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Microparticle-associated tissue factor activity in patients with acute unprovoked deep vein thrombosis and during the course of one year

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ABSTRACT

Background: Tissue factor (TF) is the main *in-vivo* initiator of blood coagulation. Microparticles (MPs) are small procoagulant membrane vesicles. Elevated TF-bearing MPs have been found in different prothrombotic conditions and MP-associated TF activity may contribute to the pathogenesis of unprovoked deep vein thrombosis (DVT).
Objective: To determine MP-TF activity levels at diagnosis of DVT and at four additional time points during the course of one year in a well-defined group of patients with unprovoked DVT of the lower limb.
Patients/Methods: In this study, 41 patients with acute unilateral symptomatic and unprovoked DVT of the lower limb were included and followed for 1 year. Venous blood samples for determination of MP-TF activity were drawn at diagnosis of acute DVT, and 1-, 3-, 6-, and 12 months later. In addition, 10 young and healthy control subjects were included.
Results: The median MP-TF activity was 0.06 pg/mL (25th-75th percentile: 0.0-0.53) in patients with acute DVT and 0.18 pg/mL (0.07-0.33) in healthy controls, and did not differ significantly ($p = 0.35$). No significant changes in MP-TF activity were found in the follow-up measurements. MP-TF activity did also not differ significantly between patients with proximal- or distal DVT and between those with- or without residual DVT after 6 months.
Conclusions: MP-TF activity is low at the acute event in patients with unprovoked DVT of the lower limb and remains unchanged during the course of the disease. Our data do not support the hypothesis that TF-bearing MPs play a determining role in the pathogenesis of unprovoked DVT.

Introduction

More than 150 years ago Virchow published his thoughts on the pathogenesis of deep vein thrombosis (DVT), centered on the triad of stasis, changes in the vessel wall and hypercoagulability [1]. To date, it is well established that DVT most frequently occurs on the surface of activated but largely intact endothelium [2]. It was demonstrated that particularly the valve pockets of the large deep veins of the lower limbs are prone to thrombosis, due to irregular patterns of blood flow and hypoxia-induced endothelial activation [3]. Increasing evidence also indicates a central role of prothrombotic changes in the blood, referred to as thrombophilia, in the pathogenesis of unprovoked DVT [4]. However, most of the to date established contributors to thrombophilia, like the presence of heterozygous factor V Leiden- or prothrombin mutation, have been shown to increase the propensity of developing venous thromboembolism (VTE) only moderately [5].

Tissue factor (TF) is a transmembrane receptor and the main *in-vivo* initiator of blood coagulation. It was hypothesized that DVT is triggered by increased amounts of active circulating TF, which is bound to small procoagulant membrane vesicles, so-called microparticles (MPs). Indeed, elevated levels of MP-associated TF activity were found in different prothrombotic conditions with significant activation of monocytes like cancer-related VTE [6–8], disseminated intravascular coagulation [9,10] and sickle cell disease [11]. Since MPs are known to fuse with activated platelets and neutrophils, TF-bearing MPs may also contribute to diseases like antiphospholipid syndrome [12] and the formation of neutrophil extracellular traps [13].

Data on the course of plasma MP-TF activity after acute unprovoked DVT are missing, so far. We therefore sought to determine MP-TF activity levels at the time of diagnosis and at four different time points in the following year in a well- defined group of patients with unprovoked DVT of the lower limb. We also investigated whether MP-TF activity levels differ between patients with acute proximal- or distal DVT, and between those with- or without residual DVT after 6 months. In addition,

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we determined TF antigen levels, and investigated whether they correlate with MP-TF activity levels.

Methods

Study population

In this prospective longitudinal study 41 patients with acute unilateral symptomatic DVT of the lower limb were included and followed for 1 year. All patients suffered acute unprovoked DVT, which was defined as thrombosis without a triggering event such as surgery, trauma causing immobilization, pregnancy, delivery, or malignancy. All patients were diagnosed at the outpatient department of the Division of Angiology of the Medical University of Vienna, Austria. DVT was confirmed in all patients by color duplex sonography. Events in women taking contraceptives were also considered unprovoked, because such treatment had been continued for a long time and was probably not the trigger for DVT.

In addition, 10 young and healthy control subjects were recruited for this study. These control subjects came from the same geographic region and ethnic background, and were neither related to the patients nor related to each other. Healthy individuals were hospital staff or relatives and friends of hospital staff. Five controls were females.

Young and healthy individuals were included for defining MP-TF activity levels in physiologic conditions. Controls with an age of 18 years or older underwent a structured interview with a detailed standardized questionnaire focusing on the detection of events that indicate alterations of the hemostatic system. Specific exclusion criteria for healthy controls were arterial or venous thromboembolism or a bleeding tendency in the medical history, routine blood coagulation tests below or above the normal range at study inclusion, surgery within one month preceding study inclusion, clinically overt bacterial or viral infections and smoking. In addition, women who were pregnant, following delivery, lactating or taking hormonal contraceptives were not included in the control group. The median age of controls was 29 years (25th-75th percentile: 26-31 years) and the median body mass index was 23 kg/m^2 (25th-75th percentile: 22-26 kg/m^2).

