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Studies of fibrin formation and fibrinolytic function in patients with the antiphospholipid syndrome ☆

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ABSTRACT

Objective: The antiphospholipid syndrome (APS) is defined by persistent antiphospholipid antibodies together with thrombosis and/or pregnancy morbidity. We investigated the tightness of fibrin clot and fibrinolytic function in plasma samples from APS patients compared with two control groups.
Material and Methods: APS patients (n = 49), healthy controls (HC) (n = 19) and warfarin-treated nonAPS thrombosis controls (nonAPS-TC) (n = 39) were investigated. Fibrin permeability was assessed as the permeability coefficient (Ks) by a flow measurement technique. Additionally, clot density and fibrinolytic function was analysed by a turbidimetric clotting and lysis assay. Fibrin structure was visualised using scanning electron microscopy. Finally, the number of cell-derived microparticles (MPs) in the samples were correlated to fibrin permeability
Results and Conclusions: The Ks value was lower in samples from APS-patients compared to HC and nonAPS-TC ($p < 0.0001$ for both) indicating a less permeable fibrin clot in APS patients. Scanning electron microscopy images confirmed compact fibrin with smaller intrinsic pores and thinner fibers in samples from APS patients as compared to HC. Prolonged fibrinolysis (clot lysis) times were present in the subgroup of APS patients with previous arterial thrombosis (n = 15) as compared to HC and to nonAPS-TC (all p-values < 0.05). In conclusion, tighter fibrin clots were formed in plasma from APS patients compared with healthy controls and warfarin treated patients with thrombosis of "nonAPS origin". This new observation presents a possible mechanism contributing to the thrombotic predisposition of APS patients. Impaired fibrinolysis, selectively present among APS patients with previous arterial thrombosis, may further aggravate the pro-thrombotic state in this APS subgroup.

Introduction

The antiphospholipid syndrome (APS) is a heterogeneous but potentially life-threatening disorder. It is characterized by antiphospholipid antibodies (aPL), thrombosis, which can affect arterial, venous or micro vessels, and/or pregnancy morbidity [1]. APS is common among patients with systemic autoimmune diseases, in particular among patients with Systemic lupus erythematosus (SLE), but it can also occur as an isolated condition [2]. Both the disorder itself and the anticoagulant treatment used in APS can cause substantial morbidity and mortality. Taken together, this stresses the need for reliable risk assessments to guide individualised treatment [3]. As good tools for risk assessment are lacking further studies of disease pathogenesis and possible biomarkers in APS are urgent.

Formation of thrombi with an abnormal architecture and decreased fibrin permeability, is a feature of many conditions with augmented risk for cardiovascular and thrombotic events [4–9]. Such thrombi are

Abbreviations: APS, Antiphospholipid syndrome; HC, Healthy controls; nonAPS-TC, NonAPS thrombosis controls; aPL, Antiphospholipid antibodies; SLE, Systemic lupus erythematosus; PAI, Plasminogen activator inhibitor; SEM, Scanning electron microscopy; MPs, Microparticles; aCL IgM and aCL IgG, Anticardiolipin IgM and IgG; anti-β_2GPI IgG, Anti-β_2 glycoprotein-I IgG; LA, Lupus anticoagulant; NSAID, Non-steroidal anti-inflammatory drugs; ASA, Acetylsalicylic acid; EDTA, Ethylenediamine tetraacetic acid; CV, Coefficient of variation; hsCRP, High sensitive C-reactive protein; Lag, Lag time; MaxAbs, Maximum absorbance; HBS, Hepes-buffered saline; PS, Phosphatidylserine; PMPs, Platelet derived MP; MMPs, Monocyte derived MP; EMP, Endothelial derived MP; TF, Tissue factor; OR, Odds Ratio; NR, Non-reported; M, Male; F, Female; N, Number; Ns, Non-significant.

