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Circulating microparticles in patients with antiphospholipid antibodies: Characterization and associations

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ABSTRACT

The antiphospholipid syndrome is characterized by venous or arterial thrombosis and/or recurrent fetal loss in the presence of circulating antiphospholipid antibodies. These antibodies cause activation of endothelial and other cell types leading to the release of microparticles with procoagulant and pro-inflammatory properties. The aims of this study were to characterize the levels of endothelial cell, monocyte or platelet derived, and tissue factor-bearing microparticles in patients with antiphospholipid antibodies, to determine the association of circulating microparticles with anticardiolipin and anti-β_2-glycoprotein antibodies, and to define the cellular origin of microparticles that express tissue factor. Microparticle content within citrated blood from 47 patients with antiphospholipid antibodies and 144 healthy controls was analyzed within 2 hours of venipuncture. Levels of Annexin-V, CD105 and CD144 (endothelial derived), CD41 (platelet derived) and tissue factor positive microparticles were significantly higher in patients than controls. Though levels of CD14 (monocyte-derived) microparticles in patient plasma were not significantly increased, increased levels of CD14 and tissue factor positive microparticles were observed in patients. Levels of microparticles that stained for CD105 and CD144 showed a positive correlation with IgG ($R = 0.60$, $p = 0.006$) and IgM anti-beta$_2$-glycoprotein I antibodies ($R = 0.58$, $p = 0.006$). The elevation of endothelial and platelet derived microparticles in patients with antiphospholipid antibodies and their correlation with anti-β_2-glycoprotein I antibodies suggests a chronic state of vascular cell activation in these individuals and an important role for β_2-glycoprotein I in development of the pro-thrombotic state associated with antiphospholipid antibodies.

Introduction

The antiphospholipid syndrome (APS) is a multi-system disorder characterized by arterial and/or venous thrombosis or recurrent fetal loss in the presence of antiphospholipid antibodies (APLA) [1–5]. The majority of pathogenic APLA are actually directed against β_2-glycoprotein I (β_2GPI), an abundant plasma phospholipid binding protein [6,7]. However, the pathogenesis of APS remains incompletely understood. APLA/anti-β_2GPI antibodies appear not only to be an important serologic marker of disease, but to play a central role in the pathogenesis of thrombosis [8,9]. Some APLA inhibit important anticoagulant pathways, such as the activation or activity of protein C or S [10–12] or the anticoagulant activity of annexin A5 [13,14]. APLA antibodies also activate vascular cells, including endothelial cells [15–17], monocytes [18,19], and platelets, particularly in the presence of other agonists [20–22]. Cellular activation results in a pro-adhesive and procoagulant phenotype characterized by expression of adhesion molecules [15,23,24], tissue factor (TF) [25] and vWF [26]. Antibodies reactive with endothelial cells occur in many patients with APS, induce endothelial cell activation in a β_2GPI-dependent manner, and correlate with a history of thrombosis [26,27].

The release of microparticles from vascular cells activated by APLA has been implicated in the pathogenesis of thrombosis in patients with APS. Stimulation of microparticle release is a characteristic of activated cells and can be induced by stimuli such as terminal complement components, inflammatory cytokines and apoptosis [28–30]. Microparticles are submicron vesicles composed of anionic phospholipid that

Abbreviations: APS, Antiphospholipid syndrome; APLA, Antiphospholipid antibody; β2GPI, Beta-2-glycoprotein-I; TF, Tissue factor; SLE, Systemic Lupus Erythematosus; aCL, anticardiolipin antibody.

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express or contain specific cellular proteins and nucleic acids that may mediate procoagulant activity [31]. Elevated levels of circulating microparticles have been observed in several disorders including cardiovascular disease [30], venous thrombosis [32–34], systemic lupus [35,36], and cancer [37–39], among others. Microparticles released in vitro in response to APLA-mediated endothelial activation, express prothrombotic properties [40].

The prothrombotic properties of microparticles may result from the expression of tissue factor [41] and anionic phospholipid on the microparticle surface [42]. Microparticles may also mediate procoagulant effects through their expression of inflammatory mediators such as IL-1β, which induce activation of other cells through autocrine or paracrine mechanisms [43].

The ability to identify individuals at greatest risk for thrombosis remains a challenge in the management of patients with APLA. Better understanding of the pathogenesis of APS in humans may provide insight into more specific approaches to prevent the clinical manifestations of these antibodies. Microparticles may provide a potential surrogate marker of vascular dysfunction that may be useful in stratifying the risk of thrombosis associated with APS.

In this study, we have characterized circulating microparticles in a large cohort of patients with APLA, and determined their cellular origin, expression of tissue factor, correlation with clinical tests for APLA, and association with clinical events.

Materials and Methods

Materials

Antibodies to CD105-PE and CD144-PE (to detect endothelial cell-derived microparticles), CD41-PECy4 (to detect platelet-derived microparticles) and CD14-PE (to detect monocyte-derived microparticles) were obtained from Abcam (Cambridge, MA). A FITC-conjugated monoclonal antibody against tissue factor (CD142; product #4507CJ) was obtained from American Diagnostica (Stamford, CT). Annexin V-Alexafluor 647 and Alignflow flow cytometry beads (2.5 μM) were from Life Technologies (Grand Island, NY). Latex, amine-modified polystyrene, fluorescent yellow-green beads (1 μm; product # L1030) and all chemicals used for preparation of buffers were from Sigma-Aldrich (St. Louis, MO). Venipuncture tubes containing sodium citrate were purchased from BD (Franklin Lakes, NJ).

