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Impaired plasminogen binding in patients with venous thromboembolism: Association with protein carbonylation

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ABSTRACT

Introduction: Venous thromboembolism (VTE) is associated with hypofibrinolysis. Its mechanisms are poorly understood. We investigated plasminogen-fibrin interaction and its association with fibrinolytic capacity and protein oxidation/carbonylation in VTE patients.

Materials and methods: Plasma-purified plasminogen conversion to plasmin and surface plasmon resonance employed for plasminogen-fibrin interactions were individually evaluated in all healthy controls and non-anticoagulated patients following VTE, 10–23 months since the event. We also assessed plasma fibrin clot permeability (K_s), clot lysis time (LT), activators and inhibitors of fibrinolysis together with oxidation/carbonylation markers.

Results: VTE patients had impaired plasminogen binding to fibrin (apparent Kd, + 290%, $p = 0.002$), reduced rate of plasmin generation (− 4.7%, $p = 0.001$), and longer LT (+ 18.6%, $p < 0.001$) compared with controls. Fibrinogen and K_s were similar in both groups. Apparent Kd correlated with LT ($r = 0.43$, $p = 0.037$), tissue plasminogen activator-plasminogen activator inhibitor 1 (tPA-PAI-1) complexes ($r = 0.63$, $p = 0.012$), and active PAI-1 ($r = 0.49$, $p = 0.03$). Compared with controls, VTE patients had higher thiobarbituric acid reactive substances (TBARS), total protein carbonyl content (PC), and lower total antioxidant capacity (all $p < 0.001$), that all were associated with LT ($r = 0.61$, $r = 0.56$, and $r = -0.47$, respectively, all $p < 0.05$). Impaired plasminogen binding to fibrin reflected by apparent Kd positively correlated with TBARS ($r = 0.48$, $p = 0.032$) and PC ($r = 0.54$, $p = 0.013$) in the whole group.

Conclusions: Plasminogen-fibrin interactions are altered in young and middle-aged VTE patients, without known thrombophilias, except increased factor VIII. The mechanisms underlying these phenomena remain to be established.

1. Introduction

Venous thromboembolism (VTE) involving deep vein thrombosis (DVT) and pulmonary embolism (PE) is a chronic condition with 30% of patients experiencing a recurrent event within 10 years [1,2] and VTE-associated mortality ranges between 300,000 and 500,000 deaths per year [3].

It has been demonstrated that VTE patients are characterized by faster formation of denser fibrin clots displaying impaired lysability, which is at least in part genetically determined [4]. Prolonged clot lysis time (LT), a global fibrinolytic capacity measure, reported in VTE patients is largely determined by elevated plasminogen activator inhibitor 1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI), α_2-antiplasmin, and decreased plasminogen [4]. A hypercoagulable state associated with VTE is also associated with inflammation and enhanced oxidative stress, which both may contribute to thrombosis through increased posttranslational modifications (PTMs) of blood clotting proteins [5]. Fibrinogen is a main target for oxidative PTMs among human plasma proteins [6]. In the consequence of oxidative modification encountered at the physiological levels in humans, fibrin forms denser fiber networks with increased stiffness, which typically correlates with prolonged LT [5]. It is known that oxidative PTMs of fibrinogen have the potential to influence the kinetics of fibrin formation as well as the structure and biomechanical properties of fibrin, ultimately producing

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dysfunctional hemostatic clots depending on the oxidant type, dose-dependent inhibition of thrombin-induced fibrin polymerization and/or exposure time [7]. Some of oxidative PTMs have been identified *in vivo*, providing further evidence for a relationship between the production of oxidants and thrombosis [6]. It has been demonstrated that in patients after myocardial infarction fibrinogen carbonylation was increased and associated with altered fibrin polymerization and susceptibility to plasmin-induced lysis [8]. Nitration also has a potential to increase fibrin formation and stiffness, impair clot lysis, and alter fibrin structure [7,8]. However, it has been reported that a low extent of both oxidation and nitration did not affect clotting activity of fibrinogen or clot lysis [9].

Little is known about plasminogen modifications *in vivo*. Ajjan et al. showed that glycation of plasminogen in diabetic patients directly affected fibrinolysis by decreasing plasmin generation, reducing protein-specific activity as well as decreasing binding of glycated plasminogen to fibrin(ogen) [10]. It is unclear whether other PTMs of plasminogen molecules may contribute to altered plasmin generation in non-diabetic patients at risk of cardiovascular or thrombotic risks.

A single human plasminogen peptide chain is divided into a contiguous series of five homologous regions called kringles at the N-terminus and a serine proteinase catalytic domain in the C-terminus [11]. Lysine-binding site substructures present in K1 and K4 [12] display affinity for lysine residues, physiologically relevant for binding to fibrin at a thermostable part of the fibrin D fragment [13], to extracellular matrix components [14], and to cells [15]. K5 has some affinity for lysine analogues, but it is too weak to firmly anchor plasminogen to surface lysines [11]. Recent studies suggest, that the C-terminal lysine residues function as additional plasminogen-binding sites and can initiate the plasminogen conformational change [11].

Plasminogen deficiency occurs in 0.5% to 2.0% of patients with thrombosis, however its association with the increased VTE risk has not been proved [16]. Of note, the only well-recognized disorder associated with plasminogen deficiency in which reduced levels of plasminogen have a clear pathophysiological role is ligneous conjunctivitis [17].

