Increased Platelet Aggregation and Activation in Peripheral Arterial Disease

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Objectives: patients with peripheral arterial disease (PAD) have a threefold increase in cardiovascular mortality. Standard antiplatelet treatment may not confer uniform benefit in different patient groups. This study aimed to compare platelet function in patients with lower limb PAD, carotid disease and abdominal aortic aneurysm (AAA) with age- and sex-matched healthy controls.

Methods: patients with lower limb PAD (n = 20), carotid disease (n = 40), AAA (n = 13) and age/sex matched healthy controls (n = 20) were studied. Whole blood methods to detect spontaneous platelet aggregation (SPA), and adenosine diphosphate (ADP) and collagen-induced aggregation were used. The detection of platelet P-selectin and the PAC-1 antigen by flow cytometry were also used as markers of platelet activation and aggregation.

Results: patients with lower limb PAD or AAA had higher baseline SPA compared to normal controls (p < 0.01). There was significantly higher collagen-induced aggregation in IC patients compared to normal controls (p < 0.01). However, there was no difference in ADP-induced aggregation between lower limb PAD and control patients. There was no difference in PAC-1 binding between control patients and the patients with lower limb PAD, carotid disease or AAA. Patients with carotid disease had a higher expression of P-selectin compared to normal controls (p < 0.05).

Conclusions: this study provides further evidence that platelet hyperactivity is present in patients with PAD despite the use of antiplatelet therapy. Further antiplatelet strategies may be indicated to protect these patients.

Key Words: Activation; Aspirin; Platelets; Peripheral Arteries.

Introduction

Activated platelets have been demonstrated in a wide variety of clinical settings including peripheral arterial disease (PAD) despite the use of aspirin.¹⁻⁸ Patients with intermittent claudication (IC) are at particular risk of ischaemic episodes occurring in the peripheral, coronary and cerebral circulation and the presence of PAD is a strong indicator for systemic atherosclerosis.⁹⁻¹¹ Compared with age-matched controls, patients with IC have a threefold increase in cardiovascular mortality and although most patients are on some form of antiplatelet treatment, its benefit seems not to be uniform in different PAD patient groups.¹⁰⁻¹⁴ Antiplatelet therapy usually with aspirin (75–150 mg), can reduce the risk of cardiovascular events.¹⁵,¹⁶ However platelet aggregation and thrombosis can still occur in the presence of aspirin as there are other important pathways for platelet activation that are not affected by the blockade of cyclo-oxygenase.⁷,¹⁷,¹⁸ There may also be a group of patients resistant to aspirin or with significant platelet hyperactivation prior to any intervention despite being on antiplatelet therapy.⁸,¹⁹,²⁰ These patients may be at higher risk for ischaemic complications. The aim of the current study was to assess platelet function in a range of patients with PAD and compare it with healthy age and sex matched controls.

Materials and Methods

Patients and controls

Twenty control patients were studied to measure baseline spontaneous platelet aggregation (SPA) and

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* Please address all correspondence to: G. Stansby, Northern Vascular Unit, The Freeman Hospital, High Heaton, Newcastle upon Tyne, NE7 7DN, U.K.
aggregation in response to ADP and collagen. Platelet flow cytometry was also used to quantify P-selectin and PAC-1 binding. These patients were recruited from general surgical outpatient clinics and risk factors for vascular disease (i.e. smoking, hypertension, hyperlipidaemia, ischaemic heart disease, diabetes, IC, carotid disease and AAA) were excluded by history, clinical examination, routine blood testing, resting ankle/brachial systolic pressure index (ABPI) measurements and duplex scanning (ATL HDI 3000 ultrasonic device, Bothell, Wash) (Table 1). Patients on cardiac, antihypertensive, anticoagulation or antiplatelet medication were excluded from the control group.

