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Regular Article

Platelet activation in patients with peripheral vascular disease: *Reproducibility and comparability of platelet markers* ☆

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ABSTRACT

Background: Many markers of platelet activation have been described but their reproducibility and comparability in patient populations are poorly defined.
Objectives: We sought to compare markers of platelet and monocyte activation with platelet-monocyte aggregates, a proposed gold standard of *in vivo* platelet activation, and assess their reproducibility in patients with peripheral arterial disease: a population with substantial platelet activation, inflammation and risk of thrombotic events.
Patients/Methods: Thirty patients with peripheral vascular disease attended on two occasions to permit within-day and between-day comparisons. *In vivo* platelet and monocyte activation were determined by flow-cytometric quantification of platelet-monocyte aggregation, platelet surface expression of P-selectin and CD40L, platelet-derived microparticles, and monocyte surface expression of CD40 and CD11b. Plasma concentrations of platelet-derived microparticles, soluble P-selectin and CD40L were measured by enzyme-linked immunosorbant assays.
Results: Platelet-monocyte aggregation (36.7 ± 7.86%), and platelet surface expression of P-selectin (5.8 ± 1.65%) and CD40L (3.3 ± 1.45%) demonstrated comparable within-day (mean difference ± co-efficient of reproducibility; 0.9 ± 15.4%, 0.21 ± 1.65% and 0.2 ± 2.8% respectively) and between-day reproducibility (2.0 ± 12.4%, 0.10 ± 2.25% and 0.9 ± 6.4% respectively). Platelet-monocyte aggregates correlated well with other platelet ($r = 0.30$-0.50, $P < 0.02$) and monocyte ($r = 0.27$-0.47, $P < 0.03$) activation markers. Flow cytometric and assay quantified platelet-derived microparticles showed poorer reproducibility (co-efficient of reproducibility >40).
Conclusions: In patients with peripheral arterial disease, measurements of platelet-monocyte aggregates have good reproducibility and consistently reflect other markers of platelet and monocyte activation.

Introduction

Atherothrombosis is the leading cause of mortality in the western world. Platelets play a major role in the inflammatory and thrombotic progression of atherosclerosis [1,2]. Indeed, increased platelet activity is present in patients at increased risk of atherothrombotic events and predicts adverse cardiovascular events [3–8]. The measurement of platelet activity is therefore crucial to our understanding of the pathophysiology of atherothrombosis, the prediction of adverse cardiovascular events, and the development of novel therapeutic interventions.

Despite the development of several techniques, there is still no generally accepted ideal measure of platelet activation. A variety of methods exist, including platelet aggregometry, point of care devices, flow cytometric assessment of platelet surface antigens, and plasma markers of platelet activation: all have advantages and disadvantages [9]. Historically considered the gold standard, platelet aggregometry requires the preparation of platelet rich plasma and a high sample volume. Centrifugation and washing procedures may produce cell loss and artefactually activate platelets. Many of the point-of-care systems assess *ex vivo* platelet aggregation to various exogenous agonists. Although more labour intensive, flow cytometry is emerging as the new sensitive gold standard with measurement of surface expression of platelet antigens providing an assessment of *in vivo* platelet activation. It requires only a small sample volume, is performed on whole blood, and allows analysis of platelets in their physiological milieu. One of the most commonly studied markers of platelet activation is the α-granule membrane protein, P-selectin, that is present only on the surface of activated degranulated platelets. However,

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in vivo degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function [10]. In contrast, P-selectin-positive platelets very rapidly bind to leucocytes (mainly monocytes) via their constitutively expressed counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1) [6]. Hence circulating platelet-monocyte aggregates provide a more sensitive measure of *in vivo* platelet activation [11]. In addition platelet-monocyte aggregation may alter leucocyte function, causing monocyte arrest on the endothelium and potentially enhance the growth of atherosclerotic plaques [12].

Many methods and protocols for the measurement of platelet activation have been described [13–17]. Importantly, aspects of the techniques themselves can lead to artefactual platelet activation, thus altering the end result. However, there are few reports of test reproducibility. Even when studying the same platelet marker, the methodology and end unit of measurement may differ, making it extremely difficult to interpret and compare results. An appreciation of these issues is crucial for the meaningful interpretation of interventional studies, and the development of anti-platelet therapies.

In the present study we sought to assess the reproducibility of an established protocol (**3,4,6**) in measuring platelet-monocyte aggregation in a patient population expected to have elevated resting platelet activation. In addition, we wished to compare the reproducibility and correlation of other markers of platelet activation to a proposed gold standard of platelet-monocyte aggregation.

