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Elevated expression of platelet-derived chemokines in patients with antiphospholipid syndrome

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ABSTRACT

Objective: Platelet factor 4 tetramers (CXCL4 chemokine) form complexes with β2glycoprotein I (β2GPI), recognized by anti-β2GPI antibodies leading to platelet activation in antiphospholipid syndrome (APS), either primary (PAPS) or secondary (SAPS). Increased plasma levels of CXCL4 may favor this process; therefore we measured plasma levels of CXCL4, a CXCL4 variant (CXCL4L1) and as controls, platelet-derived chemokines CXCL7 (NAP-2) and CCL5 (RANTES), in APS, and disease controls such as patients with systemic lupus erythematosus (SLE) coronary artery disease (CAD) and healthy donors (HDs).
Methods: Plasma samples and platelets were isolated from patients with APS (n = 87), SLE (n = 29), CAD (n = 14) and 54 HDs. Plasma levels of CXCL4, CXCL4L1, CXCL7 and CCL5 as well as intracellular platelet CXCL4 and CXCL4L1 were measured using ELISA. Platelet CXCL4 and CXCL4L1 RNA levels were determined by RT-PCR.
Results: CXCL4, CXCL7 (NAP-2) and CCL5 (RANTES) plasma levels were significantly higher in patients with APS compared to both control groups (SLE, CAD) and HDs. CXCL4L1 plasma levels were also significantly higher in APS than in SLE and HDs, but lower from that of CAD patients. Statistically significant concordance was detected between CXCL4 and CXCL7 ($p < 0.0001$) or CCL5 ($p < 0.0001$) plasma levels in patients with APS, either PAPS or SAPS. CXCL4L1 plasma levels were inversely correlated with CXCL4 ($P = 0.0027$), CXCL7 ($p = 0.012$) and CCL5 ($p = 0.023$) in PAPS and positively with CXCL4 ($p = 0.0191$), CCL5 ($p < 0.0001$) and CXCL7 ($P < 0.0001$), in SAPS. Levels of CXCL4, CXCL4L1, CXCL7 and CCL5 were divided in "high" (exceeding a level defined as the mean of HDs and 3 SD) and "low" (below this level); The "CXCL4L1 high" group was characterized by increased IgG aCL, ($p = 0.0215$), double antibody positivity (either aCL or anti-β2GPI plus LA), ($p = 0.0277$), triple antibody positivity (aCL plus anti-β2GPI plus LA), ($p = 0.0073$) and thrombocytopenia ($p = 0.0061$), as well as with at least 1 thrombotic event or the last 5 years ($p = 0.0001$), or more than 3 thrombotic events ever ($p = 0.0151$).
Conclusions: Chemokines associated with platelet activation and immune cell chemotaxis were found to be elevated in APS patients' plasma and may contribute to the pathogenesis of the syndrome. High CXCL4L1 plasma levels are associated with the clinical expression of APS and should be prospectively evaluated as a biomarker.

1. Introduction

Antiphospholipid Syndrome (APS) is an autoimmune thrombophilic disorder defined by the occurrence of venous and/or arterial thrombosis and/or pregnancy morbidity, in association with persistently elevated levels of IgG or IgM antiphospholipid autoantibodies (aPL), detected either as anti-cardiolipin (anti-CL), anti-β2-glycoprotein I (anti-β2GPI) antibodies and/or lupus anticoagulant (LA) [1–3].

The thrombogenic properties of aPL have been proven by animal studies but the underlying pathogenic mechanisms remain obscure [4,5]. Several experimental findings indicate the following mechanisms: i) conformational changes of β2GPI due to oxidative stress [6,7]; ii) direct activation of vascular endothelial cells, monocytes and platelets by aPL leading to NFκB activation and eventually

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tissue factor expression [8–14]; iii) complement activation [4,15] and iv) inappropriate activation of the innate immunity components on the surface of endothelium [16].

CXCL4 is a chemokine released by the α-granules of platelets during their aggregation, in a protein kinase C-dependent manner, and promotes blood coagulation by modulating the antithrombotic effects of heparin-like molecules. CXCL4 appears to play a role in wound repair and the pathogenesis of systemic sclerosis (SSc) [17]. CXCL4L1 has been isolated from thrombin-stimulated human platelets [18,19]; it is synthesized and secreted through a constitutive pathway displaying a different secondary structure and a lower affinity for heparin compared to CXCL4 [20]. CXCL4 is involved in the induction of coagulation and inflammation [21–24], inhibition of angiogenesis [25,26] and the Heparin-induced Thrombocytopenia and Thrombosis syndrome (HITT) [27]. The main activities of CXCL4L1 are anti-angiogenic and anti-invasive overriding that of conventional CXCL4 [28,29].

Our laboratory demonstrated that β2GPI-anti-β2GPI complexes are more efficiently formed when β2GPI is dimerized upon interaction with platelet factor 4 (PF4) (the CXCL4 chemokine) tetramers. This interaction enhances the formation of thrombogenic immune complexes [$(CXCL4)_4$($\beta2GPI)_2$/anti-β2GPI] on platelet surface and leads to platelet activation [14]. Mass spectrometry was inefficient to distinguish whether the platelet protein selectively bound to β2GPI was CXCL4 or its non-allelic variant CXCL4L1, since the two proteins differ only in three aminoacids [18,20,30]. We hypothesized that the formation of this complex is taking place in an excess of CXCL4. Therefore we searched for differences in expression levels of CXCL4, as well as of CXCL4L1, in APS and healthy individuals.