Twenty patients with documented rest pain or IC and with a resting ABPI ≤ 0.85 were recruited from a vascular surgical ward and outpatient clinic. Exclusion criteria for all subjects included concomitant diabetes mellitus, malignant or infectious processes, venous disease, recent ischaemic cardiovascular event, significant hepatic or renal disease, and warfarin or systemic heparin therapy, as these factors are known to modify platelet function. All patients were on antiplatelet therapy, typically aspirin (75−150 mg) or dipyridamole (400 mg) for at least 1 month prior to entering the study (Table 1). Forty consecutive patients awaiting carotid endarterectomy (CEA) and 13 patients awaiting infra-renal abdominal aneurysm repair also participated in this study. All patients gave their informed consent. Approval was obtained from the local regional ethical committee.

Blood sampling

All subjects rested supine prior to and during venepuncture. Clean peripheral venepuncture was performed with minimal stasis using a 21-gauge butterfly needle with a double syringe method and a light tourniquet; conditions designed to minimise artefactual platelet activation. Blood was sampled from patients awaiting surgery the day before surgery. On each occasion, the first 3 ml of blood was discarded and thereafter, 20 ml of blood was collected into a 1/10-vol of 3.8% trisodium citrate anticoagulant for aggregometry and flow cytometric studies.

Table 1. Baseline characteristics of patients and controls.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Lower limb PAD patients (n = 20)</th>
<th>Carotid patients (n = 40)</th>
<th>AAA patients (n = 13)</th>
<th>Control patients (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (Range)</td>
<td>61 (55−81)</td>
<td>68 (50−78)</td>
<td>71 (56−81)</td>
<td>68 (50−78)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>12/8</td>
<td>30/10</td>
<td>10/3</td>
<td>15/5</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>15 (75)</td>
<td>16 (40)</td>
<td>8 (62)</td>
<td>0</td>
</tr>
<tr>
<td>High cholesterol, n (%)</td>
<td>10 (50)</td>
<td>12 (30)</td>
<td>1 (8)</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>13 (65)</td>
<td>25 (63)</td>
<td>9 (69)</td>
<td>0</td>
</tr>
<tr>
<td>Coronary disease, n (%)</td>
<td>12 (60)</td>
<td>13 (33)</td>
<td>8 (62)</td>
<td>0</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>18 (90)</td>
<td>36 (90)</td>
<td>8 (62)</td>
<td>0</td>
</tr>
<tr>
<td>Dipyridamole, n (%)</td>
<td>13 (65)</td>
<td>2 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>3 (15)</td>
<td>6 (15)</td>
<td>4 (31)</td>
<td>0</td>
</tr>
<tr>
<td>β-blockers, n (%)</td>
<td>5 (25)</td>
<td>12 (30)</td>
<td>9 (69)</td>
<td>0</td>
</tr>
<tr>
<td>ACE inhibitor, n (%)</td>
<td>2 (10)</td>
<td>16 (40)</td>
<td>7 (54)</td>
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<tr>
<td>PAD Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Claudication, n (%)</td>
<td>14 (70)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Rest pain, n (%)</td>
<td>6 (30)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carotid disease</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic, n (%)</td>
<td>0</td>
<td>35 (88)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Asymptomatic, n (%)</td>
<td>0</td>
<td>5 (12)</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

PAD = peripheral arterial disease, AAA = abdominal aortic aneurysm.

Flow cytometric analysis of platelet activation

The methodology for whole blood flow cytometric assay preparation was as outlined by Shattil et al. and in accordance with the European working group...
on cell cycle analysis consensus protocol for the flow cytometric characterisation of platelet function. Details of the methodology is described in an earlier report.

**Antibodies for flow cytometry**

The following murine monoclonal antibodies were used: fluorescein isothiocyanate (FITC) conjugated PAC-1, peridinin chlorophyll-a protein (PerCP)-conjugated CD61, FITC conjugated anti-CD42a and phycoerythrin (PE)-conjugated anti-CD62P. All were obtained from Becton Dickinson, Oxford, U.K. A double staining method was used where platelet samples were stained with combinations of CD42a/CD62P and CD61/PAC-1 FITC.

All samples were analysed within 2 h of preparation in a Becton Dickinson FACScan flow cytometer. The platelet population was identified by means of its light scatter characteristics and data was obtained with gain settings in the logarithmic mode from 5000 platelets collected from each sample. The saved list mode files were then analysed by LYSIS II software (FACScan, Becton Dickinson Immunocytometry Systems, Oxford, U.K.). Using two-colour analysis, platelet events were triggered for cells expressing CD42a (Gp1b) or CD61PerCP (GpIIb) only. Platelet activation was measured by determining the percentage of platelets (CD42a positive events) expressing CD62PE and the percentage of platelets (CD61 positive events) expressing PAC-1 FITC.