Initially all patients were treated with low molecular weight heparin and then switched to vitamin K antagonists with a therapeutic target (international normalized ratio of 2 to 3). Thirty-three patients (80.5%) received oral anticoagulation (OAC) for 6 months. Eight patients (19.5%) still received OAC at 12-months. Six patients had DVT and 3 patients had concomitant PE and DVT in their medical history.

Exclusion criteria were: age <18 years, ongoing anticoagulant treatment at study entry, active malignancy, and life expectancy <2 years. Specific exclusion criteria for healthy controls were arterial or venous thromboembolism or a bleeding tendency in the medical history, routine blood coagulation tests below or above the normal range at study inclusion, surgery within one month preceding study inclusion, clinically overt bacterial or viral infections and smoking. In addition, women who were pregnant, following delivery, lactating or taking hormonal contraceptives were not included in the control group.

The study protocol was approved by the local Ethics Committee and the study was conducted in accordance with the Declaration of Helsinki.

Blood sampling

Venous blood samples for measurements of MP-TF activity were drawn into citrate vacuum tubes by atraumatic and sterile antecubital venipuncture at diagnosis of acute DVT, and 1-, 3-, 6-, and 12 months later. The first 3 mL of blood were discarded to minimize contamination with traces of TF in the puncture wound. To avoid procedural deviations, all blood samples were taken by the same physician applying a light tourniquet, which was immediately released, and the samples were mixed adequately by gently inverting the tubes.

Plasma preparation and isolation of MPs

Cells were removed by centrifugation at 1500 *g* for 20 min (2). The centrifugation of each sample was performed within 1 h after blood sampling and the freezing of each sample within 1 h after centrifugation. Plasma aliquots were stored at −80 °C until measurements were performed in series.

Isolation of MPs was performed according to a protocol published by Khorana et al. [14]. MPs were pelleted two times by high-speed centrifugation at 20 000 x *g* for 15 min at 4 °C. After each centrifugation step supernatant was removed carefully except for 50 μL containing the MP pellet.

MP-TF activity measurement

The measurement of MP-TF activity was performed as previously described by Khorana et al. with a chromogenic endpoint assay that quantifies the MP-TF dependent factor Xa (FXa) generation [14]. After preparation of MPs the pellet was incubated with either an antibody for human TF (hTF1, 4 μg/mL; 1 μL; BD Biosiences, San Jose, USA) or a control antibody (mouse IgG: 4 μg/mL; 1 μL; Sigma-Aldrich, St. Louis, USA) for 15 min and then 50 μL aliquots were added to duplicate wells of a 96-well plate. In the next step, 50 μL of HBSA containing 10 nM factor VIIa (FVIIa), 300 nM factor X (FX) and 10 mM $CaCl_2$ were added to each sample and the mixture incubated for 2 h at 37 °C, then the FXa generation was determined. FXa generation was stopped by the addition of 25 μL of 25 mM EDTA buffer and 25 μL of the chromogenic substrate Pefachrome FXa 8595 (4 mM; Pentapharm, Basel, Switzerland) were added and incubated at 37 °C for 15 min. Finally, absorbance at 405 nm was measured using a Multiscan Spectrum microplate reader (Thermo Scientific Inc., Bremen, Germany). TF activity was calculated by reference to a standard curve that was generated using relipidated recombinant human TF (0–55 pg/mL; Innovin Dade-Behring, Marburg, Germany). The TF-dependent FXa generation (pg/mL), which represents the MP-TF activity, was determined by subtracting the amount of FXa generated in the presence of hTF1 from the amount of FXa generated in the presence of the control antibody.

TF antigen measurement

TF antigen levels were measured in plasma with a commercial ELISA (Immunobind® Tissue Factor, American Diagnostica, Stamford; CT USA) using a Multiscan Spectrum microplate reader.

Color duplex sonography

Color duplex sonography was performed at baseline, and 1-, 3-, 6-, and 12 months after acute DVT at the outpatient department of the Division of Angiology at the Medical University of Vienna. To avoid investigator-related variations of the results, color duplex sonography was performed in each patient by the same investigator at all time points.

Statistical analysis

Continuous variables were described by the median and the interquartile range (25th-75th percentile). Categorical variables were described by the absolute numbers and percentages. The Kruskal-Wallis test and the Wilcoxon rank-sum test were used for group comparisons.

Results

Patients' characteristics

The median age of 41 patients with acute unprovoked DVT of the lower limb was 51 years (25th-75th percentile: 39-64 years). Twenty-