To strengthen the evidence that platelet activation takes place in APS we also evaluated the plasma levels of CXCL7 and CCL5 chemokines. CXCL7 is identical to neutrophil-activating protein-2 (NAP-2). It activates mitogenesis, synthesis of extracellular matrix, glucose metabolism and plasminogen activator [31]. CCL5 (RANTES) is a C–C chemokine, chemotactic for T-cells, eosinophils and basophils. CCL5 is expressed by several cell types; endothelial cells stimulated by selectins express CCL5 [32,33].

2. Patients & methods

Forty ml of peripheral blood were obtained from a cohort of 184 individuals, including 87 patients with APS, 29 with systemic lupus erythematosus (SLE) negative for LA and aPL antibodies and without any APS related feature, 14 with coronary artery disease (CAD) and 54 from healthy donors (HDs), with the approval of the ethics committee of the National University of Athens. HDs had not taken any medication during the previous ten days. APS patients suffered either from primary APS (PAPS), (n = 33) with no underlying autoimmune disease, or secondary APS (SAPS), (n = 54), a clinical entity where the syndrome is present in association with other autoimmune diseases, such as SLE. Plasma samples from APS patients were obtained at least four months apart a thrombotic event or pregnancy complication. Complete blood count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), aPTT, Lupus Anticoagulant (LA), anti-CL and anti-β2GPI antibodies were studied in all participants. Thrombocytopenia was defined as platelet count less than 10^5/μl. Clinical events compatible with APS and SLE were extracted from patients' medical records. Regarding the therapy of APS patients, 80 were receiving acenocoumarol and 7 receiving low molecular weight heparin (LMWH). All the patients under LMWH and 58 of those under acenocoumarol were also receiving aspirin 100 mg daily (Table 1 Appendix). Furthermore we provide in Table 1 in the Appendix information regarding all the drugs the patients were receiving. Analysis within the groups and across the groups to find associations between each therapy and chemokine levels did not reveal any significant association. Regarding patients with APS in particular, as shown in Table 2 in the appendix anticoagulant therapies did not discriminate patients in those with "high" and "low" cytokine levels for all the cytokines tested.

Plasma was separated from the blood and kept frozen for subsequent analysis. Platelets were isolated from fresh platelet-rich plasma, derived from 20 ml of blood, as previously described [34] and pelleted into two aliquots for protein and RNA analysis respectively. For platelet purification, blood was collected only via gravity flow in order to avoid mechanical platelet activation. Once purified, platelet pellets (>1 × 10^8 cells) intended for RNA analysis were dissolved in 1 ml TRIzol reagent and kept frozen until subsequent RNA extraction. Microscopic observation of platelet preparations revealed no leukocyte or red blood cell contamination. The purity of platelet preparation was assessed through flow cytometry using the following monoclonal antibodies as markers: CD61, for platelets, CD3 for T-lymphocytes, CD19 for B-lymphocytes and CD14 for monocytes. Flow cytometry analysis revealed that more than 95% of cells are positive for CD61, whereas the rest 5% consisted predominantly by T-cells (Appendix Fig. 1).

2.1. Evaluation of CXCL4, CXCL7, CCL5 (RANTES) and CXCL4L1 plasma levels

Plasma levels of CXCL4, CXCL7, CCL5 and CXCL4L1 were determined using commercially available sandwich enzyme linked immunosorbent assay (ELISA) developing kits. CXCL4 (DY795) and CXCL7 (DY393) kits were derived from R&D Systems (Ambingdon, UK); CCL5 (440804) from Biolegend (London, UK) and CXCL4L1 from AMS biotechnology (Ambingdon, UK). Samples and reagents were diluted in PBS BSA 1% (R&D Systems DY995). TMB (Thermoscientific, Rockford, USA) was used as substrate and H2SO4 2N was used to stop the reaction. Samples were diluted 1/80 for CXCL4 measurement, 1/200 for CXCL7 measurement and 1/10 for CCL5 measurement. The CXCL4L1 ELISA kit displays high specificity, with no cross-reactivity or interference between CXCL4L1 and other CXC chemokine molecules, including conventional CXCL4, (sensitivity of 1 pg/ml). To prove further that the CXCL4L1 detection assay does not detect CXCL4, the CXCL4L1 ELISA plates (with anti-CXCL4L1 capture antibody), were incubated with various concentrations of recombinant CXCL4 and two detection antibodies were used in two parallel experiments to illustrate the reaction: an anti-CXCL4L1 and an anti-CXCL4 antibody. The anti-CXCL4L1 detecting antibody gave a reaction close to zero, while the anti-CXCL4 detecting antibody gave a reaction close to 0.2 absorbance units which for the CXCL4 detecting ELISA lies within the background of the method, (data shown in Fig. 2 in the Appendix). To exclude inter-assay variability we measured several samples in various days in different experiments. As shown in Fig. 3 in the Appendix, the variability of the results was minimal and in fact it was less than 5%.

2.2. Platelet protein extraction and intracellular CXCL4 and CXCL4L1 measurement

Platelet pellets were re-suspended in 500 μl phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (Complete Mini, Roche, Manheim, Germany) and sonicated on ice at 20 kHz (or 70% max) for 5 × 5 sec, in one minute interval. Protein extracts were centrifuged (1500 g × 15 min, 4 °C) and protein concentration was determined with Micro BCA protein assay kit (Thermo Scientific, Rockford, USA).

Intracellular CXCL4 and CXCL4L1 protein was determined, at a final concentration of 3 μg/ml and 3 mg/ml respectively of total platelet protein extracts, using the CXCL4 and CXCL4L1 ELISA kits,