**Statistical analysis**

Data analysis was performed using GraphPad Prism Software. All results are shown as medians with scatter plots. Differences between the groups were analysed using the unpaired non-parametric Mann-Whitney U-test. Differences were considered significant at \( p < 0.05 \).

**Results**

Figure 1 shows SPA at 3 min in patients with carotid disease, AAA and lower limb PAD compared with normal controls. There was higher baseline SPA in patients with lower limb PAD \(( p < 0.01 \) or AAA \(( p < 0.05 \) compared to normal controls. There was no significant difference between patients with carotid disease and normal controls.

Figure 2 shows SPA, ADP (1\( \mu \)M)- and collagen (1\( \mu \)g/mL)-induced platelet aggregation in control and lower limb PAD patients. There was significantly higher collagen-induced aggregation in lower limb PAD patients compared to normal controls \(( p < 0.01 \). However, there was no difference in ADP-induced aggregation between lower limb PAD and control patients.

Figure 3 shows the percentage of PAC-1 binding (specific for the activated form of GpIIb/IIIa) detected by flow cytometry in control patients and patients with carotid disease, AAA and lower limb PAD. There was no difference in PAC-1 binding between control patients and the patients with lower limb PAD, carotid disease or AAA.

P-selectin expression detected by flow cytometry is shown in Figure 4. Patients with carotid disease had a higher expression of P-selectin compared to normal controls \(( p < 0.05 \). There was no difference in P-selectin expression between the patients with lower limb PAD or AAA and normal controls.

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**Figure 1.** Spontaneous platelet aggregation (SPA) at 3 min in CEA \(( n = 40 \), AAA \(( n = 13 \) and lower limb PAD \(( n = 20 \) patients compared with normal controls \(( n = 20 \). Data is shown as medians with scatter plots \(( ^* p < 0.01 \), \( ^{**} p < 0.001 \) Mann–Whitney U-test). (CEA = carotid endarterectomy, AAA = abdominal aortic aneurysm, PAD = peripheral arterial disease).
Controls

Fig. 2. Graph of spontaneous platelet aggregation (SPA), ADP- and collagen-induced platelet aggregation in control (n = 20) patients compared with lower limb PAD (n = 20) patients. Data is shown as medians with scatter plots (* * p < 0.01 Mann–Whitney U-test).

Fig. 3. PAC-1 expression (%) in CEA (n = 40), AAA (n = 13) and lower limb PAD (n = 20) patients compared with normal controls (n = 20). Data is shown as medians with scatter plots (p = not significant, Mann–Whitney U-test). (CEA = carotid endarterectomy, AAA = abdominal aortic aneurysm, PAD = peripheral arterial disease).

Fig. 4. P-selectin expression (%) in CEA (n = 40), AAA (n = 13) and lower limb PAD (n = 20) patients compared with normal controls (n = 20). Data is shown as medians with scatter plots (* p < 0.05 Mann–Whitney U-test). (CEA = carotid endarterectomy, AAA = abdominal aortic aneurysm, PAD = peripheral arterial disease).
Discussion

This study has shown that patients with lower limb PAD or AAA have a higher degree of platelet aggregation in vivo compared to normal age- and sex-matched controls. This was not seen in patients with carotid disease and may reflect the greater extent of atherosclerotic disease in patients with lower limb PAD and AAA. These differences were demonstrated despite patients with PAD taking antplatelet therapy mainly in the form of aspirin. When ADP or collagen were used in small concentrations, there was greater agonist-induced aggregation in patients with PAD compared to controls. This has important implications in patients with PAD undergoing angioplasty or any surgical procedure where intimal damage is produced. This would expose the subendothelial matrix to hyperactive platelets and trigger the sequence of platelet adhesion and activation. These patients might then be at increased risk of thrombosis or embolic complications.

