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Full Length Article

Thrombin activatable fibrinolysis inhibitor (TAFI) — A possible link between coagulation and complement activation in the antiphospholipid syndrome (APS)

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ABSTRACT

Background: Thrombosis and complement activation are pathogenic features of antiphospholipid syndrome (APS). Their molecular link is Plasma carboxypeptidase-B, also known as thrombin activatable fibrinolysis inhibitor (TAFIa), which plays a dual role: anti-fibrinolytic, by cleaving carboxyl-terminal lysine residues from partially degraded fibrin, and anti-inflammatory, by downregulating complement anaphylatoxins C3a and C5a.
Aim: To investigate the levels of TAFI (proenzyme) and TAFIa (active enzyme) in relation to complement activation, fibrin clot permeability and fibrinolytic function in clinical and immunological subsets of 52 APS patients and 15 controls.
Results: TAFI ($p < 0.001$), TAFIa ($p < 0.05$) and complement factor C5a ($p < 0.001$) were increased, while fibrin permeability ($p < 0.01$) was decreased and clot lysis time (CLT) was prolonged ($p < 0.05$) in APS patients compared to controls. Furthermore, TAFIa was increased ($p < 0.01$) in samples from APS patients affected by arterial thrombosis compared to other APS-phenotypes. Positive associations were found between TAFI and age, fibrinogen and C5a, and between TAFIa and age, fibrinogen and thrombomodulin.
Conclusion: TAFI and TAFIa levels were increased in patients with APS as a potential response to complement activation. Interestingly, TAFI activation was associated with arterial thrombotic APS manifestations. Thus, TAFIa may be considered a novel biomarker for arterial thrombosis in APS.

1. Introduction

The Antiphospholipid syndrome (APS) is diagnosed when arterial, venous, small vessel thrombosis or obstetric morbidity concur with positive laboratory tests for antiphospholipid antibodies (aPL: anticardiolipin (aCL), anti-$\beta_2$glycoprotein-I (anti-β_2GPI)) or functional lupus anticoagulant (LA) [1]. Venous thromboembolism (VTE) is the most frequently reported clinical feature, whereas ischemic cerebrovascular disease is the most common arterial thrombotic manifestation [2]. Early and late fetal losses, premature births and pre-eclampsia are the most frequent obstetric complications [1].

The pathophysiology of APS is complex indicating that besides aPL, additional factors are necessary to induce the syndrome [3], like excessive activation of endothelial cells and platelets, aberrations in the clotting cascade and impaired fibrinolysis [3,4]. Although inflammation is not the most prominent characteristic of APS, there is mounting evidence that an inflammatory state is involved in the pathophysiology of both thrombotic [5–7], and obstetric events [8–10]. In particular, several reports demonstrate that the complement cascade is activated in APS [11–13]. The complement split products C5a and C3a are powerful inflammatory anaphylatoxins, considered to play a crucial role in the syndrome [14,15].

The levels of C3a and C5a, together with bradykinin and osteopontin, are regulated by the enzyme plasma carboxypeptidase-B, also known as thrombin-activatable fibrinolysis inhibitor (TAFIa) [16]. TAFIa derives from its pro-enzyme (TAFI), which is synthetized by several cell types including hepatocytes, macrophages and megakaryocytes, and

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activated by trypsin, plasmin and thrombin. The cleavage of the zymogen is further enhanced by the thrombin-thrombomodulin complex on the surface of endothelial cells [17].

TAFIa cleaves carboxyl-terminal lysine and arginine residues from partially degraded fibrin, limiting plasminogen binding and plasmin generation [23,24]. Thus, TAFIa plays dual roles: anti-inflammatory, by inactivation of complement split products C5a and C3a, and anti-fibrinolytic, through fibrin modifications [18,19].

However, to date, the role of TAFI/TAFIa in APS is unknown.

We hypothesized that TAFIa's dual activity might be a potential molecular link between complement activation and thrombosis, crucial pathogenic features of APS.

Therefore, we investigated TAFI/TAFIa levels in different subsets of APS-patients and in healthy controls. We also examined the relationship between TAFI/TAFIa levels and fibrin clot tightness, fibrinolytic function and complement activation quantified by means of C5a.

2. Material and methods

2.1. Patients

We recruited 52 patients (43 women and 9 men; mean age 44 years) fulfilling APS-criteria [1] from the Rheumatology, Haematology and Women's and Children's Health clinics, Karolinska University Hospital, between 2008 and 2012. At inclusion, patients filled out a detailed questionnaire about their daily habits, clinical history, signs, symptoms and medications; all records were verified through medical file review. The inclusion as well as consecutive blood-sampling was performed in the convalescent phase of the disease within median 3 (IQR: 7) years after diagnosis.

According to criteria [1], aPL positivity was considered when at least one test among anticardiolipin IgM/IgG isotype (aCL IgM/IgG) at medium/high titer, anti-β_2 glycoprotein-I IgG isotype (anti-β_2GPI IgG) and lupus anticoagulant (LA) was positive twice: confirmation tests were performed after a minimum of 12 weeks. We also required at least 12 weeks and no more than 5 years between positive testing and the clinical manifestation.

