Fibrin Derived from Patients with Chronic Thromboembolic Pulmonary Hypertension Is Resistant to Lysis

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Rationale: **Although acute pulmonary embolism is epidemiologically associated with chronic thromboembolic pulmonary hypertension, the factors responsible for resistance to thrombolysis and a shift toward vascular remodeling within the pulmonary arteries of patients with chronic thromboembolic pulmonary hypertension are unknown.**

Objective: **Determine whether fibrin from patients is more resistant to plasmin-mediated lysis than fibrin from healthy control subjects.**

Methods: **Fibrinogen purified from patients and control subjects was used to prepare fibrin clots, which were subsequently digested with plasmin for various periods of time. The degradation of the α-, β-, and γ-chains of fibrin and the appearance of peptide fragments over time were assessed by polyacrylamide gel electrophoresis and Western blotting.**

Measurements and Main Results: **Densitometry of Coomassie-stained gels revealed significantly slower cleavage of all three polypeptide chains of fibrin from patients compared with control subjects ($p < 0.05$). In particular, release of N-terminal fragments from the β-chain of fibrin, which promote cell signaling, cell migration, and angiogenesis, was retarded in patients compared with control subjects ($p < 0.01$).**

Conclusions: **The relative resistance of patient fibrin to plasmin-mediated lysis may be due to alterations in fibrin(ogen) structure affecting accessibility to plasmin cleavage sites. The persistence of structural motifs of fibrin, such as the β-chain N-terminus, within the pulmonary vasculature could promote the transition from acute thromboemboli into chronic obstructive vascular scars.**

Keywords: blood coagulation factors; fibrinolysis; pulmonary embolism; thrombosis; vascular diseases

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare outcome of acute pulmonary embolism characterized by the persistence of unresolved thromboemboli. For reasons that are still unclear, the emboli in patients with CTEPH do not resolve completely and instead are remodeled into fibrotic tissue that obstructs and narrows major pulmonary arteries, leading to increased pulmonary vascular resistance and right ventricular dysfunction. Without treatment, the disease is progressive and often fatal. In most instances, the only effective treatment of CTEPH is surgical resection of the chronic thromboembolic lesions (1).

No thrombotic risk factor, either inherited or acquired, has been clearly associated with CTEPH (2), although a minority (10–20%) are positive for lupus anticoagulant and/or anti–phospholipid antibodies. Similarly, no abnormality of the fibrinolytic pathway (3) or of the pulmonary endothelium (4) has been found that accounts for incomplete resolution of emboli and progression to CTEPH. One possible explanation is that the fibrin network of emboli is resistant to plasmin-mediated fibrinolysis in affected individuals.

We performed this study to determine if fibrin polymers themselves from patients with CTEPH were abnormally resistant to lysis. Fibrinogen was purified from patients with CTEPH and healthy control subjects without a prior history of thrombotic events, and fibrin clots were prepared *in vitro*. The kinetics of plasmin-mediated lysis of the α-, β-, and γ-chains of fibrin were measured by Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The persistence of the N-terminus of the β-chain, which may mediate fibrin remodeling into scar tissue, was evaluated by Western blotting using a monoclonal antibody specific for this region.

Some of the results of this study have been previously reported in the form of an abstract (5).

METHODS

Materials

Bovine thrombin (Hyphen BioMed, Andrésy, France) and human plasmin (Chromogenix, Milan, Italy) were obtained from Diapharma, Inc. (West Chester, OH). Human glu-plasminogen and a polyclonal rabbit antiserum specific for human glu-plasminogen were purchased from American Diagnostic (Stamford, CT). A murine monoclonal antibody (IgG_{2a}) specific for human α_2-antiplasmin was obtained from R&D Systems (Minneapolis, MN). A murine monoclonal antibody (IgG_1) raised against a synthetic peptide identical to the first seven residues of the β-chain of human fibrin was obtained as previously described (6). Nu-PAGE bis-tris polyacrylamide gels, reagents, and stains, as well as the Western Breeze blotting kit, were obtained from Invitrogen (Carlsbad, CA). All other chemicals were reagent grade or better.

