Platelet activation is increased in peripheral arterial disease

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Objective: **Platelet activation was assessed in patients with peripheral arterial disease compared with healthy control subjects.**

Methods: **This prospective comparative study included 100 subjects: 40 consecutive patients with intermittent claudication, 20 consecutive patients with critical ischemia and tissue loss, and 40 healthy control subjects. Whole blood flow cytometric analysis was performed to determine resting and stimulated platelet P-selectin expression and resting and stimulated platelet fibrinogen binding. Results are presented as platelet percentage and also as mean fluorescence intensity.**

Results: **P-selectin expression was significantly increased in patients with intermittent claudication (median, 0.85%; range, 0.31%-4.77%; $P = .023$) and critical ischemia (median, 1.11%; range, 0.2%-3.26%; $P = .028$) compared with control subjects (median, 0.59%; range, 0.16%-4.58%). The percentage of platelets binding fibrinogen was also significantly higher in patients with intermittent claudication (median, 2.89%; range, 1.08%-9.59%; $P < .001$) compared with control subjects (median, 1.57%; range, 0.17%-10.7%). There was no significant difference in percentage of platelet fibrinogen binding between control subjects and patients with critical ischemia. Fibrinogen binding by stimulated platelets was significantly diminished in patients with critical limb ischemia compared with control subjects (67.2% vs 77.9%; $P = .006$).**

Conclusions: **Platelet activation is increased in patients with peripheral arterial disease, suggesting an underlying prothrombotic state. Platelets from patients with critical limb ischemia are less responsive to in vitro stimulation. (J Vasc Surg 2003;38:99-103.)**

Patients with peripheral arterial disease (PAD) are at significantly increased risk for cardiovascular and cerebrovascular events. Mortality from cardiovascular disease is two to three times higher in patients with intermittent claudication compared with an age-matched and sex-matched population.[1] In the Edinburgh Artery Study almost 20% of patients with intermittent claudication were dead within 5 years,[2] 13.7% from cardiovascular causes. In 1998 the Joint British Recommendations on Prevention of Coronary Heart Disease in Clinical Practice stated that patients with peripheral vascular disease were "just as likely to die from a heart attack as many patients who have survived their first myocardial infarction" and recommended that treatment should be the same as for patients with established coronary heart disease.[3] Abnormal platelet function has been implicated in development and progression of atherosclerosis and in pathogenesis of acute ischemic events. However, although abnormalities in both coagulation and fibrinolysis have been convincingly demonstrated in patients with PAD,[4-8] the role of platelet activation in this patient group remains controversial.

Whole blood flow cytometry has emerged as a superior tool for investigation of platelet function and in vivo platelet activation, mainly because, unlike platelet aggregometry and plasma assays, it does not involve artifactual platelet stimulation with centrifugation and stirring.[9-11] However, the two major articles published on flow cytometric assessment of platelet function with P-selectin expression in patients with PAD yielded conflicting results.[12,13]

P-selectin is a granule membrane glycoprotein (Gp) that in resting platelets is found in the membrane of alpha granules.[14] After platelet stimulation, a release reaction from the granules occurs, and P-selectin is transferred to the plasma membrane through membrane fusion.[15]

A P-selectin–specific monoclonal antibody binds only to activated degranulated platelets. By binding a fluorochrome dye to this antibody, flow cytometric analysis can enable identification of those platelets in the population that are activated.

Platelet activation also causes transformation of the surface GpIIb/IIIa from an inactive to an active state, which enables ligand binding.[16-18] Through distinct amino acid sequences, fibrinogen is bound to GpIIb/IIIa.[19] With a labeled specific anti-fibrinogen antibody, platelet fibrinogen binding can be reliably measured with flow cytometry[20] as a marker of platelet activation.

The goal of this study was to determine whether platelet activation is increased in patients with PAD compared

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Competition of interest: none.

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with control subjects, with flow cytometric assessment of P-selectin expression and fibrinogen binding.