Methods

The study was performed with the approval of the local ethics committee, in accordance with the Declaration of Helsinki and the written informed consent of all participants.

Subjects

Peripheral venous blood was obtained from 30 patients with peripheral arterial disease. Inclusion criteria were (i) symptoms of claudication, without rest pain or ulceration, (ii) reduced ankle brachial pressure ratio, and (iii) evidence of arterial stenosis on Doppler scanning.

Study design

In order to assess reproducibility, four samples were taken from each subject – two were taken on the same day one hour apart (within-day reproducibility), and two were taken the following day at the same time points (between-day reproducibility).

Platelet activation was assessed by measuring percent platelet-monocyte aggregation andplatelet expression of P-selectin and platelet-derived microparticles (no./μL) using flow cytometry. Platelet-derived microparticles, and plasma soluble P-selectin and CD40L concentrations were also measured by enzyme-linked immunosorbant assays (ELISAs). Monocyte activation was measured via flow cytometric measurement of percent monocyte CD40 expression and mean fluorescent intensity of monocyte CD11b expression.

Blood collection

Blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood samples for assessment of platelet-monocyte aggregates, platelet expression of P-selectin and monocyte CD40 and 11b expression, were collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Flow cytometric measurement of platelet microparticles was performed on platelet poor plasma (PPP). Blood was collected into 10 mL sodium citrate tubes and PPP prepared by centrifugation at 2000 *g* at 4 ^{0} C for 10 min and confirmed by a platelet count of $<10^{9}$/L/(dilution with autologous plasma as required). Blood for the assay assessment of platelet microparticles and soluble P-selectin and CD40L was collected into tubes containing sodium citrate.

Immunolabelling and flow cytometry

Flow cytometric measurements of platelet-monocyte aggregation, platelet surface expression of P-selectin, and monocyte CD40 and 11b expression were performed as described previously [3,4,6]. Immunolabelling was performed in whole blood within 5 min of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). In order to assess platelet-monocyte aggregates, 60 μL of blood were incubated for 15 min with a FITC-conjugated anti-CD42a monoclonal antibody (GRP-P, platelet marker) and a PE-conjugated anti-CD14 monoclonal antibody (Tuk-4, monocyte marker) before fixation and erythrocyte lysis with 500 μL of FACSLyse solution (**6**). Samples were processed using a BeckmanCoulter flow cytometer and at least 2,500 cell events were analysed by EXPO32 software. Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody (platelet marker) and PE conjugated anti-CD62P monoclonal antibody (TRAP 1, IgG1). Isotype controls were used to reduce error from non-specific binding. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min.

Platelet microparticles were identified by both size and expression of platelet markers CD41 (GPIIb) and CD31 (GPIIa; PECAM). Aliquots (25 μL) of PPP were incubated for 30 min with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate buffered saline to form a volume of 1 mL. Platelet microparticles were gated according to their size (events <1.0 μm) by assessment of their forward light scatter. TruCOUNT beads of 1.0 μm (Becton Dickenson) of a known concentration were used to calculate the volume of sample analysed over 120 s at medium flow rate. This allowed the absolute number of platelet microparticles to be measured. Isotype controls were used to reduce error from non-specific binding. Platelet microparticles were detected by gating for events that were <1 μm in size (based on forward scatter) and positive for both CD31 and CD41.

ELISAs

Platelet-derived microparticles

Platelet-derived microparticles were assessed using a time-resolved immunofluorometric assay previously reported by Michelsen *et al.* [18]. This method quantifies the amount of platelet-microparticle-located CD41 (GPIIb) antigen in detergent-treated platelet-free plasma (μg/L). In brief, PPP is filtered to remove any platelet micro-particle larger than 0.1 μm (Ultra-free-MC Filter Units, Millipore, Billerica, MA, USA). The GPIIb/GPIIIa complex (CD41/CD61) is then released from the microparticle membrane and solubilized by mixing 1 volume PPP and 1 volume Delfia Assay buffer containing 1% of the non-ionic detergent Igepal CA-630. Two different monoclonal antibodies to GPIIb (CD41) are used, one labelled with Europium (Diatec, Oslo, Norway), and the other conjugated with biotin (clone DD4.1, Southern Biotechnology, Birmingham, AL, USA). Samples (50 μL) of the solubilized GPIIb/IIIa were then added to a Delfia streptavidin-coated plate (Perkin-Elmer Life Sciences, Boston, MA, USA) and 150 μL of antibody mixture added. Following incubation for 2 hours at room temperature, the wells are washed and Delfia Enhancement solution (200 μL/well) added prior to measurement of time-resolved fluorescence in a Victor^{2}1420 (Perkin-Elmer Life Sciences, Boston, MA,USA).