Study Population

Consecutive patients in whom the diagnosis of CTEPH was confirmed histologically after pulmonary thromboendarterectomy at the University of California, San Diego, Medical Center were enrolled in the study. This criterion distinguished patients with bona-fide CTEPH from those with more unusual causes of chronic proximal pulmonary artery obstruction, such as pulmonary artery sarcoma and pulmonary arteritis. The control group consisted of healthy subjects without a history of thrombotic events. The University of California, San Diego, Institutional Review Board approved this study.

Blood Collection and Processing

After obtaining informed, written consent, blood samples (20 ml) were collected into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing one-tenth volume of buffered sodium citrate (3.8%) and

(*Received in original form June 14, 2005; accepted in final form February 28, 2006*)

This work was supported by National Institutes of Health grants R21-HL080302 and R21/33-CA099835. T.A.M. is also supported by the American College of Chest Physicians as the GlaxoSmithKline Distinguished Scholar in Thrombosis.

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Am J Respir Crit Care Med Vol 173. pp 1270–1275, 2006
Originally Published in Press as DOI: 10.1164/rccm.200506-916OC on March 2, 2006
Internet address: www.atsjournals.org

immediately placed on wet ice. Plasma was obtained by centrifugation at 2,000 × *g* for 10 min at 4°C and stored at −70°C until used.

Fibrinogen Purification

Fibrinogen was purified using an ethanol precipitation method (7). Fibrinogen concentration was determined by ultraviolet spectroscopy at 280 nm using an extinction coefficient of 1.51 $(mg/ml)^{-1}$. Clottable protein was determined by measuring the protein content of the fluid phase (by absorbance at 280 nm) before and after clotting a sample of fibrinogen with thrombin for 1 h at room temperature. The purity of the fibrinogen preparations was assessed by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis under reducing conditions. Fibrinogen purified according to this method is typically more than 90% pure and contains more than 90% clottable protein.

Formation of Fibrin and Digestion with Plasmin

Fibrin clots were prepared in microcentrifuge tubes by incubating thrombin (2 NIH units/ml final concentration) with fibrinogen (2 mg/ml final concentration) in 25 μl of 50 mM Tris (pH, 7.4), 100 mM NaCl, and 20 mM ethylenediaminetetraacetic acid for 1 h at room temperature. Plasmin was then added (50 μl of 25 μg/ml), and the fibrin clots were digested for various time periods (up to 6 h) at 37°C. The digestion reaction was terminated by adding 25 μl of 4X lithium dodecyl sulfate (LDS) gel electrophoresis sample buffer.

Electrophoretic Analysis of Plasmin Digests

Samples were heated at 70°C for 10 min under reducing conditions (50 mM dithiothreitol), and aliquots from each digest (equivalent to 1 μg of fibrin) were loaded onto duplicate 4–12% polyacrylamide gels. After electrophoresis, one gel was stained with Coomassie blue and the other was used for Western blotting. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes and probed with a monoclonal primary antibody specific for the N-terminus of the β-chain of fibrin, followed by visualization using a Western Breeze chromogenic kit. Band intensities on Coomassie-stained gels and Western blots were quantified by densitometry using a ChemiImager 4400 imaging system (Alpha Innotech, San Leandro, CA). An investigator blinded to the identity of the samples performed all densitometry analyses.

Determination of Plasminogen and α_2-Antiplasmin Content of Fibrinogen

Aliquots of purified fibrinogen (30 μg) and human plasminogen (8, 40, and 200 ng) were subjected to SDS-PAGE on 4–12% polyacrylamide gels under nonreducing conditions, and then electroblotted onto PVDF membranes. Blots were developed with a primary polyclonal rabbit antibody specific for human plasminogen, followed by visualization using a Western Breeze chromogenic kit. Band intensities were quantified by densitometry and plasminogen content of the fibrinogen samples was interpolated from a curve generated from the plasminogen standards. The plasminogen content was expressed as a percentage of the total protein concentration of each purified fibrinogen preparation. The same approach was used for determining the α_2-antiplasmin content except that human plasma with a known α_2-antiplasmin concentration (70 μg/ml) was used for the standard curve, and the blots were developed with a monoclonal antibody specific for human α_2-antiplasmin.