METHODS

Subject selection. Participants in the study were recruited from patients referred to the Vascular Unit, Aberdeen Royal Infirmary. Patients were eligible for inclusion if they had symptoms of intermittent calf claudication or critical limb ischemia with tissue loss and supportive findings at clinical examination and noninvasive laboratory investigations of peripheral vascular disease. Exclusion criteria included hemoglobin concentration less than 100 g/L; platelet count less than 150×10^9/L; creatinine concentration more than twice the upper limit of normal; aspartate aminotransferase, alkaline phosphatase, or γ-glutamyl transferase concentration more than three times the upper limit of normal; and body mass index exceeding 33. In addition, patients with a history of hematologic malignancy, acute illness unrelated to PAD within 14 days, transfusion of whole blood within 14 days, known or suspected alcohol or drug abuse, or receiving warfarin sodium, steroid, or clopidogrel therapy were excluded. Patients with diabetes were excluded from the claudication group. Forty patients with intermittent calf claudication and 20 patients with tissue loss were identified and recruited for the study. All patients with intermittent claudication and critical ischemia were using aspirin when their blood was sampled.

Healthy control subjects were recruited from patients referred to the Vascular clinic with varicose veins but no history of ischemic heart disease, cerebrovascular disease, or peripheral vascular disease. All control subjects had a normal ankle-brachial pressure index (ABI). Forty control subjects were identified and recruited for the study. None of the control subjects were using aspirin.

Informed consent was obtained from each subject, and full ethical approval for the study was sought and granted by the Grampian Research Ethics Committee.

Sample preparation and flow cytometry. Blood samples were collected with a 21-gauge needle inserted into an antecubital vein, with the cuff applied to the upper arm. The cuff was removed when the first trickle of blood appeared in the first of two 1:10 3.2% sodium citrate Vacutainers. For measurement of platelet activation, 50 μL of blood was immediately transferred from the second sodium citrate container with a micropipet and diluted in 450 μL of HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) magnesium buffer (10 mmol/L of HEPES, 145 mmol/L of sodium chloride, 5 mmol/L of potassium chloride, 1 mmol/L of magnesium sulfate, pH 7.4), and thus blood used for flow cytometry was taken without a cuff.

The method of analysis was adapted from that described previously.[20] Samples were treated in a standard manner within 1 hour of collection, as follows: 40 μL aliquots of whole blood in HEPES magnesium buffer were incubated with monoclonal antibodies for 20 minutes at room temperature in the dark. Platelets were labeled with fluorescein isothiocyanate (FITC)–conjugated anti-CD61 (Dako, Glostrup, Denmark), which binds to GpIIIa. P-selectin expression was measured with dual staining with FITC–anti-CD61 and phycoerythrin (PE)–conjugated anti-CD62P (Immunotech, Marseilles, France). Fibrinogen binding was measured with single-color flow cytometry with FITC-conjugated rabbit polyclonal anti-human fibrinogen antibody (Dako). An optimal final concentration of 2 ng/μL of each antibody was used. Reactions were stopped with addition of 1 mL of phosphate-buffered saline solution. Samples were analyzed within 4 hours of preparation with a Coulter XL-MCL flow cytometer (Coulter Electronics, Luton, England). Platelets were gated by their side and forward light scatter characteristics and enclosed in an electronic bitmap. Listmode data were stored and processed with a personal computer and SYSTEM II software version 1.0 (Coulter Electronics), and converted to scatterplots and histograms. Ten thousand events were analyzed for fluorescence, and the results were expressed as percentage of platelets positive for P-selectin and binding of fibrinogen. Mean cell fluorescence as a measure of the average density of antibody binding per platelet was recorded. Basal activation status was assessed in resting samples and platelet reactivity after incubation with adenosine diphosphate (ADP) at a final concentration of 1×10^{-5} mol/L for 5 minutes (Sigma, St Louis, Mo). The flow cytometer was aligned daily with Flowcheck and Immunobrite beads (Coulter Electronics) to calibrate light scatter and fluorescence parameters, respectively. The flow cell was cleaned thoroughly between individual subject samples to exclude carryover of platelets to subsequent analyses with Coulter Clenz cleansing solution (Coulter Electronics). Murine FITC-conjugated and PE-conjugated isotype controls (Becton Dickinson, Oakville, Canada,) were used to adjust for nonspecific antibody binding.

Statistical analysis. Calculations were performed with SPSS for Windows, version 10.0, statistical software (SPSS, Chicago, Ill). Differences between data for patients and control subjects were analyzed with the Mann-Whitney *U* test. $P < .05$ was considered significant. Values greater than 3 SD from the mean were excluded to avert the influence of artifactual platelet activation.