Patients with carotid disease had higher P-selectin expression but not PAC-1 binding (which indicates activation of the GPIIb/IIa receptor). Most of these patients had been recently symptomatic from their carotid disease and this may explain the higher expression of platelet P-selectin since Grau et al. have previously shown an increased fraction of circulating activated plasma P-selectin in acute and previous cerebrovascular ischaemia. There was no difference in PAC-1 binding or P-selectin expression in patients with lower limb PAD or AAA compared to normal controls. This may be due to the reversible expression of some or all of the surface neoepitopes associated with the platelet activation. Surface expressed P-selectin is rapidly cleaved to release soluble P-selectin into the plasma. Thus, the levels of P-selectin on the platelet surface may not be a true reflection of the in vivo activation state of the circulating platelets. Several clinical studies have demonstrated elevated plasma levels of soluble P-selectin associated with acute and chronic atherosclerotic syndromes including PAD.

Galt et al. have also compared platelet function in patients with severe PAD and age-matched controls. Like us no they found no difference in baseline P-selectin expression between PAD and healthy age-matched control subjects. However, since α-degranulation is a late event in the process of platelet activation, P-selectin expression may be a late marker of platelet activation. In contrast to the findings of Galt et al., we found that whole blood platelet aggregation was significantly enhanced in PAD patients. This difference may be explained by the use of whole blood methods to study platelet aggregation and avoiding the limitations described with using PRP.

The results of SPA and agonist-induced aggregation in lower limb PAD patients in this study suggest that these circulating platelets maintain a heightened activation status. It may be inferred that these platelets possess a lower threshold for aggregation. There are a number of possible explanations for this hyperactivated status despite the use of aspirin. Firstly, it may be a consequence of multilevel atherosclerosis inducing platelet activation through increased shear stresses generated at sites of arterial stenosis or via elevated plasma concentrations of platelet agonists secondary to platelet-plaque interactions. Secondly, it may reflect the influence of risk factors such as smoking, type IIa hypercholesterolaemia or hypertension which by themselves persistently activate platelets. Davi et al. provide data suggesting that the metabolic and haemodynamic disturbances exerted by these risk factors and not diffuse atherosclerotic lesions per se provide the major triggers of persistent platelet activation in PAD. Thirdly, it is possible that this hyperactivated status may result from the increased presence of larger, younger reticulated platelets in PAD as a result of increased consumption at the level of the atherosclerotic plaque. Increased reticulated platelet counts have been demonstrated in some conditions associated with hyperactivated platelets and these reticulated platelets have been shown to be hypereactive. Finally it may be that a proportion of these patients have platelets which are relatively resistant to aspirin. Unfortunately, the present study was not designed to identify the relative contributions of these mechanisms but platelet hyperactivation in PAD is most likely to result from a complex combination of these factors.

Platelet inhibition is not a uniform process. In this study platelet aggregation was increased in PAD patients despite the use of aspirin. Only some aspects of platelet activity are inhibited by aspirin which acts mainly on endoperoxide synthesis, reducing TXA2 release from platelets. In contrast, ticlopidine and clopidogrel inhibit ADP-induced platelet aggregation, which is a relatively aspirin resistant process. Clopidogrel also inhibits platelet aggregation induced by thrombin, TXA2 and collagen. This multiplicity of actions reflects the central role of ADP (released from activated platelets) in mediating and amplifying the aggregation induced by several agonists.

In summary, the results of the current study suggest there may be a group of patients with significant platelet hyperactivation prior to any intervention despite being on aspirin. These patients may be at higher risk of thromboembolic complications. The increased
effectiveness of clopidogrel compared to aspirin in the CAPRIE study may be related to its inhibition of ADP-induced platelet aggregation.14 This form of platelet activation may be important in PAD and may explain the observed aspirin resistance in these patients. Since TXA2 and ADP contribute to thrombogenesis, simultaneous inhibition of the two pathways, with clopidogrel plus aspirin, might add significantly to the proven benefit of aspirin alone. Evidence of the efficacy of the combination of aspirin and ticlopidine or clopidogrel has recently been found in recent trials of patients undergoing coronary stenting.43–45 Similar clinical studies in PAD are still awaited.

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References


