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A new cytofluorimetric approach to evaluate the circulating microparticles in subjects with antiphospholipid antibodies

Elena Niccolai [a,1], Danilo Squatrito [a,1], Giacomo Emmi [a], Elena Silvestri [a], Lorenzo Emmi [a,b], Lucia Ciucciarelli [a], Federica Ricci [a], Daniele Manganaro [c], Amedeo Amedei [a,b,*], Domenico Prisco [a,b]

[a] Department of Experimental and Clinical Medicine, University of Florence, Italy
[b] SOD Interdisciplinary Internal Medicine, Center for Autoimmune Systemic Diseases, Behçet Center and Lupus Clinic, AOU Careggi, Florence, Italy
[c] BD Biosciences Italy S.p.A, Buccinasco, Italy

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ABSTRACT

Introduction: Growing evidence supports the idea that microparticles (MPs) could contribute to the pathogenesis of the thrombotic phenomena associated with antiphospholipid antibody syndrome (APS), inducing a hypercoagulable state. But, to date, different approaches to evaluate circulating MPs and conflicting results have been reported.
Materials and methods: We have characterized the different circulating subpopulations of MPs in APS patients, and in asymptomatic aPL-positive subjects (carriers) by examining the correlation between the amount and phenotype of MPs and the clinical parameters. Forty-eight subjects were enrolled: 16 with primary APS, 16 aPL-positive, but without clinical criteria for APS (carriers), and 16 healthy subjects. The levels of MPs were evaluated using a new cytofluorimetric approach based on BD Horizon Violet Proliferation dye (VPD) 450.
Results and conclusions: Using a new detection cytofluorimetric approach, we demonstrated that the AnnV-negative MPs, underestimated/or excluded in the previous studies, are a large subset of circulating MPs. Also, the levels of MPs in the plasma of aPL positive subjects indicate a state of cellular activation, which is much more pronounced in patients with APS compared to aPL carriers. Moreover, the preliminary data of our pilot study suggest that the evaluation of circulating MPs, in particular PMPs and EMPs, could be used as a surrogate biomarker for platelet and vascular damage monitoring and, if confirmed in a more numerous cohort of patients, it could be used as a prognostic factor to identify aPL positive subjects at higher risk of developing thrombosis.

1. Introduction

Antiphospholipid antibody syndrome (APS) is defined as the association of at least one persistently positive test for antiphospholipid antibodies (aPL) with arterial or venous thrombosis (VT), and/or pregnancy-related morbidity (PM) [1]. A growing evidences support the idea that microparticles (MPs) could contribute to the pathogenesis of the thrombotic phenomena associated with APS, inducing an hypercoagulability state [2,3]. MPs are small vesicles of heterogeneous size (100–1000 nm) released from the plasma membrane after cell activation, apoptosis or exposure to shear stress [4]. Blood cells, such as erythrocytes, leukocytes, platelets and endothelial cells, are capable of generating MPs and typically, MPs harbor membrane and cytoplasmic components that reflect their specific cellular origin [5]. They are implicated in various biological processes [6,7] and an increased concentration of a distinct MPs population may be considered a sign of cell activation or damage, so representing an important parameter/marker in the evaluation of systemic or inflammatory diseases [8], such as APS [3]. The potential role of endothelial cells in the APS pathogenesis has been recognized [9,10]. In particular, aPL can activate endothelial cells, resulting in a prothrombotic state. An increased number of MPs of endothelial origin (EMPs) in aPL positive subjects with or without a clinical history of thrombosis compared to healthy subjects has been reported [9,11]. Moreover, also leukocyte (LMPs) and platelet- (PMPs) derived MPs could have a pathogenic role in APS thrombotic complications [12,13]. Nevertheless, the presence of aPL does not necessarily mean APS, since the aPL positivity can be detected in healthy subjects without history of VT or PM. In addition, there are some aPL positive patients that, despite the absence of the clinical criteria necessary for APS diagnosis, present some "features associated with APS" [1,14]. For these "non-criteria" APS patients the correct therapeutic strategy is not clear, and in them the finding of MPs might be of importance to decide the treatment due to the MPs prothrombotic potential.