RESULTS

Study population. One hundred persons were recruited for the study. Mean age was 52.7 years for control subjects, 66.6 years for patients with claudication, and 74.5 years for patients with critical ischemia. Sex distribution, smoking habits, and mean ABI for the study population are shown in the Table.

P-selectin expression. Percentage of platelets expressing P-selectin was significantly higher in patients with intermittent claudication (median, 0.85%; range, 0.31%-4.77%; $P = .023$) and critical ischemia (median, 1.11%; range, 0.2%-3.26%; $P = .028$) compared with control subjects (median, 0.59%; range, 0.16%-4.58%). These differences were still significant when the only extreme value was included in the analysis (claudication group, no

Patient characteristics

	Control subjects (N = 40)		*Patients with claudication (N = 40)*		*Patients with critical ischemia (N = 20)*	
	N	*%*	*N*	*%*	*N*	*%*
Age (y)						
Mean	52.7		66.6		74.5	
Range	24-79		43-80		63-80	
Sex						
Male	16	40	34	85	12	60
Female	24	60	6	15	8	40
Smoking history						
Smoker	10	25	13	32.5	8	40
Stopped smoking <1 y	0		8	20	2	10
Stopped smoking >1 y	15	37.5	14	35	8	40
Never stopped	15	37.5	5	12.5	2	10
Ankle-brachial pressure index	0.95		0.60		0.46	

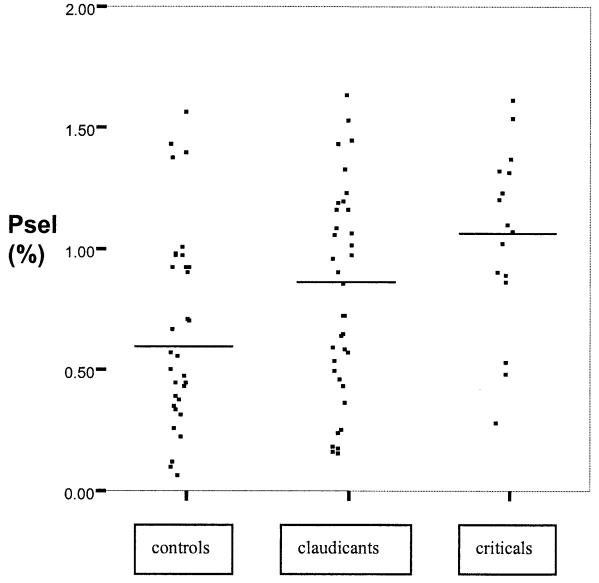

Fig 1. Scatterplot of percentage platelet P-selectin (*Psel*) expression in control subjects, patients with claudication, and patients with critical ischemia. *Horizontal lines*, median values; one extreme value has been excluded.

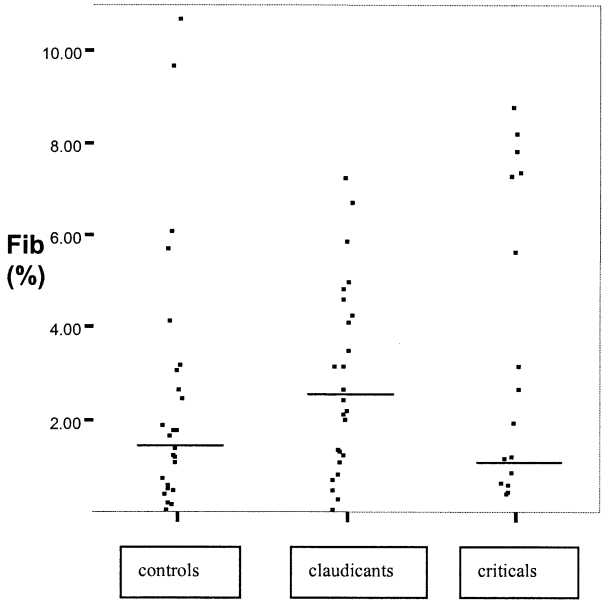

Fig 2. Percentage platelet bound fibrinogen (*Fib*) in control subjects, patients with claudication, and patients with critical ischemia. *Horizontal lines*, median values; five extreme values have been excluded.

change; critical ischemia group, no change; control group, median, 0.6%, range, 0.16%-12.9%; $P = .039$ and $P = .045$, respectively). Results for P-selectin expression are shown in Fig 1. Median P-selectin mean fluorescence intensity was 1.585 for control subjects, 1.44 for patients with claudication, and 1.505 for patients with critical ischemia. There was no difference in mean fluorescence intensity between the three groups. Although percentage of platelets expressing P-selectin was higher in patients with critical ischemia compared with patients with claudication, the difference was not statistically significant.

