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Regular Article

Chronic thromboembolic pulmonary hypertension-associated dysfibrinogenemias exhibit disorganized fibrin structure ☆

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A B S T R A C T

Introduction: Mechanisms contributing to the pathogenesis of chronic thromboembolic pulmonary hypertension (CTEPH) are poorly understood. This disorder is characterized by incomplete resolution of pulmonary perfusion defects resulting from acute venous thromboembolism. We previously identified several dysfibrinogenemias in some patients with CTEPH. The purpose of this study was to determine whether fibrin clot architecture might be implicated in the thrombolytic resistance in patients with these CTEPH-associated dysfibrinogenemias.
Materials and Methods: Purified fibrinogen from patients and healthy controls was clotted with thrombin in the presence of calcium. Clot turbidity, porosity, and susceptibility to fibrinolysis were evaluated by spectrophotometric and permeation analyses. Fibrin network structure was assessed by laser-scanning confocal microscopy.
Results: Compared to normal fibrinogen, CTEPH-associated dysfibrinogenemias exhibited low clot turbidity, decreased porosity, and fibrinolytic resistance. In addition, the dysfibrinogenemias exhibited a more disorganized fibrin network structure characterized by thinner fibers, greater network dispersal and more extensive fiber branching.
Conclusions: Abnormal clot architecture and fibrinolytic resistance may contribute to incomplete clot resolution following acute venous thromboembolism in patients with CTEPH-associated dyfibrinogenemia.
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Introduction

After acute venous thromboembolism, thrombi either resolve completely or are replaced by chronic intravascular scars, which may have various clinical effects. In the case of acute pulmonary emboli, residual lung perfusion defects are common [1–6] and probably represent minor degrees of intravascular scarring. However, more extensive scars within the pulmonary arteries may result in chronic thromboembolic pulmonary hypertension (CTEPH), a life-threatening disease [7]. Likewise, residual intraluminal venous thickening is common after acute deep vein thrombosis [8], whereas extensive scarring within the deep veins produces chronic thrombotic venous disease (CTVD), a cause of post-thrombotic syndrome [9].

The factors that contribute to poor resolution of venous thromboemboli and the intravascular scar formation that leads to CTEPH and CTVD are not fully understood. Polymerized fibrin is a major component of acute venous thromboemboli, and resistance to lysis is characteristic of some abnormally formed fibrin clots [10]. We previously reported an unusually high prevalence of dysfibrinogenemias among CTEPH patients [11], which may contribute to a relative resistance to plasmin-mediated fibrinolysis in some cases [12].

We undertook the current series of experiments to determine if CTEPH-associated dysfibrinogenemias lead to abnormal fibrin network formation. In particular, we are interested in alterations that might be implicated in delayed fibrinolysis, abnormal cellular responses to thrombi and subsequent remodeling of thrombotic material into scar tissue.

Materials and Methods

Materials

Human α-thrombin and glu-plasminogen were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant human tissue-type plasminogen activator (tPA), 2-chain form, was obtained from Burroughs Wellcome (Research Triangle Park, NC). Human fibrinogen conjugated with Alexa Fluor 488 (9 moles dye/mole protein) was purchased from Molecular Probes (Grand Island, NY). Glass bottom 96-well plates (No. 1.5, uncoated) were obtained from MatTek Corporation (Ashland, MA).

Abbreviations: CTEPH, chronic thromboembolic pulmonary hypertension; CTVD, chronic thrombotic venous disease.

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Subjects

The cohort of patients with CTEPH as well as healthy controls without a history of venous thromboembolic disease is described elsewhere [11]. Fibrinogen from five CTEPH patients with previously identified dysfibrinogenemias, one CTVD patient with γ 114 Tyr/His dysfibrinogenemia, and six healthy controls (including two that were heterozygous for the Aα 312 Thr/Ala polymorphism and two that were heterozygous for the Bβ 448 Arg/Lys polymorphism) was isolated and characterized as previously described [11]. The University of California, San Diego Institutional Review Board approved this study and all participants gave written informed consent.

Clot Turbidity and Fibrinolysis

Fibrin clots were simultaneously formed and lysed as previously described [11,12]. Briefly, fibrinogen was mixed with plasminogen, tPA and thrombin in 50 mM Tris (pH 7.0) containing 150 mM NaCl and 10 mM $CaCl_2$. The mixture was immediately added to a microplate well and the turbidity of the solution was monitored at 1-minute intervals for 1 hour in a microplate reader at room temperature. Each experiment included up to three patients and one control. Each curve was characterized by an increase in absorbance (polymerization) followed by a brief plateau and then a decrease in absorbance (lysis) to baseline (Supplementary Material, Fig. S1). Maximum turbidity and lysis rate were calculated as described [11].