In the present study, since currently methods to analyze MPs in blood samples are still not completely standardized [12,15–17], we

* Corresponding author at: Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla, 3, 50134 Firenze, Italy.
E-mail address: aamedei@unifi.it (A. Amedei).
[1] E. Niccolai and D. Squatrito equally contributed to the preparation of the manuscript.

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have set up a new strategy to quantify MPs in plasma samples using a BD FACScanto flow cytometer and a multiparametric approach based on BD Horizon Violet Proliferation dye (VPD) 450, in combination with fluorescent mAbs to specific cell surface markers and Annexin V. In addition, we have characterized the different circulating subpopulations of MPs in APS patients, and in aPL-positive subjects (carriers) comparing the quantity and the phenotype of MPs with the clinical parameters.

2. Methods

2.1. Patients

We enrolled 48 subjects with serum aPL: 16 patients with a diagnosis of primary APS (PAPS), 16 aPL-positive subjects, but without the clinical criteria for APS (carriers), and 16 healthy subjects (HS). In particular, carriers were considered a) all the asymptomatic subjects occasionally screened for aPL and resulted positive (these subjects, screened elsewhere, were examined by our dedicated Center to exclude or confirm APS or other autoimmune diseases such as Systemic Lupus Erythematosus); b) all the patients referred to our Center due to aPL positivity associated with suggestive clinical features, such as non-ischemic neurological manifestations, *livedo reticularis* and thrombocytopenia (these patients, according to Miyakis consensus, presented "non-criteria features of APS")..

Fully informed consent and approval of the local Ethical Committee was obtained. Carriers and PAPS had demonstrated positive testing for one or more aPL test (lupus anticoagulant, IgG/IgM anti-cardiolipin antibodies, or IgG/IgM anti-β2-glycoprotien I antibodies) on two or more occasions greater than 12 weeks apart [1]. A PTT based screening test and diluite Russell viper venom (DRVTT) confirmatory test were used to detect LAC. IgM/IgG anticardiolipin antibodies (aCL) were quantified using EliA Cardiolipin assay (Phadia GmbH, Germany) and results were expressed, respectively, as GPL-, MPL-U/ml. According to Sydney Criteria (1) positive cut-off values were considered >40 GPL/MPL, while results between 10 and 40 GPL-,MPL-U/ml are classified as low positive. Determination of IgM/IgG anti-β2-glycoprotien I antibodies was conducted using EliA β2-Glycoprotein I test (Phadia GmbH, Germany) and due to the lack of international standardization, EliA β2-Glycoprotein I results are given in arbitrary EliA Units. The cut-off is defined at 10 EliA U/ml.

All the APS patients had developed an episode of VT at least one year before our analysis, while no one reported obstetric manifestations; of them 4 showed an aPL single test positivity (APS s), 4 a double test positivity (APS d) and 8 were triple positive (APS t). Regarding the aPL carriers, 5 showed an aPL single test positivity (CAR s), 4 a double test positivity (CAR d) and 7 were triple positive (CAR t). 7/16 aPL carriers (1 CARs, 2 CARd and 4 CARt) had features associated with APS (but not included in the revised criteria of Miyakis), such as neurological manifestations, thrombocytopenia and *livedo reticularis*, while the remaining 9 subjects (4 CARs, 4 CARd and 1 CARt) were completely asymptomatic. The characteristics of patients and healthy subjects are summarized in Table 1.

2.2. MP preparation and labeling

Blood samples for MP preparation were collected into sodium citrate anticoagulant at 3.2% (0.105 M) final concentration and processed within 1 h [18] Platelet-free plasma (PFP) was prepared at room temperature by serial centrifugations, (15 min at 1500 ×*g*, and 3 min at 3000×*g*) [19], frozen in 1.5-ml tubes as 500 ml aliquots, and stored at −80 °C until analysis.

