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Regular Article

Procoagulant activity, but not number, of microparticles increases with age and in individuals after a single venous thromboembolism

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ABSTRACT

The Calibrated Automated Thrombogram (CAT), a plate-based assay that measures thrombin generation and inhibition in plasma samples, is modified to measure the procoagulant activity of phospholipid associated with plasma microparticles (MP). The assay uses a tissue factor trigger without addition of 4 μM exogenous phospholipid (PL) used in the standard CAT. Calibrated with 4:1 posphatidylcholine- phosphatidylserine (PCPS) liposomes, the assay defines a median of 40 nM procoagulant phospholipid (PPL) equivalents in plasma containing MPs from 50 normal donors, with a range from 20 – 200 nM. Like the standard CAT, the modified assay detected no difference in plasma from 36 individuals with a history of a single episode of venous thromboembolism. However the male cases had double the PPL activity, as measured by rate of thrombin generation, of females; and there was a significant correlation among cases of increased thrombin generation with age. In contrast, there were no gender disparities or age correlations among control plasmas. The findings suggest that procoagulant activity of plasma microparticles, facilitated by a simplified, one-stage plate-based assay, offer a promising avenue of investigation of mechanisms and management of venous thromboembolic disorders.

Introduction

Thrombosis can occur when a vascular lesion activates the hemostasis system, or when the hemostasis system itself becomes hypersensitive to an otherwise benign vascular anomaly. This hyper-responsiveness of the system, viewed as "thrombophilia" or "propensity to thrombosis" [1] is clearly dependent upon components within blood [1,2] but is otherwise undefined [1,3]. The presymptomatic diagnosis and subsequent prediction of thrombosis after disease is established remain elusive. Elucidation of mechanisms of thrombosis, development of sensitive and specific diagnostics, and design of effective but benign therapeutics calls for better insight into the biochemistry and cell physiology of hemostasis, the pathobiology of vascular disease, and the pathophysiology of the thrombotic process itself.

The risk of recurrence after an initial VTE is the highest within six months and as high as 30% within 10 years. Standard medical practice is that all VTE patients are prescribed prophylactic anticoagulants [4,5]. Complications of prolonged anticoagulation therapy can be serious, so patients who would not require treatment are exposed to unnecessary risk [6,7]. Known risk factors for recurrence of VTE include indications of ongoing thrombosis, such as a high D-dimer level, residual thrombosis detected by ultrasound and increased thrombin generation [8–13]. Male gender has also been found to be associated with a higher risk for VTE recurrence, but a plausible mechanism is not apparent [14–17].

The presence of increased circulating procoagulant phospholipid (PPL) has been detected in a number of pre-thrombotic conditions, including myocardial infarction (MI), cancer and trauma but has not been reported for VTE [18,19]. Triggers for venous thrombosis are not known, but the process of clot formation requires PPL and definition of thrombophilia could most certainly include increased levels of circulating procoagulant phospholipids [20]. Most PPL detected in other disease states appears to be associated with microparticles derived from platelets. PPL-positive microparticles shed from platelets are thought to result as a consequence of platelet activation and therefore an indicator of ongoing thrombosis (reviewed in [21]). Although platelets do not generally appear to be prevalent within venous thrombi [22,23], microparticles derived from circulating platelets have been shown to be very potent activators for both factor X and prothrombin [19,24]. To determine whether procoagulant phospholipids associated with microparticles are present in VTE patients, we modified an established thrombin generation assay, the calibrated automatic thrombogram (CAT) [25], to be sensitive to the

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PPL associated with procoagulant microparticles. Since annexin V binding has been equated to procoagulant activity, we also assayed isolated platelet microparticles by flow cytometry. We find that procoagulant phospholipid associated with microparticles is increased in VTE patients with increasing age but not with the number of annexin V-positive microparticles derived from platelets. Furthermore, significantly higher rates of thrombin generation are supported by microparticles in male compared to female VTE patients.

Methods

Patient and Control subjects

Mayo Clinic outpatients age 18 years or older with objectively-diagnosed DVT or PE (confirmed by venography, pulmonary angiography, compression venous duplex ultrasonography, ventilation/perfusion lung scan interpreted as high probability for PE, computed tomographic pulmonary angiography, magnetic resonance imaging or pathology examination of thrombus removed at surgery) who resided in the upper Midwest United States and who were referred to the Mayo Clinic Special Coagulation Laboratory or Mayo Clinic Thrombophilia Center were recruited over the study period, (10/13/2008 – 11/06/2009, 12 months), for study participation. The time between VTE and blood draw ranged from <1-33 years, with a median of 5 years. We excluded patients with VTE related to active cancer, an indwelling central venous catheter, transvenous pacemaker or other mechanical cause for thrombosis, or a lupus anticoagulant or other antiphospholipid antibodies. Controls were selected prospectively from persons undergoing outpatient general medical examinations during the same time period. VTE cases and controls were not receiving anticoagulation (heparin, warfarin) or an antithrombotic (aspirin, non-steroidal anti-inflammatory drug, thienopyridine) within the four weeks prior to blood sample collection. We collected 51 controls (27 females and 23 males) and 36 VTE patients, (15 females and 21 males) to be assayed for thrombin generation. Ages ranged from 19-81, and all were white of European ancestry. Except for one female control, no VTE cases or controls were receiving hormone therapy.

