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Endothelial and platelet microparticles in patients with antiphospholipid antibodies

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ABSTRACT

Background: The antiphospholipid syndrome (APS) is the association of thrombosis and recurrent pregnancy loss and/or pregnancy morbidity with persistent antiphospholipid antibodies (aPL). Previous studies of microparticles in patients with APS/aPL have mainly been small and findings, contradictory.
Objectives: To quantify endothelial and platelet microparticle levels in patients with isolated antiphospholipid antibodies or primary antiphospholipid syndrome (PAPS).
Patients/Methods: We measured endothelial and platelet microparticle levels by flow cytometry in 66 aPL/PAPS patients and 18 healthy controls.
Results: Levels of circulating platelet (CD41 and CD61) and endothelial microparticles (CD51 and CD105) were significantly increased in patients with PAPS and aPL compared to healthy controls. There were correlations between platelet and endothelial microparticles levels in all patients with aPL.
Conclusions: Platelet and endothelial microparticles are increased in all patient groups within this cohort of patients aPL. Whether they may have a role in the pathogenesis of APS merits further study.

Introduction

Microparticles (MP), first described in 1967 [1], are small membrane fragments of 0.1-1 μm in diameter released from cell surfaces of endothelial cells, platelets, leucocytes and trophoblast cells with platelet microparticles constituting the majority of circulating microparticles [2]. MP express antigens specific to their cell origin providing a method for detection. MP play a role in inflammation, thrombosis and angiogenesis through cell-cell interaction and signalling and although present in healthy individuals are elevated in many disease states including inflammatory disorders, prothrombotic disorders, malignancy and infection.

Cell activation and apoptosis by varied stimuli such as cytokines, thrombin or hypoxia, leads to microparticle release from the cell surface. Microparticles express antigens according to the cell from which they are derived and their size and composition can differ according to stimulus for release. Expression of negatively charged phospholipids by microparticles provides a procoagulant surface, which allows binding of coagulation factors and promotes formation of prothrombinase complexes [3]. Numerous adhesion molecules, receptors, enzymes and major histocompatibility complexes (MHC) are expressed on leucocyte, endothelial and platelet microparticles and this, in addition to their size, forms the basis for microparticle detection. Microparticles have been implicated in the pathogenesis of inflammation [4], thrombosis [4] and malignancy [5]; and have been studied in conditions of cardiovascular disease such as coronary artery disease and stroke [6], haemolytic states such as TTP [7] and sickle cell disease, inflammatory conditions including Crohn's disease in addition to pregnancy, miscarriage [8,9], pre-eclampsia and venous thrombosis [10,11].

Antiphospholipid syndrome (APS) is an immune disorder associated with thrombotic and obstetric complications in association with persistent antiphospholipid antibodies [12]. To date, there have been a small number of studies of microparticles in patients with PAPS or healthy individuals with antiphospholipid antibodies (aPL) and findings have been variable. Studies have usually included only patients with a history of thrombosis or patients with isolated aPL in association with other immune disorders, and only one study to date has included patients with obstetric complications associated with aPL [13]. Three studies of endothelial microparticles levels in patients with isolated aPL and thrombotic complications of aPL demonstrated increased endothelial microparticle levels in patients but study numbers were small [14–16]. Another study including only patients with obstetric complications of aPL ($n = 9$) showed no difference in EMP compared to healthy controls [13]. Platelet microparticles were found to be elevated in a study of patients with both thrombotic and obstetric complications of aPL [17] and in another

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study including patients with thrombotic complications of aPL [16]. However two other studies found no difference in platelet microparticles in patients with thrombotic and obstetric complications of aPL [13,18].

The primary aim of this study was to measure levels of circulating endothelial and platelet microparticles in a large group of patients with thrombotic and obstetric complications of aPL, and isolated aPL and compare to healthy controls. The secondary aim was to assess if there was a correlation between platelet and endothelial levels.

Materials and Methods

Patients

Ethical approval was obtained from London Surrey Borders Research Ethics Committee. Samples were obtained from non-pregnant female patients attending outpatient clinics at our institution who had APS according to International Consensus statement criteria [12], or had persistent aPL without associated complications. Patients with SLE and other inflammatory disorders, acute illness, intercurrent infection or malignancy were excluded in view of potentially increased microparticle levels in these patient groups. The control group was recruited from healthy hospital staff who were not known to have aPL or any of their associated complications.

