fully automated (Best 2000, Biokit Elisa systems, Barcelona, Spain) and performed according to the manufacturers instructions.

**Statistical analysis**

Data was analysed using Minitab (release 13.1). Deviations from Gaussian distribution were assessed using the Anderson-darlington test. A Mann-Whitney test was used for inter-group analysis.

**Results**

12 normal controls, 19 patients with PAD and 10 patients with CLI were recruited. The characteristics, PAD risk factors and medication are summarised in
Table 1. As platelet function varies with age the groups were analysed to assess any significant difference in age and no significant difference was identified \((p > 0.05,\) Mann-Whitney U test). There were significantly more males in the PAD and CLI groups \((p < 0.05,\) Chi^2). 2 of the CLI patients had leg ulcers. All patients with CLI were identified to have significant infra-inguinal disease. Half of this group also had evidence of supra-inguinal disease but confined to the iliac arteries. In the PAD group 11 of the patients had isolated infra-inguinal occlusive disease, 2 had occlusive diseases isolated to the supra-inguinal region and 6 had combined infra and supra-inguinal disease.

**P-selectin (Fig. 1)**

The median percentage positive cells expressing P-selectin was significantly higher in the population with CLI (3.48; IQR 1.9–9.07) compared to the claudicants (1.36; IQR 0.7–2.65; \(p = 0.011\)) and the controls (1.76; IQR 1.38–2.19; \(p = 0.025\)). Despite the claudicant population exhibiting a lower median value compared to the control population. The difference was not statistically significant \((p = 0.53)\).

**PMA (Fig. 2)**

The median percentage positive cells was higher for the population with CLI (44.5; IQR 30.83–80.68) compared to the claudicant population (20.48; IQR 17.34–28.59) and the control population (28.33; IQR 20.63–35.93). The CLI population were significantly higher than the claudicant population \((p = 0.004)\) and the control population \((p = 0.036)\). There was no statistically significant difference between the control and claudicant populations \((p = 0.103)\).

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**Table 1. Baseline characteristics claudicants, CLI and controls**

<table>
<thead>
<tr>
<th></th>
<th>Age Matched Controls (n = 12)</th>
<th>Claudicants (n = 19)</th>
<th>CLI (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, range)</td>
<td>66.5 (49–82)</td>
<td>64 (53–81)</td>
<td>68.5 (56–81)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>6/6</td>
<td>16/3</td>
<td>10/1</td>
</tr>
<tr>
<td>Ankle brachial pressure index (range)</td>
<td>1.0 (1.0–1.2)</td>
<td>0.6 (0.5–0.8)</td>
<td>0.4 (0.0–0.5)</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, (n,) %</td>
<td>0 (0)</td>
<td>10 (53)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Hypercholesterolaemia, (n,) %</td>
<td>2 (17)</td>
<td>11 (58)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>Hypertension, (n,) %</td>
<td>3 (25)</td>
<td>9 (47)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Ischaemic Heart disease, (n,) %</td>
<td>0 (0)</td>
<td>5 (26)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-blockers, (n,) %</td>
<td>0 (0)</td>
<td>3 (16)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Calcium channel blockers, (n,) %</td>
<td>3 (25)</td>
<td>6 (32)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Statins, (n,) %</td>
<td>1 (8)</td>
<td>9 (47)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>Ace inhibitors, (n,) %</td>
<td>0 (0)</td>
<td>4 (21)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Diuretics, (n,) %</td>
<td>2 (17)</td>
<td>5 (26)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>Nitrates, (n,) %</td>
<td>0 (0)</td>
<td>2 (11)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0 (0)</td>
<td>19 (100)</td>
<td>10 (100)</td>
</tr>
</tbody>
</table>
MCP-1 (Fig. 3)

The median value for MCP-1 expression was maximal for the critical ischaemic population (175.4 pg/mL; IQR 156.4–255.2). The control population (117 pg/mL; IQR 81.91–132.9) showed higher median values than the claudicant population (76.1 pg/mL; IQR 72.04–85.38). The CLI population was significantly higher than the claudicant ($p < 0.0001$) and the control population ($p = 0.0005$). The difference between the claudicant and control populations was also statistically significant ($p = 0.0013$).