Eight patients also fulfilled criteria for other rheumatic diseases (6 rheumatoid arthritis, 1 ankylosing spondylitis with ulcerative colitis, and 1 Sjögren's syndrome); the remaining 44 were primary APS patients. Patients with systemic lupus erythematosus (SLE) were excluded from the study.

All thrombotic episodes were confirmed by imaging techniques (ultrasound, computed tomography-CT and/or magnetic resonance-MR). Pregnancy associated morbidity was defined according to classification criteria for APS [1]. Arterial hypertension was considered present if patients were receiving antihypertensive treatment or if arterial blood pressure was >140/90 mm Hg at inclusion; hyperlipidemia, if the patient answered positively to the questionnaire or if the available values of total or fractioned cholesterol were not in the reference range at the time of enrollment.

As controls, we recruited 15 healthy individuals through our laboratory. All of them were instructed to avoid non-steroidal anti-inflammatory drugs (NSAID) and acetylsalicylic acid (ASA) for at least two weeks before blood sampling. The local Ethics Committees approved the study and written informed consent was obtained from all participants.

2.2. Blood sampling

Blood samples from the APS-patients and healthy controls were drawn into 0.129 mol/L trisodium citrate, pH 7.4 (1 part trisodium citrate + 9 parts blood). Within 60 min of sampling, the citrated blood was centrifuged at room temperature (for 20 min at 2570 and 2000*g* respectively). Obtained plasma and serum were deep frozen in aliquots of 0.5 mL at −70 °C until the assays were made. Samples were thawed in water bath at 37C before testing.

2.3. Laboratory assays

Antibodies against cardiolipin (aCL IgM/IgG) as well as antibodies against β_2 glycoprotein-I (anti-β_2GPI IgG) were analyzed by a routinely performed ELISA (Organtec, Mainz, Germany). Lupus anticoagulants were performed using Dilute Russell Viper Venom (DRVVT) method according to manufacturer's instructions. Reagents from Biopool, Umeå, Sweden and Gradipore, North Ryde, Australia.

TAFI (proenzyme) was determined by a chromogenic substrate method using the STA® - Stachrom® TAFI kit (Diagnostica Stago, France).

TAFIa (enzyme) was quantified by measuring TAFIa/TAFIai complex (complex of active and inactive forms of the enzyme corresponding to the concentration of the active form that cannot be measured because of instability) using an ELISA (Asserachrom TAFIa/TAFIai antigen kit; Diagnostica Stago, France).

C5a was analyzed using MicroVue C5a Enzyme Immunoassay, which is a direct-capture immunoassay for the measurement of C5a in human serum or plasma (Quidel Corporation, San Diego, USA).

Thrombomodulin was determined using Human Thrombomodulin/BDCA-3 Quantikine ELISA Kit, R&D Systems Inc. USA, employing the quantitative sandwich enzyme immunoassay technique.

Fibrin clot structure was studied in citrated plasma samples by measurement of the permeability coefficient (Ks) [20], and the results have been published elsewhere by our group [21]. 200 μL of plasma was supplemented with $CaCl_2$ and thrombin at final concentrations of 20 mmol/L and 0.2 U/mL respectively, and 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 (Fisher 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. Analyses were performed in duplicate. The permeability coefficient (Darcy constant, Ks), providing information on the fibrin network porosity, was calculated [20]. Low levels of Ks indicate reduced fibrin permeability. The inter-assay coefficient of variation was 9.5%.

Turbidimetric clotting and lysis assays Fibrin formation and degradation was assessed with turbidimetric clotting and lysis assays respectively, according to the methods described by Carter et al. [22]. In brief, 75 μL of assay buffer (pH 7.4, 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl) was added to 25 μL of citrated plasma (in duplicate) in a microtiter plate. 50 μL of a mixture of thrombin (final concentration 0.03 IU/mL) and $CaCl_2$ (final concentration 7.5 mMol/L) was added to the plasma samples and the absorbance at 340 nm was read every 18 s (240 cycles for each sample). In the turbidimetric lysis assay, recombinant tPA (Technoclone, GmbH, Vienna, Austria) was added to the Tris-HCl buffer at a final concentration of 83 ng/mL. CLT was defined as the time from the midpoint of the clear–to–maximum-turbidity transition (which is defined as clotting time), to the midpoint of the maximum turbid–to–clear transition. Inter-assay CV: 11.4%, intra-assay CV: 2.3%.

Fibrinogen concentration Analyses of 29 samples were performed using a BN Prospec nephelometer (Dade Behring, Deerfield, IL, USA) with reagents from the same manufacturer. In the samples from 23 APS patients and healthy controls analyses were performed using a Sysmex® CS2100i (Sysmex, Kobe, Japan) with reagent Dade Thrombin from the Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA.

2.4. Statistical analysis

Data are presented as mean values ± standard deviation (SD) for normally distributed variables, and as median (interquartile range (IQR)) for non-normally distributed variables. Data with skewed distributions were logarithmically transformed to obtain a normal distribution, if possible. Fisher's exact test was used to compare categorical variables between independent groups. Continuous variables were compared with independent sample *t*-test when normally distributed

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