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associated with an increased resistance to fibrinolysis due to, for example, decreased permeability of fibrinolytic factors through the fibrin clot [10–13]. Fibrin formation includes the construction of fibrin monomers that polymerise and then laterally aggregate to form fibrin fibres, as well as factor XIII-mediated stabilisation of the fibrin structure through cross-linking of the fibres. Concentrations of fibrinogen and thrombin are well-studied factors, which influence fibrin permeability [14,15]. Moreover the fibrin structure is affected by common antithrombotic therapeutic approaches [16–19].

Though relatively rarely studied, there are some reports of impaired fibrinolysis contributing to the pro-coagulant state in APS. Hypofibrinolysis has been demonstrated using global assays in both obstetric [20] and thrombotic populations [21] but the underlying mechanisms for impaired fibrinolysis in APS are not clear. It is likely that the high levels of plasminogen activator inhibitor (PAI-1) (antigen and activity) in plasma reported in patients with systemic autoimmune conditions where aPL are common [22,23] contribute. Clot architecture is another possible factor, which may affect fibrinolysis and coagulation equilibrium, but its role has not yet been established in patients with APS.

We hypothesised that a less permeable (i.e. tight) fibrin clot with increased resistance to fibrinolysis contributes to the pro-coagulant state observed in patients with APS. As a first objective, we thus investigated fibrin permeability in 49 APS-patients, 19 healthy controls (HC) and 39 nonAPS-thrombotic, Warfarin-treated controls (nonAPS-TC). For confirmation we performed a turbidimetric clotting assay where clot density is estimated through measurements of maximal light absorbance [24]. Moreover, we studied scanning electron microscopy (SEM) images of fibrin structure in APS patients compared to HC and statistically investigated if the number of cell-derived microparticles (MPs) were related to fibrin permeability. As a second objective, we examined if there was a difference in overall fibrinolysis function between patients and controls using a turbidimetric lysis assay. Finally we investigated whether fibrin clot characteristics and fibrinolysis profile could separate subsets of APS patients as determined by clinical and/or immunological characteristics.

Materials and Methods

We included 49 patients fulfilling APS-criteria [1] from the Rheumatology, Haematology and Women's and Children's Health clinics, Karolinska University Hospital, between 2009–2010. At inclusion, patients filled out a detailed questionnaire about symptoms and medications. Initially 54 patients consented participation but 5 patients were finally not included in the study because their blood samples were damaged during sample transport or handling and thus not possible to analyse. According to criteria [1] aPL-positivity was defined as at least two positive tests of medium/high titer for anticardiolipin IgM/IgG (aCL IgM/aCL IgG), a titer > 99th percentile for anti-β_2 glycoprotein-I of IgG isotype (anti-β_2GPI IgG) or a positive lupus anticoagulant (LA) test according to routine procedure. Confirmation tests were performed after a minimum of 12 weeks. We also required at least 12 weeks and not more than 5 years between positive testing and the clinical manifestation. Three patients fulfilled the criteria for SLE according to the 1982 American College of Rheumatology criteria [25] and nine patients fulfilled the criteria for other rheumatic diseases but the majority were primary APS patients.

As a primary control group we used 19 healthy individuals recruited through our laboratory. All of them were instructed to avoid non-steroidal anti-inflammatory drugs (NSAID) or acetylsalicylic acid (ASA) at least two weeks prior to the study. A second control group constituted of 39 nonAPS thrombosis-patients, all treated with warfarin at our coagulation unit. The control groups are referred to as healthy controls (HC) and nonAPS thrombotic controls (nonAPS-TC) respectively.

Written informed consent was obtained from patients and control subjects. The local Ethics Committees approved the study.

Handling of Blood Samples

Blood samples from the 49 APS-patients and 19 HC were centrifuged at room temperature to obtain platelet poor plasma (for 20 min at 2570 and 2000G respectively) which was then dispensed into aliquots and stored at -70 °C. Blood samples from the 39 nonAPS-TC were centrifuged (15 min at 2000G) prior to storage at -70 °C.