Discussion

Although platelet hyperactivity has been previously documented in PAD, this is the first study to compare the expression of MCP-1, PMA and p-selectin amongst these populations.\textsuperscript{27,28}

In this study platelet P-selectin was seen to be elevated in patients with CLI, despite treatment with aspirin. In the claudicant population treated with aspirin, the expression was comparable to age matched controls not treated with aspirin. The pattern of platelet monocyte aggregate expression mirrored that of p-selectin with the highest levels being seen in the population of CLI and the claudicants displaying levels comparable to age matched controls. P-selectin is known to be important in the PMA interaction and hence both tests yielding comparable results adds strength to this finding. However, these findings suggest that aspirin may offer some benefit in preventing platelet monocyte aggregate formation, possibly via inhibition of p-selectin in the claudicant population. In more advanced PAD the expression of p-selectin may not be so well suppressed and hence PMA expression is elevated. The effect of aspirin on PMA requires further study. Although platelet-leukocyte aggregates have not been shown to be suppressed by aspirin a single study in CVA patients suggests that PMA expression is reduced in aspirin treated patients.\textsuperscript{29,30}

The expression of P-selectin did not differ significantly between claudicants and controls which is comparable to previously published results.\textsuperscript{28}

The expression of MCP-1 was significantly higher in the CLI population but surprisingly lower in the claudicant population. Although MCP-1 levels have been studied in the context of CAD, only three studies have compared this chemokine in PAD and non PAD controls, and all showed elevated levels in PAD.\textsuperscript{24,31,32} None of these studies fully define the proportion of their population treated with aspirin and contained populations with varying severity of lower limb PAD. In our study the PAD population is separated into claudicants and CLI and both PAD populations were treated with aspirin. Furthermore, the use of statin therapy is less clearly defined in other published work which may offer some explanation to the differences seen between this study and other published work.\textsuperscript{34}

The effect of hyperlipidaemia and statin therapy on MCP-1 expression has been previously published.\textsuperscript{33} A greater proportion of the CLI population had a history of hypercholesterolaemia compared to the PAD group. However, treatment with statin therapy was also greater in this population and hence it is unlikely that the MCP-1 levels can be attributed to the incidence of hypercholesterolaemia alone. The incidence of smoking was also greater in the population with CLI. This may offer some explanation towards the elevated PMA levels seen in this group.

The action of aspirin is not limited to the platelet. It has been shown to alter the expression of chemokines involved in atherosclerosis. For example the endothelial expression of MCP-1 is suppressed by aspirin in the animal model.\textsuperscript{34,35} Measurement of aspirin resistance has always focused on its antiplatelet effects but no work has described resistance in terms of its other actions. In this study as well as elevated platelet markers in the CLI population we have shown significantly elevated MCP-1 and its relevance needs further investigation. MCP-1 has been linked to atherosclerotic disease and atherosclerotic risk factors and hence the high levels seen with advanced lower limb PAD are to be expected.\textsuperscript{36} However we would

![Fig. 3. MCP-1 expression in claudicants, CLI and age matched controls. The box extends from the 25th percentile to the 75th percentile, with a horizontal line to represent the median. The whiskers show the highest and lowest values (*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$ Mann-Whitney test).](image-url)
have expected the intermittent claudication group to also show high levels compared to the control group, unless aspirin was able to significantly suppress the expression of MCP-1.

This study suggests that patients with CLI have greater platelet activation than controls and claudicans and is in agreement with other studies using different methods to assess platelet activation. Furthermore, aspirin is able to suppress some of the markers of platelet activation but with increasing severity of disease its actions are overwhelmed with evidence of increasing platelet activation and elevated levels of the pro-atherosclerotic chemokine MCP-1. Whether this is a result of increase atherosclerotic disease load or a greater incidence of atherosclerotic risk factors cannot be concluded from this study. However, the results provide an argument for additional antiplatelet agents in patients with CLI and further work is required to assess the potential benefits of both alternative and combination antiplatelet therapy.

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References


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