Clot Permeability

The permeability of fibrin clots was assessed using a modification of the method previously described [13]. Briefly, fibrin clots were formed as described above except that plasminogen and tPA were omitted. The clot mixture (300 µl) was immediately added to a disposable 1-ml column that had been previously primed with deaerated permeation buffer (50 mM Tris (pH 7.0) containing 150 mM NaCl and 0.1% (w/v) bovine serum albumin) and sealed at the bottom with parafilm. The column was placed in a humid chamber for 2 hours at room temperature. Pilot studies indicated that enough factor XIII co-purifies with the fibrinogen preparations to enable complete crosslinking of the fibrin clot within this time period (Supplementary Material, Fig. S2). Flow measurements with permeation buffer were performed at constant hydrostatic pressure and the volume of buffer collected in 1 hour was measured gravimetrically. This system was shown to obey Darcy's Law (i.e., flow is inversely proportional the length of the clot). The Darcy constant K_s, which is a direct measure of the clot surface area available for flow and gives information on the size of pores within the fibrin network, was calculated as previously described [13]. Each experiment included one patient and one control, both done in duplicate.

Laser Scanning Confocal Microscopy

Fibrin clots were formed as described above except that plasminogen and tPA were omitted and Alexa Fluor 488-conjugated fibrinogen was added (10% final fibrinogen concentration) to enable visualization of the fibrin fibers, as previously described [14,15]. The mixture was immediately added to duplicate wells of a glass bottom 96-well plate (100 µl per well) and incubated for 2 hours at room temperature in a humid chamber. Analysis of fibrin networks was performed with an Olympus FV1000 inverted laser scanning confocal microscope equipped with a 100X (1.4 numerical aperture) objective and controlled by FlowView software (version 3.0.2.0). Images were collected in the XYZ scan mode using 3% laser power with filters automatically set for detection of Alexa Fluor 488. Image size was 512x512 pixels in the XY dimension (0.248 µm/pixel), and 41 optical sections were collected in the Z dimension (0.38 µm/slice). Total scan time was about 3 min. Care was taken to start the optical sectioning of each clot approximately 35 µm from the surface of the cover glass, with subsequent sections penetrating deeper into the clot. This region represents approximately the mid-point of the total depth of field. Fully reconstructed images represent an area of about 127x127 µm and a thickness of about 15 µm. Each experiment included up to four patients and one control.

Image Analysis

Fully reconstructed images were used for visual analysis. NIH ImageJ software (version 1.45) was used to estimate fiber diameter, the dispersal of the fiber network throughout the observed area, and the extent of fiber branching. The fiber diameter of each clot was measured on 10 randomly selected fibers from 3 reconstructed images, each consisting of 2 sequential slices (0.76 µm total thickness). To measure fibrin network dispersal, analysis of each clot was performed on 4 reconstructed images, each consisting of 10 sequential slices (3.8 µm total thickness). The extent of dispersal was represented by the proportion of the studied area that contained filaments of the fibrin network. The coefficient of variation within each sample was typically $<10\%$ indicating that the extent of dispersal was consistent throughout the fully reconstructed image. To estimate the extent of fiber branching (junctions), analysis of each clot was performed on 20 reconstructed images, each consisting of 2 sequential slices (0.76 µm total thickness). "Thin" sections were used for branching measurements to guard against the possibility of counting overlapping fibers as branch points. The coefficient of variation within each sample for this measurement was also typically $<10\%$ indicating that the extent of fiber branching was consistent throughout the fully reconstructed image.

All measurements were made by an operator blinded to the identity of the clots. Values from each well were expressed as a percentage of the average value obtained from normal control fibrinogen wells included in each experiment. The normal controls used in these experiments did not differ significantly from one another with respect to fiber diameter, dispersal, or branching (one-way ANOVA, $P > 0.05$).

Results

Fibrinogen Regions Affected by Dysfibrinogenemias

The five dysfibrinogenemias previously reported in CTEPH patients [11] are listed in Table 1. Each occurred in a different patient and all result from heterozygous missense mutations in one or more of the fibrinogen genes. Two patients had compound mutations. None of these mutations were found in a group of 20 healthy controls without a history of venous thromboembolism [11]. The patient with Bβ 235 Pro/Leu dysfibrinogenemia was also heterozygous for the common Bβ 448 Arg/Lys polymorphism, and the patient with Aα 554 Arg/His dysfibrinogenemia was also heterozygous for the common Aα 312 Thr/Ala polymorphism.

Two of the amino acid substitutions (Aα 69 Leu/His and γ 114 Tyr/His) occur at proximal and distal sites, respectively, within the coiled

Table 1
CTEPH-associated dysfibrinogenemias.*

Dysfibrinogenemia	Region(s) affected
Bβ 235 Pro/Leu plusγ 375 Arg/Trp	D and sialylation†
Bβ 235 Pro/Leu plus γ 114 Tyr/His	D and coiled coil
Bβ 235 Pro/Leu‡	D
Aα 69 Leu/His	Coiled coil
Aα 554 Arg/His§	αC

* Previously designated San Diego I-V [11], top to bottom.

† Fibrinogen containing mutant γ chains does not reach the circulation; however the γ 375 Arg/Trp mutation causes excessive sialylation of the Bβ and γ chains in circulating fibrinogen.

‡ Also heterozygous for the common Bβ 448 Arg/Lys polymorphism.

§ Also heterozygous for the common Aα 312 Thr/Ala polymorphism.

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