Evaluation of Fibrin Porosity and Fibrinogen Concentration

Evaluation of fibrin permeability was carried out as previously described [26]: 49 APS-patient plasma samples, 19 samples from HC and 39 samples from nonAPS-TC (total volume 500 μL) were dialysed at 4 °C against dialysing buffer (pH 7.4, 0.05 mol/L Tris, 0.1 mol/L NaCl (VWR, number: 16404–1) 1 mmol/L ethylenediamine tetraacetic acid (EDTA) (Ficher Scientific, Stockholm, Sweden) aprotinin (standard drug ordered from Swedish pharmacy) 5 KIU/mL) for 3 hours, with change of outer fluid at 1-hour intervals (the aprotinin content of the dialysing buffer inhibits fibrinolysis in the examined plasma and thereby enables stability of the fibrin clot over-night). Subsequently, 200 μL of dialysed plasma were transferred into plastic test tubes and 10 μL of $CaCl_2$ (conc. 440 mmol/L) and 10 μL of thrombin (Sigma Aldrich, St. Louis, MO, USA (conc. 4.5 IU/mL) were added. Plasma was mixed and immediately transferred into small plastic cylinders where the fibrin clots were formed. The final concentrations of $CaCl_2$ and thrombin were 20 mmol/L and 0.2 IU/mL, respectively. The clots were left in a moisture atmosphere overnight in order to allow clot formation in all samples. Thereafter, percolating buffer (pH 7.4, 0.02 mol/L Tris, 0.02 mol/L imidazole (Ficher Scientific, Stockholm, Sweden), 0.1 mol/L NaCl) was allowed to pass through the clots at 5 different hydrostatic pressures and the volume of collected eluate was measured after an indicated time [26]. The permeability coefficient (Darcy constant, Ks), providing information on the fibrin network porosity, was calculated from the equation given by Carr et al. 1977 [27]. A low Ks value indicates less permeability and a tighter fibrin clot. For 49 APS-patients, 19 HC and 38 nonAPS-TC the permeability coefficient could be calculated and used in the sub sequential analysis. The intra-assay coefficient of variation (CV) for the method is 9.6% and the inter-assay CV is 9.5% [28].

For the 49 APS-patients fibrinogen concentrations in plasma were analysed using a BN Prospec nephelometer (Dade Behring, Deerfield, IL, USA) with reagents from the same manufacturer. The assay had a total coefficient of variation of 5.7% at 2.23 g/L and 4.4% at 4.73 g/L. For the 39 nonAPS-TC and 17 HC, fibrinogen concentrations in plasma were analysed using a Sysmex® CS2100i (Sysmex, Kobe, Japan) with reagent Dade Thrombin from the Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA. The assay had a total coefficient of variation of 4.0% at 1.24 g/L and 3.47 at 3.16 g/L.

High sensitive C-reactive protein (hsCRP) was analyzed using a Beckman Coulter Synchron LX system Chemistry Analyser with reagents and calibrators supplied by Beckman Coulter Inc. (Sydney, NSW, Australia). Albumin was analyzed by means of a bichromatic digital endpoint methodology using bromcresol purple reagent ALBm®, Beckman Coulter Inc Ireland (Mervue, Galway, Ireland).

Turbidimetric Clotting and Lysis Assays

For the 49 APS-patients, 19 HC and 39 nonAPS-TC a turbidimetric clotting assay and a turbidimetric lysis assay were performed as previously described [24]. Two APS-patients (one with previous arterial thrombosis) both treated with dalteparin, were excluded from the analysis since no clotting reaction was initiated in the analysis of their plasma samples. Subsequently, plasma samples from 47 APS-patients, 19 HC and 39 nonAPS –TC were finally included in the analyses.

In brief, for the turbidimetric clotting assay 75 μl of assay buffer (pH 7.4, 0.05 mol/L Tris–HCl, 0.15 mol/L Na Cl) was added to 25 μl of citrated plasma (in duplicate) in a microtiter plate. Fifty μl of a mixture