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Original Contribution

Nitrated fibrinogen is a biomarker of oxidative stress in venous thromboembolism

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ABSTRACT

The pathogenesis of venous thromboembolism (VTE) is linked to inflammation and oxidant production, although specific markers for these pathways with pathological relevance to VTE have not been explored. The coagulant protein fibrinogen is posttranslationally modified by nitric oxide-derived oxidants to nitrated fibrinogen in both acute and chronic inflammatory states. Therefore, nitrated fibrinogen may serve as a marker of inflammation and oxidative stress in VTE. To test this hypothesis we enrolled subjects ($n=251$) presenting with suspected VTE at the University of Pennsylvania Hospital emergency department, 50 (19.9%) of whom were positive by imaging or 90-day follow-up. Mean nitrated fibrinogen was elevated in VTE-positive (62.7 nM, 95% CI 56.6–68.8) compared to VTE-negative patients (54.2 nM, 95% CI 51.4–57.1; $P<0.01$). Patients in the highest quartile of nitrated fibrinogen had an increased risk of VTE compared with patients in the lowest quartile (OR 3.30; 95% CI 1.25–8.68; $P<0.05$). This risk persisted after univariate adjustment for age, active cancer, and recent surgery, but not after multivariate adjustment. Mean fibrinogen levels measured either by the Clauss assay or by ELISA were not different between VTE-negative and VTE-positive patients. These data indicate that nitrated fibrinogen is an oxidative risk marker in VTE, providing a novel mechanistic link between oxidant production, inflammation, and VTE.

Introduction

Venous thromboembolism (VTE) is a common thrombotic disease that encompasses both deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE affects around 1 per 1000 people per year within the United States, with increasing incidence for congenital and acquired thrombophilic risk factors [1–3]. VTE is a chronic disease with 30% of patients experiencing a recurrent event over 10 years [4,5] and is estimated to account for between 100,000 and 180,000 deaths each year [6]. Thus, VTE represents a significant health problem that requires considerable attention.

The precise molecular and biochemical mechanisms of thrombus initiation in VTE remain unclear. Virchow proposed that changes in blood rheology, induction of a hypercoagulable state, or endothelial injury are conditions required for venous thrombosis [7]. More recent studies indicate that inflammation plays an important role in VTE. However, the interplay between inflammation and Virchow's triad is still unresolved. One possibility is that components of the triad activate or damage endothelial cells within the vein wall resulting in upregulation of receptors for inflammatory and procoagulant molecules. Electron micrographs of early thrombus formation show leukocyte adhesion to the vein wall [8], presumably through the binding of P- and E-selectins that are expressed on activated endothelial cells [9,10]. Leukocytes shed tissue factor-bearing microparticles, which induce coagulation and the production of a fibrin clot [11]. Neutrophils and monocytes, two of the most prevalent leukocytes in venous thrombi, are also able to generate nitrating intermediates, capable of lipid peroxidation and nitration of proteins [12,13].

One protein known to be modified by tyrosine nitration is the coagulant protein fibrinogen. Upon activation of the coagulation cascade, circulating plasma fibrinogen is cleaved by thrombin to fibrin monomers, which polymerize to form a fibrin clot. Recent studies demonstrate an emerging role for nitrated fibrinogen that links inflammation and oxidant production to coagulation.

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Nitrated fibrinogen is elevated in the plasma of patients with coronary artery disease (CAD) and in smokers [14,15]. Additionally, in humans injected with small amounts of lipopolysaccharide, plasma levels of nitrated fibrinogen increase and remain elevated for at least 72 h postinjection [16]. This increase follows an elevation of circulating myeloperoxidase, an enzyme localized to the azurophilic granules of neutrophils, which is released during neutrophil activation and is capable of generating nitrating intermediates [12]. Although these data suggest that nitrated fibrinogen plays a key role linking inflammation, oxidant production, and arterial thrombotic diseases, its role in VTE remains undefined.

Moreover, nitrated fibrinogen is not simply a marker of inflammation and oxidative stress, but also has functional effects on fibrinogen and fibrin clotting. Fibrinogen nitration was shown to increase the rate of fibrin clot formation, diminish clot lysis rate, and alter fibrin clot structural and viscoelastic properties [14–16]. These functional effects suggest that nitrated fibrinogen may represent a risk factor for increased thrombotic tendency during inflammation and oxidant stress. However, the levels of nitrated fibrinogen in VTE have not been ascertained. Herein we quantified the levels of nitrated fibrinogen in patients presenting to the emergency department with suspected VTE and evaluated if nitrated fibrinogen is a biochemical risk factor that could explain associations of oxidative stress and inflammation with thrombotic complications in VTE.