Patients and Controls

Forty-seven patients with APLA and 144 healthy controls were studied. Patients were recruited from the hematology clinics of the Cleveland Clinic and Case Western Reserve University. This study included patients with antiphospholipid antibodies, not specifically patients who met clinical criteria for APS. However, since the patients were largely recruited from hematology clinics, most of them met criteria for clinical APS. Complete clinical information was available for 46 of 47 patients. Thirty-eight met clinical criteria for APS (either thrombosis or pregnancy loss). Of the remaining 8 patients, 6 were referred for evaluation on the basis of a persistent APLA without clinical manifestations. In two cases this was detected during evaluation of SLE and in another case it was detected during evaluation of persistent migraine with aura. Patients with a history of thrombosis were enrolled at least three months after their most recent thrombotic event to minimize confounding of acute thrombosis on microparticle levels. Control subjects consisted of normal, healthy individuals who did not have APLA, a history of thrombosis or other congenital or acquired thrombophilia and who did not smoke. The Institutional Review Boards at Cleveland Clinic and Case Western Reserve University approved this study.

Microparticle Isolation and Flow Cytometry

Blood samples from patients and controls were collected by venipuncture into citrated tubes and processed within 2 hours; the first 3 ml were discarded and not used for microparticle measurements. Blood was processed as previously described by Lee et al. [44] and Dignat-George et al. [45]. Briefly, blood was centrifuged at 1500 x G for 15 minutes. The supernatant "platelet poor plasma" was collected and centrifuged again at 13,000 x G for 2 minutes to remove residual platelets and cell fragments, yielding "platelet-free plasma". Platelet-free plasma was added to individual tubes containing isotype and label-specific control IgG, specific fluorochrome-labeled antibodies to CD105, CD144, CD41, CD14-PE and tissue factor, or annexin V. After incubation for 60 minutes, 200 μl of a solution of 2.5 μM μm Alignflow flow cytometry beads (concentration $= 3 \times 10^6$ beads/ml) were added to each tube and used for calibration to ensure analysis of an identical volume of each sample.

Samples were analyzed by flow cytometry using an LSRII flow cytometer (BD Biosciences). On a log forward scatter (FSC) vs. log side scatter (SSC) plot, 1 μm latex beads (Sigma-Aldrich) were used to define the MP gate. MP within this gate labeled with either annexin V or control or antigen-specific fluorochrome-labeled antibodies were counted; data collection for each sample was terminated following counting of 50,000 Alignflow beads in a separate gate distinct from that of microparticles. Background staining due to control antibodies was generally <5% of that observed with antigen-specific antibodies and was subtracted from that caused by the latter.

In a randomly selected cohort of 19 control individuals and 12 patients, we also determined the contribution of microparticles from specific cell types to tissue factor expression by double staining microparticles using cell-specific and anti-tissue factor antibodies. These studies were performed in an identical-manner as those utilizing single antibodies, except for the inclusion of anti-mouse Ig, /negative control compensation beads to adjust for spectral overlap between the flourochrome emission spectra of the two labeled antibodies.

Measurement of APLA

A PTT based screening test (positive >32.4 seconds) and dilute Russell viper venom (DRVVT) confirmatory test (positive >46.9 seconds with DRVVT confirm ratio >1.20) were used to detect LAC. Determination of anticardiolipin antibodies (aCL) was performed by standardized ELISA for both IgG and IgM isotypes (INOVA Diagnostics Inc., USA) with bovine calf serum in the sample diluent as the source of β_2GPI. Results are expressed as GPL units for the IgG (positive ≥23 GPL) and MPL units for the IgM (positive ≥11 MPL) aCL antibodies, with 1 GPL or MPL unit being equivalent to 1 μg/mL of an affinity-purified standard IgG or IgM aCL antibody sample. Determination of IgM and IgG anti-β_2GPI antibodies was performed by ELISA with irradiated and chemically activated plastic microwell plates containing purified human β_2GPI (INOVA Diagnostics Inc., USA). Results are expressed in standardized units, SGU for the IgG (positive ≥20 SGU) and SMU for the IgM (positive ≥20 SMU) anti-β_2GPI antibodies. All tests met the quality control standards as determined by the manufacturer.

Statistical Analysis

Statistical analysis was performed using SPSS Software version 20.0 (IBM Corp. 2011). Descriptive statistics were calculated for MP measurements (mean, standard deviation). The distribution of MP and antiphospholipid antibody measurements was skewed, therefore a logarithmic transformation was performed prior to parametric analyses. Wilcoxon rank sum test was used to compare MP levels between patients and controls and between sub-groups of patients (with or without DVT/pregnancy morbidity). Additional adjustment of covariates was performed through the analysis of covariance method. Correlations