Bound fibrinogen. Percentage of platelets binding fibrinogen was significantly higher in patients with intermittent claudication (median, 2.895%; range, 1.08%-9.59%) compared with control subjects (median, 1.57%; range, 0.17%-10.7%; $P < .001$). This difference was still highly significant when the five extreme values were included in the analysis (claudication group, median, 2.9%, range, 1.08%-78.3%; control group, median, 1.58%; range, 0.17%-76.3%; $P = .001$). However, there was no significant difference between percentage of platelets binding fibrinogen in patients with critical ischemia (median, 1.3%; range, 0.09%-8.27%) compared with control subjects ($P = .45$). Results of fibrinogen binding to platelets in the three groups is shown in Fig 2. Median bound fibrinogen mean fluorescence intensity was 2.75 for control subjects, 2.55 for patients with claudication, and 2.355 for patients with

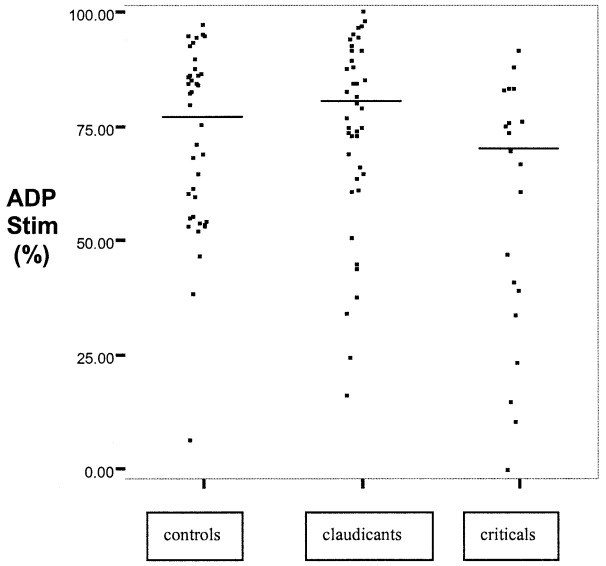

Fig 3. Percentage platelet bound fibrinogen at adenosine triphosphate stimulation (*ADP Stim*) in control subjects, patients with claudication, and patients with critical ischemia. *Horizontal lines*, median values.

critical ischemia. On ex vivo stimulation of platelets with ADP, patients with critical ischemia exhibited significantly diminished platelet fibrinogen binding (median, 67.15%; range, 1.52%-89.8%) compared with control subjects (median, 77.95%; range, 8.63%-97.6%; $P = .006$) (Fig 3). There was no difference between control subjects and patients with claudication (median, 78.3%; range, 18.6%-97.6%; $P = .31$) in platelet fibrinogen binding after stimulation with ADP.

DISCUSSION

The increased incidence of major vascular events in patients with PAD and the central role of platelets in these events raise the question of whether platelets in these patients circulate in a "primed" or activated state. We found a significantly higher percentage of platelets expressing P-selectin in patients with PAD compared with control subjects. In addition, the proportion of platelets binding fibrinogen in patients with intermittent claudication was significantly higher than in control subjects. There was, however, no difference in resting platelet fibrinogen binding between control subjects and patients with critical ischemia.

Flow cytometric studies of platelet activation in patients with PAD have yielded conflicting results.[12,13] Only the study by Galt et al[12] used percentage platelet expression of P-selectin as a platelet activation marker. Those authors found no difference in percentage platelet P-selectin expression between 15 control subjects and 16 patients with severe PAD. However, the technique used for sample preparation involved significant blood handling, which inevitably results in excessive platelet activation in vitro. Indeed, the results obtained by Galt and colleagues for percentage of platelets expressing P-selectin (11% for patients with PAD, 13% for control subjects) is more than 10-fold higher than those obtained in our study. Other researchers using flow cytometric analysis of P-selectin expression in patients with stroke[21] and coronary heart disease[22] have reported percentage platelet expression between 0.35 and 4.7, similar to our figures. Use of whole blood flow cytometry of platelets minimizes the contribution of in vitro activation and may reflect more closely the in vivo condition. Extreme care was taken during blood sampling and processing to minimize blood handling and artifactual platelet activation.

