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Regular Article

Normal Platelet Activation Profile in Patients with Peripheral Arterial Disease on Aspirin☆

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A B S T R A C T

Background: Peripheral arterial disease (PAD) is a progressive vascular disease associated with a high risk of cardiovascular morbidity and death. Antithrombotic prevention is usually applied by prescribing the antiplatelet agent aspirin. However, in patients with PAD aspirin fails to provide protection against myocardial infarction and death, only reducing the risk of ischemic stroke. Platelets may play a role in disease development, but this has not been tested by proper mechanistic studies. In the present study, we performed a systematic evaluation of platelet reactivity in whole blood from patients with PAD using two high-throughput assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on spotted microarrays.
Methods: Blood was obtained from 40 patients (38 on aspirin) with PAD in majority class IIa/IIb and from 40 age-matched control subjects. Whole-blood flow cytometry and multiparameter thrombus formation under high-shear flow conditions were determined using recently developed and validated assays.
Results: Flow cytometry of whole blood samples from aspirin-treated patients demonstrated unchanged high platelet responsiveness towards ADP, slightly elevated responsiveness after glycoprotein VI stimulation, and decreased responsiveness after PAR1 thrombin receptor stimulation, compared to the control subjects. Most parameters of thrombus formation under flow were similarly high for the patient and control groups. However, *in vitro* aspirin treatment caused a marked reduction in thrombus formation, especially on collagen surfaces. When compared per subject, markers of ADP- and collagen-induced integrin activation (flow cytometry) strongly correlated with parameters of collagen-dependent thrombus formation under flow, indicative of a common, subject-dependent regulation of both processes.
Conclusion: Despite of the use of aspirin, most platelet activation properties were in the normal range in whole-blood from class II PAD patients. These data underline the need for more effective antithrombotic pharmacoprotection in PAD.

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Introduction

Peripheral arterial disease (PAD) is a systemic vascular disorder with manifestations of atherosclerosis in particular in the lower extremities, resulting in obstruction of the arterial blood flow, and strongly associated with cardiovascular events [1–3]. In the Western world, a considerable part of the elderly population appears to have characteristics of PAD, albeit the affected subjects often remain asymptomatic [4,5]. Symptomatic patients, already at an early stage (Fontaine class IIa/IIb), suffer from intermittent claudication and cannot walk a long distance without feeling pain [2]. At later stages (Fontaine classes III/IV), PAD patients progressively suffer from chronic and critical leg ischemia [2].

While it is recognized that systemic atherosclerosis is one of the underlying diseases of PAD, the pathophysiology of this disease is still not well understood. One of the early concepts was that aberrant coagulation, inflammation and platelet activity in these patients lead to ongoing thrombogenesis in the lower extremities [6]. In agreement with this, markers of systemic coagulation such as levels of D-dimer are increased in patients with PAD, but it is unclear whether this also reflects causality

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[7–10]. Consistently, also plasma levels of the inflammation marker C-reactive protein are elevated [10,11]. There is also ample evidence in the literature for increased platelet activation and aggregation in PAD [12–15], although this is not an undisputed finding [8,16,17]. The role of platelets in PAD may indeed be complex. On the one hand, antiplatelet treatment with aspirin, inhibiting thromboxane formation, is a standard therapy for PAD patients, with inhibitors of the platelet $P2Y_{12}$ receptors (clopidogrel, prasugrel) as effective alternatives [18]. On the other hand, there is evidence that in these patients, even after aspirin treatment, platelet functions remain high [19], platelet-monocyte aggregates are still formed [20], and platelet cleavage products such as soluble CD40L accumulate in the plasma [21]. Since PAD in many patients is a progressive disease with high cardiovascular risk, the question is whether residual high on-treatment platelet activity may contribute to the disease progression.

In recent years, a number of whole-blood based tests for detailed and overall platelet phenotyping in bleeding and thrombosis have been developed and validated. Plate-wells based flow cytometric tests allow simultaneous determination of key platelet responses, *i.e.* fibrinogen binding (integrin $\alpha_{IIb}\beta_3$ activation) and secretion (P-selectin exposure) in response to a panel of receptor agonists [22,23]. Such flow cytometric tests are advantageous in detecting changed platelet activation in thrombotic patients [24]. Furthermore, flow chamber devices for whole-blood perfusion over a thrombogenic surface like collagen have appeared to be highly valuable in detecting a loss or gain of platelet function, supporting their use as *ex vivo* assays reflecting arterial thrombus formation [25–27]. Recently, we have extended this method into a multi-parameter platelet function test to assess thrombus formation on arrays of microspotted platelet-adhesive surfaces [28].

In the present paper, we used both high-throughput whole-blood assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on microarray spots, for detailed evaluation of the platelets in patients with PAD receiving aspirin. The data show that platelet function in these patients is in general high despite aspirin treatment, thus not pointing to a decreased prothrombotic propensity.