Fibrinogen Mixing Experiment

Four pairs of fibrinogen samples from patients with CTEPH and control subjects were chosen for analysis from previous experiments based on maximal differences between the amounts of intact β-chain remaining after a 3-h plasmin digest. For each experiment, three fibrinogen samples were prepared: control fibrinogen alone (50 μg), CTEPH fibrinogen alone (50 μg), and a 50:50 mixture of control and CTEPH fibrinogen (25 μg each). Each sample was clotted with thrombin and then incubated for 3 h at 37°C in the presence or absence of plasmin as described above. The digestion reaction was terminated by the addition of LDS gel electrophoresis sample buffer, and aliquots from each sample were subjected to Western blotting. The fraction of intact β-chain remaining in the control sample (C), the patient sample (P), and the mixture (M) was determined by densitometry. The mixing index for each control subject/patient with CTEPH pair was calculated as follows: mixing index = (M − C)/(P − C). According to this formula, the mixing index will theoretically vary between the values of 0 and 1 depending on whether the mixture mimics the digestion behavior of the control or patient fibrinogen, respectively. The mixing index will have a value of 0.5 if the control and patient fibrinogens behave independently in the mixture. Each mixing experiment was repeated a total of three times and the data were averaged.

RESULTS

Characteristics of the Subjects

The characteristics of the 10 consecutive patients with CTEPH (two males, eight females) enrolled in the study are presented in Table 1. Most patients were in New York Heart Association functional class III or IV at the time of evaluation. The patients also exhibited hemodynamic abnormalities characteristic of CTEPH. The diagnosis of CTEPH was confirmed by removal of substantial amounts of thromboembolic material during the thromboendarterectomy procedure. The patients were roughly matched to the healthy control subjects with regard to age and sex. The median age of the patients was 47 yr (range, 21–69 yr), and the median age of the control group was 43 yr (range, 21–53 yr; *t* test for age differences between groups, p = 0.08).

Fibrinogen Purity

The characteristics of fibrinogen purified from patients with CTEPH and control subjects are presented in Table 2. Preparations from the two groups were similar with respect to purity (∼ 95%) and clottable protein (∼ 91%). A Coomassie-stained polyacrylamide gel showing Aα-, Bβ-, and γ-chains of a representative fibrinogen sample from each group is shown in Figure 1. Protein bands comigrating with plasminogen or with thrombin-activatable fibrinolysis inhibitor or other fibrinolytic inhibitors (e.g., α_2-antiplasmin, plasminogen activator inhibitor-1 [PAI-1]) were not detected in any of the samples on Coomassie-stained gels.

Kinetics of Fibrinolysis by Plasmin

Fibrin clots from control subjects and patients with CTEPH were formed by incubation of fibrinogen with thrombin. The clots were then digested for various periods of time by incubating with plasmin, and the resulting peptide fragments were resolved by SDS-PAGE. A representative Coomassie-stained gel from a

TABLE 1. CHARACTERISTICS OF PATIENTS WITH CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

Subject	Age (*yr*)	Sex	NYHA Class	PVR ($dyn \cdot s^{-1} \cdot cm^{-5}$)	CO ($L \cdot min^{-1}$)	Mean PAP (*mm Hg*)
1	53	M	III	1,040	3.0	44
2	68	M		1,055	2.4	40
3	42	F	II/III	614	4.3	39
4	49	F	III/IV	729	2.9	38
5	21	F		900	3.5	43
6	45	F	III	926	2.9	41
7	43	F	IV	889	2.7	38
8	58	F		1,059	3.9	65
9	69	F	III/IV	606	3.2	37
10	35	F		772	4.9	53
Median	47			895	3.1	41
(range)	(21–69)			(606–1,059)	(2.4–4.9)	(37–65)

Definition of abbreviations: CO = cardiac output; F = female; M = male; NYHA = New York Heart Association; PAP = pulmonary artery pressure; PVR = pulmonary vascular resistance.

Values are from a cohort of consecutive patients with chronic thromboembolic pulmonary hypertension undergoing surgery for removal of chronic obstructive thromboembolic material from proximal pulmonary arteries.