Blood Sample Collection and Processing

Blood was drawn by flawless venepuncture into Vacuette® evacuated collection tubes (Greiner Bio-One, Stonehouse, UK) containing citrate 0.105M in a ratio of 9:1 and stored at room temperature for up to 3 hours prior to analysis. Samples for measurement of microparticle procoagulant activity were centrifuged at 2000g for 15 min at 4°C. Plasma was removed and centrifuged again at 2000g for 15 min at 4°C. Following this step, plasma was divided into 500μl aliquots and further centrifuged at high-speed (12,000g) for 2 minutes at 4°C and then stored at −80°C until use. Samples for measurement of platelet and endothelial microparticles were centrifuged at 2000g for 15 min at 4°C, plasma removed and centrifuged again at 2000g for 15 min at 4°C. Plasma was then divided into 500μl aliquots, and stored at −80°C until use. Samples from patients with aPL/APS and controls were processed in the same manner.

aPL Determination

Patients with PAPS or isolated aPL had demonstrated positive testing for aPL (lupus anticoagulant, IgG/IgM anti-cardiolipin antibodies, or IgG/IgM anti-β2-glycoprotein I antibodies) on two or more occasions greater than 12 weeks apart [12].

Solid Phase Assays

Anticardiolipin antibodies (isotypes IgG and IgM) were quantified by indirect ELISA using AEUSKULISA® Cardiolipin-GM reagents (Grifols UK, Cambridge, UK). Anti-β2-glycoprotein I antibodies (isotypes IgG and IgM) were quantified by indirect ELISA using QUANTA Lite® reagents (INOVA Diagnostics Inc. San Diego, CA, USA). Positive cut-off values for both were determined according to Sydney Criteria (>40 GPL/MPL or >99th percentile)[12].

Lupus Anticoagulant Detection

Lupus anticoagulant (LA) detection in compliance with published guidelines [19] was determined by dilute Russell's viper venom time (DRVVT) and dilute APTT (DAPTT), accompanied by appropriate confirmatory tests. DRVVT was performed with Life Diagnostics LA Screen and LA Confirm reagents (Life Therapeutics, Clarkston, GA, USA). DAPTT was performed using PTT-LA (Diagnostica Stago, Asniéres, France) in the screen with a platelet neutralisation procedure employing Biodata Platelet Extract Reagent (Alpha Laboratories, Hampshire, UK) in the phospholipid-dependence confirmatory test. Patients on oral anticoagulation additionally received screening with Taipan snake venom time (TSVT)[20] employing Diagen Taipan venom (Diagnostic Reagents, Thames, UK) with an Ecarin time confirmatory test using *E. carinatus* venom (Diagnostic Reagents)[21,22]. All elevated screens received the confirmatory test plus a screen and confirmatory test on 1:1 mixing studies with normal plasma. Technoclone Lyophilised Platelet Poor Plasma, (Pathway Diagnostics Ltd, Dorking, UK) was used as the normal plasma throughout. LA assays were performed on a Sysmex CS2000i analyser, (Sysmex UK, Milton Keynes, UK). CRYOcheck™ Normal Reference Plasma, (Alpha Laboratories, Hampshire, UK) was used as the normal plasma throughout.

Microparticle Procoagulant Activity Assay

Microparticle procoagulant activity was also measured by a specific ELISA (Zymuphen MP-Activity ELISA kit, Hyphen Biomed, Surrey, UK). Briefly, microparticles present in a plasma sample bind to Annexin A5 coated wells and expose their phospholipid surface. On addition of FXa-FVA, calcium ions and purified prothrombin, thrombin generation occurs and is measured by cleavage of a chromogenic thrombin substrate, which produces absorbance at 405nm, phospholipid concentration being the rate-limiting step. Intra- assay and inter-assay CVs were 3-8% and 5-10% respectively.

Isolation and Quantification of Platelet and Endothelial Microparticles

To determine if microparticles were of platelet or endothelial origin, samples were labeled with a CD41 or CD61 fluorescently labeled antibody to detect platelet microparticles and CD51 or CD105 labeled antibodies to detect endothelial microparticles by flow cytometer. Two different antigens were used to confirm microparticle cell origin since microparticles are known to express different antigens according to the stimulant of their release [5].

Method

A 500μl aliquot of platelet-free plasma was thawed at room temperature. 10μl of sample was incubated with CD41 PECy5, CD61 PECy7, CD51 FITC, and CD105 PE fluorescently labeled antibodies (Beckman Coulter, California, USA) in each of 2 polypropylene tubes. Samples were incubated in the dark at room temperature for 30 minutes. Nine hundred μl of filtered PBS based enzyme free cell dissociation solution was added, followed by 100 μl of flow count fluorospheres (renumeration beads) of a known concentration to tube 2 only and samples immediately analysed. Data was acquired using a FC500 flow cytometer. Tube 1 was used to gate the negative population. This gate was then used to collect positive events from analysis of tube 2. Regions corresponding to microparticles were defined using forward light scatter versus side angle light scatter intensity dot plot representation. Microparticles were defined as elements positively labeled by CD41, CD61, CD51 or CD105 monoclonal antibodies. Microparticle quantification (absolute counts) was determined using the following formula (previously described [15]):

$$\begin{aligned}\text{Absolute Count (cells/}\mu\text{L)} &= (\text{Total Number of Cells Counted} \\ &\quad / \text{Total Number of Fluorospheres Counted}) \\ &\quad \times \text{Flow-Count Fluorospheres Conc.}\end{aligned}$$