Methods

Patients and Controls

Included were 40 patients with established PAD. Patients were selected based on a decrease in the ankle brachial index <0.9 [29,30] and most of them suffered from a stage IIa/IIb disorder according to the Fontaine classification [2]. As a control group, 40 healthy control subjects were included with similar age and gender. Fitness (*i.e.,* non-disease state) of the control subjects was assessed with the Edinburgh claudication questionnaire [31]. Medication use was checked in all patient and control subjects by personal interview. Exclusion criteria for patients and controls were: proved coagulation or chronic inflammatory disorders, active infection, malignancy, anti-phospholipid syndrome, pregnancy and/or prescribed antiplatelet or anticoagulant medicines other than aspirin. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. All patients and healthy subjects gave written informed consent for participation according to the declaration of Helsinki.

Blood Collection

Blood was drawn from subjects in resting condition, and collected into 10 mL tubes containing 3.2% trisodium citrate. Portions of the whole-blood were directly used for flow cytometric and flow chamber assays. Separate blood samples were collected into 4 mL EDTA tubes (BD Vacutainer, Breda, the Netherlands) for determination of haemoglobin and blood cell counts with a Sysmex XN-9000 analyzer (Kobe, Japan).

Whole-Blood Flow Cytometry

Agonist-induced platelet activation was assessed by an optimized and validated test using diluted whole-blood samples without centrifugation steps [22]. In brief, a 96-well plate was thawed, containing phycoerythrin (PE)-conjugated anti-P-selectin mAb (BD 555524, Pharmingen, Franklin Laker, NJ) and FITC-conjugated anti-fibrinogen Ab (F011102, Dako, Denmark) together with varying concentrations of ADP (01897-1G, Sigma-Aldrich, Zwijndrecht, the Netherlands), collagen peptide (CRP-xl, Cambridge University, UK, a generous gift) [22] or TRAP-6 (H2936.0025, Bachem, Weil am Rein, Germany); volume was 50 μL in buffer A (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$). Activations were started by adding to each well 5 μL of blood, while mixing. Final agonist concentrations were: 0.1-2.7 μM ADP, 5-135 ng/mL collagen peptide or 0.4-3.2 μM TRAP-6. A separate row did not contain platelet agonists and served as vehicle. After 20 minutes of activation, 500 μL fixation solution (0.9% NaCl, 0.2% formaldehyde) was added to each well. Integrin activation and P-selectin expression in platelets were analyzed on an Accuri C6 flow cytometer (Becton and Dickinson, CA; 10,000 events), as described [24]. Data are represented as mean fluorescence intensities.

Whole-Blood Thrombus Formation on Microspots

Whole-blood thrombus formation under flow was investigated with the Maastricht flow chamber (depth 50 μm, width 3 mm, length 30 mm), employing a newly developed and validated multi-parameter assay in combination with three microspots [28]. Where indicated, control blood samples were pre-incubated with lysine acetylsalicylate for 10 minutes at 37 °C (100 μM, Aspegic, Sanofi, Gouda, the Netherlands). Microspots on glass coverslips were prepared by applying 0.5 μL collagen type I Horm (100 μg/mL), von Willebrand factor (vWF, 50 μg/mL) combined with CLEC-2 agonist rhodocytin (250 μg/mL), and vWF plus fibrinogen (250 μg/mL). Sources of microspots were as described before [32]. Citrate whole-blood perfusion was at arterial wall-shear rate of 1600 s^{-1} for 3.5 minutes. Brightfield images were taken from all three microspots, while chambers were stained with Alexa Fluor (AF) 647-labeled annexin A5 (1:200, A23204, Life Technology, Eugene, OR) and FITC-labelled anti-P-selectin mAb (1:40, A07790, Beckman Coulter, Woerden, the Netherlands) in buffer B (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% glucose, 0.1% bovine serum albumin, 5 U/mL fragmin (low-molecular weight heparin) (RVG20607, Pfizer, Capelle a/d IJssel) and 1 U/mL unfractionated heparin (1760 U/mL, 125 K1336, Sigma), pH 7.45). The anticoagulants were added to all Ca^{2+}-containing perfusion buffers to prevent thrombin and fibrin generation. Images of FITC and AF647 fluorescence were captured after a short rinse with buffer

Table 1
Baseline characteristics of control subjects and PAD patients. Data are represented as medians (ranges).

Subject characteristics	Controls	Patients	*P*
General			
Age, *years*	67 (60 - 71)	67 (65 - 72)	
Male gender, *n*	22/40	24/40	
Body mass index, *kg/m²*	26 (23.4 - 27.8)	26.1 (23.7 - 29.3)	
Diagnosis PAD (Fontaine), *n*			
I, IIa/IIb, III	0, 0, 0	1, 37, 2	
Medical history, *n*			
Diabetes mellitus	1	5	
Hypertension	13	29	
Aspirin intake	0	38	
Anticoagulant intake	0	0	
Hemostatic variables			
Platelet count, *x10⁹/L*	255 (205 - 291)	249 (213 - 284)	n.s.
Mean platelet volume, *fL*	10.8 (10.2 - 11.4)	11.1 (10.4 - 11.9)	n.s.
Hematocrit, *L/L*	0.44 (0.42 - 0.47)	0.43 (0.39 - 0.45)	n.s.
Leukocyte count, *x10⁹/L*	6.15 (5.60 - 7.45)	7.50 (6.03 - 9.13)	0.003
D-dimers, *ng/mL*	366 (259 - 520)	528 (363 - 835)	0.005

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