TABLE 2. CHARACTERISTICS OF PURIFIED FIBRINOGEN

Group	Yield* (*mg*)	Purity (%)	Clottable Protein (%)
CTEPH	6.24 ± 1.20	96.0 ± 1.9	91.2 ± 1.2
Control	4.72 ± 1.38	94.7 ± 2.6	91.6 ± 3.0

Definition of abbreviation: CTEPH = chronic thromboembolic pulmonary hypertension.

Data are mean ± SD of 10 subjects in each group.

* Based on starting volume of 4 ml of plasma.

digest of control and CTEPH fibrin is presented in Figure 2A. A progressive and essentially complete loss of the intact α-, β-, and γ-chains of control fibrin was observed after 6 h of digestion with plasmin. A concomitant appearance of a characteristic pattern of peptide fragments was also observed. In contrast, lysis of CTEPH fibrin was delayed, with noticeable amounts of intact α-, β-, and γ-chains remaining after the 6-h digestion period. However, the pattern of peptide fragments did not differ noticeably between control and CTEPH fibrin (Figure 2A). Group differences between the plasmin-mediated lysis of the intact α-, β-, and γ-chains of control and CTEPH fibrin over time were assessed by densitometric scanning of Coomassie-stained gels. As shown in Figure 2B, the α-, β-, and γ-chains of the CTEPH group were significantly more resistant to lysis compared with the control group ($p < 0.05$ for each chain).

The fate of the intact β-chain of fibrin during digestion with plasmin was also investigated by Western blotting using a monoclonal primary antibody specific for the N-terminus of the β-chain of fibrin. A blot of the gel depicted in Figure 2A is shown in Figure 3A. In agreement with the Coomassie-stained gel, there was a near complete loss of the intact β-chain of control fibrin over the 6-h digestion period. A transient appearance of a pair of closely migrating peptide fragments (~ 14 kD) derived from the N-terminus of the β-chain was also observed. By 6 h, the larger of these two fragments disappeared completely. In contrast, noticeable amounts of the intact β-chain and 14-kD peptide fragment pair from CTEPH fibrin persisted throughout the 6-h digestion period. Group differences, as determined by densitometric scanning of Western blots, are shown in Figure 3B.

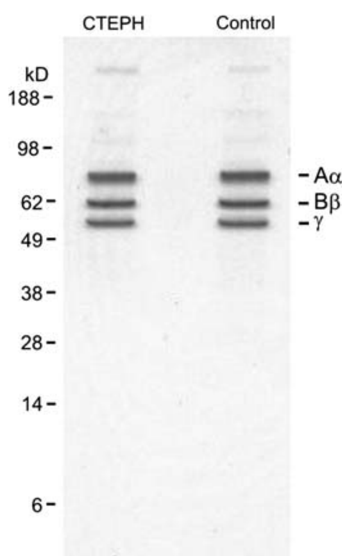

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of purified fibrinogen. Samples of purified fibrinogen (1 μg) from a representative patient with chronic thromboembolic pulmonary hypertension (CTEPH) and a healthy control subject were reduced with dithiothreitol and loaded onto a 4–12% SDS polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue and analyzed for purity by quantitative densitometry. Results indicated purities of 93.4 and 94.5%, respectively, for these samples. Molecular mass markers and the Aα-, Bβ-, and γ-chains of fibrinogen are indicated on the *left* and *right side* of the figure, respectively.

The intact β-chain of the CTEPH group was more resistant to lysis than that of the control group ($p < 0.02$). In addition, the concomitant release of β-chain N-terminal fragments (~ 14 kD) in the CTEPH group was also retarded compared with the control group ($p < 0.01$).