Sample Collection and Processing

Blood was collected by venipuncture using an 18 gauge winged-infusion set (Becton Dickinson, New Jersey, USA) into Vacutainer tubes containing 3.2% sodium citrate. Platelet-poor plasma (PPP) was prepared by an initial centrifugation at 1500 ×*g* for 10 min to remove platelets followed by a second centrifugation at 2750 ×*g* for 15 min. PPP for thrombin generation or microparticle analysis experiments was stored at -70 °C.

Isolation of blood microparticles for flow cytometric analysis

Frozen samples were thawed in a 37 °C water bath for 5 min, vortexed, and then centrifuged at 3,000 ×*g* for 15 min. The plasma sample was then centrifuged at 20,000 ×*g* for 30 min. Supernatants were removed, and the remaining pellets (microparticles) were reconstituted with Hanks' solution buffered (pH 7.4) with 20 mM HEPES. Tubes containing reconstituted microparticles were vortexed and centrifuged again at 20,000 ×*g* for 30 min. After centrifugation, the pellets containing microparticles were reconstituted again and vortexed for 1 to 2 min before analysis. All buffers were filtered twice through a 0.2-μm membrane filter before use.

Experimental Procedures

Thrombin Generation Assay

Thrombin generation was measured with the Calibrated Automatic Thrombinogram Assay (CAT Assay, Thrombinoscope BV, Maastricht, The Netherlands) [26]. Either of two 20 μL triggers, the proscribed PPP-Reagent 5 pM (5 pM tissue factor/4 μM phospholipid, Stago, US) or diluted Innovin (Dade Behring, Newark, DE) was added to polypropylene 96-well microtiter plates (Nunc, Thermo Fischer Scientific, Rochester, NY). Then, citrated platelet poor plasma (80 μL), containing 50 μg/mL of corn trypsin inhibitor (CTI, Haematologic Technologies Inc. Essex Junction, VT) was added and the plate was warmed to 37 °C for 5 minutes in the pre-equilibrated plate-reader. Thrombin generation was initiated with pre-warmed Fluo-substrate (Stago, US) that contained calcium and buffer (Fluo-buffer, Stago, US). Thrombin progress curves were recorded continuously with a Fluoroskan FL instrument (Thermo Labsystems, Helsinki, Finland) under control of Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands), filtered for excitation at 390 nm and emission at 460 nm. For each plasma sample, a calibrator assay was included in which the tissue factor/PL trigger was replaced with a solution that contained a known concentration of thrombin-α2-macroglobulin complex (thrombin calibrator, Stago, US). The calibrator corrects for inner filter effects and quenching variation among individual plasmas. Each trigger and calibrator was assayed in triplicate for each plasma sample analyzed.

Data Analysis

Raw fluorescence data were converted to thrombin activity (nM), relative to the calibrator by the Thrombinoscope software [27]. For each plasma sample and trigger combination, four parameters were derived by the software: lagtime (LT, min), endogenous thrombin potential, (area under the curve; ETP, nM-min), peak thrombin activity (Peak, nM) and time to peak (TTP, min). Rate of thrombin generation (nM/min) was calculated by: $\frac{Peak}{TTP-LT}$.

PPL Standard Curve

A standard curve was constructed from dilutions of synthetic phospholipid (1.3 mM) composed of 20 mol% phosphatidylserine and 80 mol% phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) [28] combined with dilute Innovin (approximately 5 pM tissue factor). Approximate TF concentration was determined by titration of diluted Innovin (Dade Behring, Newark, DE) against the PPP-Reagent 5 pM, as judged by equivalent peak height. The PC/PS-Innovin reagent was added, from zero to 4 μM PC/PS, constant Innovin, into Cryocheck control pool plasma (Precision BioLogic, Dartmouth, NS, Canada) that had been depleted of MP by centrifugation at 49,000 ×*g* for 10 minutes, room temperature, in an Airfuge (Beckman Coulter, Brea, CA). The CAT assay was initiated with the substrate-calcium reagent and resulting Peak height (nM thrombin) was plotted versus concentration of PC/PS (μM).

Procoagulant Phospholipid Assay

To measure endogenous procoagulant phospholipid content in individual plasma samples, the CAT assay was triggered only with the diluted Innovin reagent *in absentia* of excess, exogenously added phospholipid. As demonstrated by the addition of purified PC/PS to MP-depleted plasma, under these conditions the extent of thrombin generation becomes dependent upon the concentration of PPL in the plasma samples. In order to define the contribution of MP to the total plasma PPL, a sample that had been depleted of MP by centrifugation at 49000 ×*g* for 10 minutes is also assayed with the diluted Innovin trigger. The PPL derived from microparticles is determined by subtracting the amount of thrombin generated from the MP-depleted sample from that of the non-centrifuged plasma.

Flow Cytometric Analysis of MP

Flow cytometry (FACS-Canto, BD Biosciences) was used to define microparticles by size and positive fluorescence using marker-specific antibodies and annexin V as described previously [29].