Koksch et al[13] used an improved technique for flow cytometric analysis of platelet activation. They found that P-selectin expression on both ADP-stimulated and nonstimulated platelets was significantly increased in patients with PAD compared with control subjects. However, that group reported only fluorescence intensity of platelets rather than actual percentage of activated platelets in the peripheral circulation, and their conclusions were based on these measurements. Mean fluorescence intensity alone is not a realistic reflection of proportion of activated platelets in the circulation, because it is simply a measure of the mean quantity of antibody bound to each platelet, and in our study no difference was noted between the three groups. The control subjects in the study by Koksch et al[13] were younger than the patients with PAD, as was also the case in our study. Platelet function and coagulation activity, however, are independent of age,[23] and differences in mean age between groups should not have significantly affected results. Our goal was to find control subjects who were as close to the study groups as possible, yet did not have evidence of arterial disease. Percentage of patients who smoked was similar in all groups. Smoking increases platelet activation.

All patients with PAD in the study were receiving low-dose aspirin therapy (75-150 mg); none of the control subjects had been taking aspirin. Aspirin does not appear to influence platelet P-selectin expression and fibrinogen binding in healthy control subjects,[24] and it is also noteworthy from our results that aspirin alone is insufficient to completely suppress in vivo platelet activation in patients with PAD. While there is considerable overlap in values between PAD and control groups, measurement of platelet activation may allow individual patient- directed antiplatelet therapy in the future. More effective anti-platelet treatment strategies, including newer anti-platelet drugs and combinations of drugs, may be more effective in achieving this. We are currently investigating the effect of the combination of aspirin and clopidogrel in patients with claudication undergoing peripheral angioplasty, in a randomized controled trial.

Platelet fibrinogen binding increased with ADP stimulation in both control subjects and patients with PAD. There was no difference between ADP-stimulated platelet fibrinogen binding between control subjects and patients with claudication. However, ADP-stimulated platelet fibrinogen binding was significantly less in patients with critical ischemia compared with control subjects. This suggests that platelets from patients with critical ischemia are

less responsive to in vitro stimulation. This has also been shown by Galt et al,[12] whose patients all had severe PAD with ABI below 0.45, which would match our group of patients with critical ischemia. Reduced platelet responsiveness to stimulation has also been found in patients who have had atherothrombotic stroke.[21] One possible explanation for these findings is that, as a result of removal from the circulation of those activated platelets that have formed platelet-platelet and platelet-leukocyte aggregates, the remaining circulating platelets are less susceptible to stimulation. In other words, the platelet population is made up of subpopulations of platelets with a different propensity for activation. In patients with severe PAD those platelet groups that are more easily activated will be removed from the circulation as they adhere and aggregate at sites of diseased endothelium, leaving those platelets that are less responsive in circulation and platelets that have already been activated and are no longer susceptible to further stimulation. The failure of platelets in patients with critical ischemia to demonstrate increased fibrinogen binding is difficult to explain and requires further study.

This finding highlights the problem with sampling of peripheral blood to assess platelet activation. Although we used whole blood flow cytometry and were extremely careful to standardize preanalytical procedures (blood withdrawal, specimen handling, exact and frequent instrument calibration) to avert artifactual platelet stimulation, the study assessed function in circulating platelets. This does not necessarily reflect activity of platelets at the site of atherosclerotic plaque throughout the vascular system, and therefore it is only an indirect assessment of platelet function. Attempts have been made to assess platelet function at the site by measuring platelet activation in arterial blood taken distal to lesions treated at angioplasty through an arterial catheter.[25] This may introduce artifact from platelet activation induced by the procedure itself.

In conclusion, our study provides evidence for enhanced platelet activation in patients with PAD. Platelet activation is a marker of a prothrombotic state, which may be partly responsible for the increased incidence of major vascular events and disease progression in this group of patients.

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