Plasminogen and α_2-Antiplasmin Content of Purified Fibrinogen

To investigate the possibility that copurifying plasminogen or α_2-antiplasmin may have influenced the lytic profiles of the fibrin clots, we quantified the amount of these proteins in our purified fibrinogen preparations by performing densitometry on Western blots using primary antibodies specific for plasminogen and α_2-antiplasmin. Plasminogen accounted for less than 0.1% of the total protein in the fibrinogen preparations (range, 0.02–0.06%), and there was no significant correlation between the plasminogen content and the amount of intact β-chain remaining after a 3-h plasmin digest of fibrin clots ($r = 0.17$, $p = 0.48$, Pearson correlation test). Furthermore, the average amount of plasminogen in the purified fibrinogen samples accounted for only 1.6% (range, 0.8–2.5%) of the plasmin used in the subsequent digestion experiments. Similarly, α_2-antiplasmin accounted for less than 0.1% of the total protein in the fibrinogen preparations (range, 0–0.05%), and there was no significant correlation between the α_2-antiplasmin content and the amount of intact β-chain remaining after a 3-h plasmin digest of fibrin clots ($r = 0.36$, $p = 0.12$). Moreover, the average amount of α_2-antiplasmin in the purified fibrinogen samples accounted for only 0.8% (range, 0–2.0%) of the plasmin used in the subsequent digestion experiments. Thus, it is unlikely that the differences observed in the plasmin-mediated lysis of control and CTEPH fibrin were due to differences in either the plasminogen or α_2-antiplasmin content of these fibrinogen preparations.

Mixing Studies

To determine whether other substances copurifying with fibrinogen may have influenced the plasmin digestion results, we performed mixing experiments with four pairs of control and CTEPH fibrinogen samples. Equal mixtures of control and patient fibrinogen were made before clotting (with thrombin) and digesting (with plasmin). The fraction of intact β-chain remaining after a 3-h digest was determined by Western blotting/densitometry, and compared with the amounts obtained using control and CTEPH fibrinogen alone. The data were used to calculate a mixing index for each pair as described in METHODS. If the different types of fibrinogen (control and patient) were acting independently in the mixture, without the influence of copurifying activators or inhibitors, the mixing index should have a theoretic value of 0.5. Significant deviations from this value would be strongly suggestive of the presence of an interfering substance in one of the fibrinogen preparations. For instance, a mixture that behaved exactly like the control would have a mixing index of 0, whereas a mixture that behaved exactly like the patient would have a mixing index of 1. The mixing index for the four mixing pairs analyzed was 0.485 ± 0.205 (mean ± SD). This value was significantly different from either extreme (0 or 1) when compared using a two-tailed, one-sample *t* test ($p < 0.02$). The results of this experiment suggest that the group differences in plasmin-mediated fibrinolysis are not due to the presence of copurifying activators or inhibitors, at least those that would have their full effect on fibrinolysis when present in the mixture.

DISCUSSION

These *in vitro* experiments demonstrate that fibrin clots from patients with CTEPH are resistant to fibrinolysis, when compared