The aim of the current study was to investigate plasminogen-fibrin interactions and its association with fibrinolysis and protein oxidation/carbonylation in VTE patients.

2. Patients and methods

Blood samples were collected from 19 patients (9 men, 10 women) aged from 18 to 61 years (median 34) following the first-ever documented VTE episode, 15 (10 − 23) months since the index event. All patients stopped anticoagulation before enrolment. There were 13 subjects with isolated DVT and 6 individuals with PE and concomitant DVT. Age and sex-matched 16 healthy subjects served as controls (5 male, 11 female; median age 39 years). The diagnosis of DVT of the lower or upper limb was established by a positive finding of color duplex sonography (visualization of an intraluminal thrombus in calf, popliteal, femoral, or iliac veins). The diagnosis of PE was based on the presence of typical symptoms and positive results of high resolution spiral computed tomography (CT). Unprovoked (idiopathic) VTE episode was defined as having no history of cancer, surgery requiring general anesthesia, major trauma, plaster cast or hospitalization in the last month, pregnancy or delivery in the last 3 months. A proximal DVT event was defined as thrombosis in the popliteal vein, including the trifurcation, the femoral or iliac veins.

The following exclusion criteria were acute infection, known malignancy, vitamin K antagonist (VKA) treatment in last 3 months, acute coronary syndromes or stroke within preceding 6 months, international normalized ratio (INR) > 1.2 at the day of enrolment, indication for long-term anticoagulation due to other reasons than VTE [18], end-stage kidney disease, any recurrent VTE events, inherited and acquired thrombophilias, including factor V Leiden mutation, prothrombin G20210A, or antithrombin III deficiency, and the refusal to provide a consent. The Jagiellonian University Medical College Ethical Committee approved the study, and participants provided informed consent in accordance with the Declaration of Helsinki.

2.1. Laboratory investigations

Blood samples were drawn from an antecubital vein with minimal stasis using atraumatic venipuncture at 8:00 to 10:00 AM. Blood samples were collected into citrated tubes (9:1 of 0.106 M sodium citrate), centrifuged at 2500*g* at 20 °C for 10 min, snap-frozen within 60 min, and stored in small aliquots at − 80 °C until analysis. Samples were thawed at 37 °C for 5 min, again centrifuged, and the supernatants were used for analyses of fibrinolytic proteins and inhibitors. Complete blood count, glucose, creatinine, activated partial thromboplastin time and INR were assayed by routine laboratory techniques. Fibrinogen was determined using the Clauss assay. High-sensitivity C-reactive protein (CRP) was measured by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany). Plasma D-dimer was measured with the Innovance D-dimer assay (Siemens, Marburg, Germany). All VTE patients were screened for thrombophilia [19], including factor V Leiden mutation, prothrombin G20210A, protein C and S deficiencies, and presence of antiphospholipid syndrome. Plasma levels of factor (F)VIII were evaluated using the Behring Coagulation System (Siemens Healthcare Diagnostics, Marburg, Germany). Commercially available immunoenzymatic assays were used to determine fibrinolytic proteins, including plasma PAI-1 (Technoclone, Vienna, Austria), tissue-type plasminogen activator (tPA) (American Diagnostica, Stamford, CT, USA) as well as activated and inactivated TAFI (TAFIa/ai) antigen (Imubind TAFIa/ai antigen ELISA; American Diagnostica) and tPA-PAI-1 complexes (tPA-PAI-1 Complex ELISA Kit, Technoclone, Vienna, Austria). Plasminogen was measured by a chromogenic assay (STA-Stachrom plasminogen, Diagnostica Stago, Asniéres, France).

2.2. Fibrin clot permeation

Fibrin clot permeation was determined using a pressure-driven system [20,21]. Briefly, 20 mM calcium chloride and 1 U/mL human thrombin (Merck KGaA, Darmstadt, Germany) were added to 120 μL citrated plasma. After 2 h of incubation in a wet chamber, tubes containing the clots were connected *via* plastic tubing to a reservoir of a buffer (0.01 M Tris, 0.1 M NaCl, pH 7.4) and its volume flowing through the gels was measured within 60 min. A permeation coefficient (K_s), which indicates the pore size, was calculated from the equation: $K_s = Q \times L \times \eta/t \times A \times \Delta p$, where Q is the flow rate in time t; L, the length of a fibrin gel; η, the viscosity of liquid (in poise); A, the cross-sectional area (in cm^2); Δp, a differential pressure (in dyne/cm^2) and t is percolating time. The interassay and intraassay coefficients of variation were < 7%.

2.3. Lysis assays

To assess efficiency of clot lysis, we used the method described by Carter et al. [22] with slight modifications. Briefly, 75 μL of assay buffer (5 mM TRIS-HCl, 15 mM NaCl, pH 7.4) with addition of recombinant tPA (rtPA, Boehringer Ingelheim, Ingelheim, Germany) at a final concentration of 83 ng/mL was added to 25 μL of citrated plasma in a microtiter plate. Then, 50 μL of a mixture of thrombin (final concentration 0.03 U/mL) and $CaCl_2$ (final concentration 7.5 mM) were added with a multichannel pipette. The turbidity was measured at 405 nm at 37 °C. LT was defined as the time from the midpoint of the clear-to-maximum-turbid transition, which represents clot formation, to the midpoint of the maximum-turbid-to-clear transition (representing the lysis of the clot). The interassay and intraassay coefficients of variation were < 8%.