Briefly, for each analysis 50 μl of freshly thawed PFP was labeled by adding: 4 μl of BD Horizon Violet Proliferation Dye 450 (VPD450, BD Biosciences, San Jose, CA, USA), 5 μl APC Annexin V (BD Pharmingen, San Diego, CA, USA), 5 μl 7-Amino-Actinomycin (7-AAD, BD Pharmingen). Because APC Annexin V binding is calcium dependent, the staining was performed in 200 μl of Buffer with Ca^{2+} (Binding buffer 2×, BB2×, BD Pharmingen). The best Ca^{2+} concentration was determined with titration experiments. To identify the cellular origin of MPs, either of the following fluorescent mAbs to specific cell surface markers were added: 5 μl APC-H7 Mouse anti-human CD45 (BD Pharmingen) to detect LMPs, 5 μl FITC Mouse anti-human CD31 and 5 μl PerCP-Cy5.5 Mouse anti-human CD41a (BD Biosciences) to identify EMPs and PMPs. After 1 h of incubation (4 °C in the dark) BB2× were added and then cytofluorimetric analysis performed. The optimal concentration was experimentally determined for each antibody or dye by titration experiments. VPD450 is a violet laser excitable dye; the non-fluorescent VPD450 dye passively diffuses across cell membranes and is cleaved by esterase activity within the cytoplasm of viable cells, or intact vesicles. The cleaved dye becomes highly fluorescent and covalently binds to protein amine groups within the cells. This allows to identify closed membrane vesicles, like MPs, excluding cellular fragments or debris [20,21]. Moreover, viable cells with intact membranes exclude 7-AAD; this molecule allows the discrimination of MPs from membranes of dead and damaged cells and apoptotic bodies, that are permeable to 7-AAD and AnnV positive.

2.3. Analysis by flow cytometry

Plasma samples were analyzed immediately after labeling using a BD FACSCanto equipped with the BD FACSDivaTM software. An unstained sample was acquired to detect the auto-fluorescence and set the photomultiplier for all the considered channels. MPs were firstly identified by relative size. Forward and side scatter channels (FSC and SSC) were used on a logarithmic scale. The setup of the FSC and SSC photomultiplier was calculated using background noise as the lower limit. The FSC photomultiplier was increased until the background noise filled the dots available of dot plots. As previously reported [22–24], a MPs gate was established on the FACSCanto instrument by preliminary standardization experiments using Megamix (Biocytex, Marseille, France), a mix of fluorescent beads of varied diameters to cover the MPs (0.5–0.9 μm) and platelet size ranges (0.9–3 μm). Because Megamix identifies only MPs with size between 0.5–1 μm but MPs is ranging from 0,1 to 1 μm, we used threshold as lower side. So the threshold, set on the FSC channel, was progressively increased to reduce the noise, in order to detect MPs smaller than 0,5 μm that were discriminated from background noise through the VPD450 positivity. In this way we hypothesize to reach 0.1 μm as lower limit. Using a reverse pipetting technique, 50 μL of the diluted stained sample were pipetted into a TruCount tube (BD Biosciences, San Jose, CA, USA) to which 450 μL of binding buffer were then added. TruCount tubes, containing a standardized number of fluorescent beads, were used to quantify MPs.

We defined MPs as particles that were less than 1.0 mm in diameter and, regardless of the Annexin V expression, VPD450-positive and 7-AAD-negative. In addition, we identified the phenotype of MPs by the expression of cell specific markers (Fig. 1). In short, the $AnnV^{+/-}/CD31^{+}/CD41a^{-}/CD45^{-}$ MPs were defined as EMPs, the $AnnV^{+/-}/CD31^{dim}/CD41a^{+}/CD45^{-}$ as PMPs and the $AnnV^{+/-}/CD31^{-}/CD41a^{-}/CD45^{+}$ as LMPs. Sample acquisition was stopped when the number of TruCount beads in the relative region reached 5000 events. The number of MPs per microliter was calculated by the formula:

[(N.of events in gating containing microparticles/N.of events in absolute count bead region) × (N.of bead counts per test/Sample volume (μL))] × Dilution factor.

2.4. Statistical analysis

The data were evaluated and processed using the "GraphPad Prism 5" software. Continuous values with a normal distribution were

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