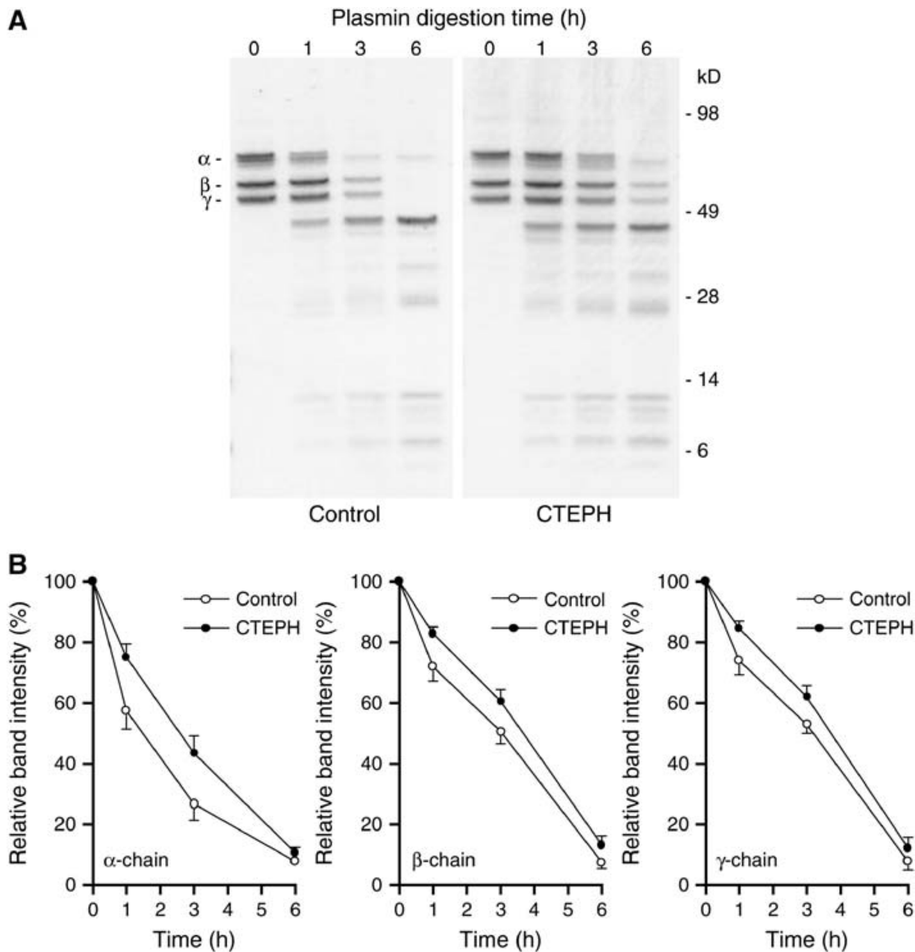

Figure 2. Fibrin from patients with CTEPH is resistant to plasmin-mediated lysis. (*A*) Fibrin polymers from a representative control subject and patient with CTEPH were analyzed at the indicated times after digestion with plasmin. Samples (equivalent to 1 μg of fibrin) were applied to each *lane* and subjected to SDS-PAGE followed by Coomassie staining of the gel. Molecular mass markers and the intact α-, β-, and γ-chains of fibrin are indicated on the *right* and *left side* of the figure, respectively. (*B*) Summary data on control subjects and patients with CTEPH (n = 10 each) from densitometric analysis of Coomassie-stained gels showing delayed degradation of the intact α-, β-, and γ-chains of CTEPH fibrin. Results are expressed as the mean percentage of the band intensity at t = 0. *Error bars* indicate standard error of mean. Group differences were evaluated by two-way analysis of variance.

with fibrin clots from healthy control subjects. The lysis resistance was not due to the presence of copurifying plasminogen or α_2-antiplasmin, both of which accounted for only a small fraction of the final plasmin concentration used in the digestion experiments. The lysis resistance also could not be explained by the presence of other potent fibrinolytic activators or inhibitors as detected by the mixing experiment. Because the lysis experiments were performed with plasmin, rather than plasminogen, the resistance could not be due to the presence of thrombin-activatable fibrinolysis inhibitor, which confers resistance by removing plasminogen binding sites from fibrin (8).

These findings have important implications for the understanding of the mechanisms responsible for CTEPH. Epidemiologically, the association between acute pulmonary thromboembolism and CTEPH is compelling. Approximately one-half of patients with CTEPH have a previously documented acute pulmonary thromboembolism (9). In the remainder, the previous thromboemboli are believed to have been clinically unrecognized, because even emboli large enough to be fatal often evade clinical detection *antemortem* (10). Furthermore, CTEPH has been reported in as many as 3.8% of patients with acute pulmonary thromboembolism (11), but is otherwise extremely rare in the general population (12). However, despite the strength of the clinical association between the two disorders, no mechanism has previously been identified through which the emboli in some patients evade normal thrombolytic processes to become large intravascular scars characteristic of CTEPH.

Although CTEPH can be induced in animals after acute pulmonary embolism by inhibiting fibrinolysis with tranexamic acid (13), no abnormality of coagulation enzymes, fibrinolytic enzymes, or pulmonary endothelial cell fibrinolytic function has been identified previously that accounts for the incomplete thrombus resolution in patients with CTEPH. In a prospective study of 46 patients with CTEPH, the frequencies of antithrombin III, protein C, and protein S deficiencies, and factor V and factor II mutations were low and similar to those of control subjects (2), a finding confirmed by others (12, 14). With regard to the fibrinolytic pathway, a study of 32 patients with CTEPH revealed neither high resting plasma levels of PAI-1 activity nor a blunted response of tissue-type plasminogen activator (TPA) to venous occlusion (3). In a separate study of 13 patients, TPA and PAI-1 levels in conditioned medium from primary cultures of pulmonary artery endothelial cells obtained from patients with CTEPH during thromboendarterectomy did not differ significantly from those of cells obtained from organ donor control subjects (4). Moreover, endothelial cells from patients with CTEPH and control subjects did not differ in their ability to secrete TPA and PAI-1 in response to stimulation with thrombin (4). However, a study of occlusive organized tissue removed from patients with CTEPH during thromboendarterectomy revealed a prevalence of smooth muscle cells and endothelial cells exhibiting elevated PAI-1 antigen and mRNA levels, which the authors concluded could contribute to the stabilization of vascular thrombi during remodeling (15). One practical consequence of all of these findings is an inability to predict which patients with acute pulmonary thromboembolism will go on to develop CTEPH.