Materials and methods

Patient population

We performed a prospective cohort study from January 2010 to March 2011 of consecutive subjects 18 years of age or older presenting to the Hospital of the University of Pennsylvania emergency department with suspected acute lower extremity DVT or PE. Exclusion criteria included history of VTE within the prior 4 weeks, unavailable for 90-day follow-up, and inability to provide informed consent. DVT was diagnosed by compression ultrasonography and PE by computed tomographic pulmonary angiography. Patients with negative imaging were contacted by telephone 90 days after enrollment to confirm that they did not develop clinically apparent VTE. Those who reported development of VTE over this interval despite negative imaging were also considered positive for DVT and/or PE. Secondary variables were also collected, including demographic information, thrombotic risk factors, comorbidities, and medications. Before initiation of anticoagulant therapy in the emergency department, blood was collected from patients in 3.2% sodium citrate (BD Vacutainer; Franklin Lakes, NJ, USA). Blood was centrifuged at 150*g* for 15 min, and the supernatant, platelet-rich plasma, was transferred to a new tube. Platelet-rich plasma was then centrifuged at 10,000*g* for 10 min to obtain platelet-poor plasma, followed by storage at −80 °C for future analysis. Study design was approved by the Institutional Review Board Involving Human Subjects at the University of Pennsylvania and written informed consent was obtained from all study participants.

Plasma nitrated fibrinogen levels

Nitrated fibrinogen levels were measured in plasma using an ELISA described previously [16]. Briefly, 96-well plates were coated with 10 μg/ml anti-nitrotyrosine antibodies generated and described elsewhere [17] in 50 mM carbonate buffer, pH 9, and incubated at 4 °C under constant rocking overnight. The next day, the plates were washed with 50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TBS-T). The plates were blocked with 3% bovine serum albumin (BSA; Roche Diagnostics, Mannheim, Germany) (wt/vol) in TBS-T for 2 h at 37 °C, followed by incubation with 1% BSA in TBS-T for 1 h at 37 °C. A standard curve was created from purified fibrinogen (American Diagnostica, Stamford, CT, USA) that had been chemically nitrated and the levels of nitration were independently determined [16]. Plasma samples were diluted (1:10, 1:20) in 1% BSA in TBS-T, added to the plate in duplicate, and incubated 2 h at room temperature under constant rocking. Plates were washed with TBS-T and coated with 0.3 μg/ml horseradish peroxidase (HRP)-conjugated rabbit anti-human fibrinogen polyclonal antibody (DakoCytomation, Glostrup, Denmark) in 1% BSA in TBS-T. After 2 h incubation at room temperature under gentle rocking, the plates were washed with TBS-T and developed with TMB substrate (KPL, Gaithersburg, MD, USA) followed by quenching of the reaction with 2 M sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA). The absorbance was measured at 405 nm (Molecular Devices, Sunnyvale, CA, USA). Nitrated fibrinogen values were normalized to quality control plasma run with each plate. Each patient sample was analyzed a minimum of three times and samples that exceeded 15% coefficient of variation were excluded from analysis. The normalized mean intra-assay and interassay variations were 8.23 and 9.32%.

Plasma fibrinogen level

Fibrinogen levels were measured in plasma using an ELISA previously described [16]. Ninety-six well plates (Maxisorb; Nunc, Rochester, NY, USA) were coated with 10 μg/ml mouse anti-fibrinogen monoclonal antibody [16] in carbonate buffer, pH 9, and incubated at 4 °C, rocking overnight. The next day, the plates were washed with TBS-T and blocked with 3% BSA in TBS-T for 2 h at 37 °C. A standard curve was created from purified fibrinogen (American Diagnostica) in TBS-T. Plasma was diluted (1:5000, 1:10,000), added to the plate in triplicate, and incubated for 1 h at 37 °C. The plates were washed with TBS-T, then coated with 0.3 μg/ml HRP-conjugated rabbit anti-human fibrinogen polyclonal antibody (DakoCytomation) in 1% BSA in TBS-T, and incubated 1 h at room temperature. The plate was developed with the TMB peroxidase substrate as described above. Fibrinogen values were normalized to quality control plasma run with each plate. The normalized mean intra-assay and interassay variations were 11.23 and 14.0%.

Plasma fibrinogen levels were measured by the Clauss method [18] using a Destiny Max coagulation analyzer according to the manufacturer's protocol (Trinity Biotech, County Wicklow, Ireland).

D-dimer

Plasma D-dimer levels were measured at the University of Pennsylvania Hospital Coagulation Laboratory using the Vidas D-dimer exclusion assay (bioMérieux, Lyon, France). This assay measures the fibrin degradation product (FDP) D-dimer by an enzyme-linked fluorescence assay. This sandwich ELISA-based method uses two anti-FDP monoclonal antibodies (P10B5E12C9 and P2C5A10) coupled with fluorescence detection [19,20]. In accordance with the manufacturer's instructions, a D-dimer level of < 0.5 μg/ml was considered negative.

The laboratory personnel and technologists performing all assays were blinded to patient outcome. Laboratory results obtained on the archived plasma samples were not made available to clinicians or patients.