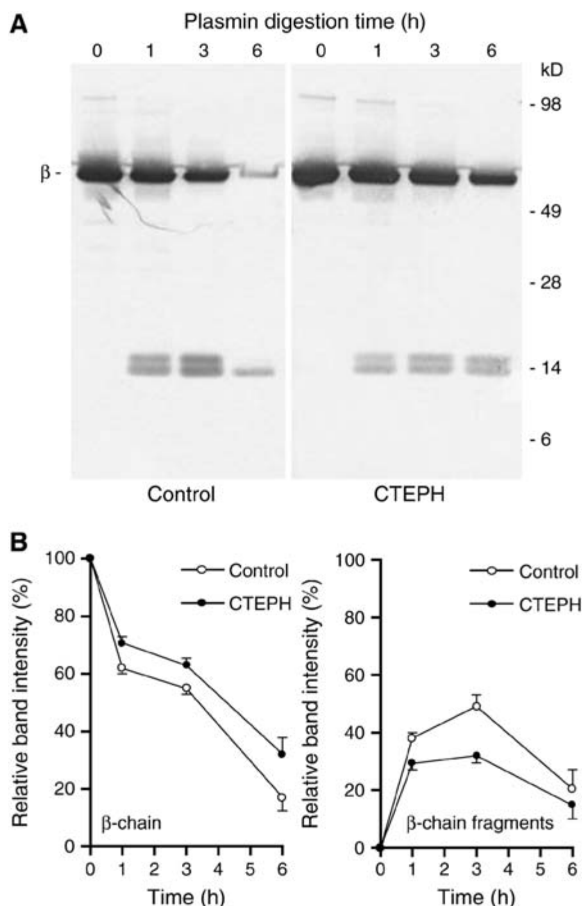

Figure 3. Persistence of the β-chain N-terminus after plasmin-mediated lysis of CTEPH fibrin. (*A*) Proteins from the gel shown in Figure 2 were electroblotted and probed with an antibody specific for the fibrin β-chain N-terminus. The degradation of the intact β-chain and appearance of a pair of closely migrating pair of β-chain N-terminal fragments are shown. (*B*) Summary data on control subjects and patients with CTEPH (n = 10 each) from densitometric analysis of Western blots showing persistence of the intact β-chain N-terminus as well as N-terminal β-chain fragments of CTEPH fibrin. Results are expressed as the mean percentage of the band intensity at t = 0. *Error bars* indicate standard error of mean. Group differences were evaluated by two-way analysis of variance.

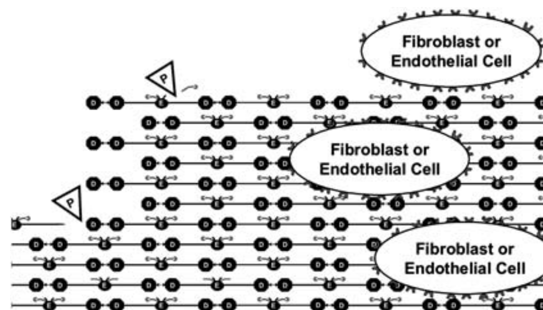

Figure 4. The balance between plasmin-mediated lysis and vascular remodeling of the fibrin polymer in pulmonary emboli. Plasmin (*triangles*) lyses the fibrin polymer by cleaving the peptide coils connecting the E and D regions. Plasmin also cleaves the signaling peptide at the N-termini of the two β-chains (*gray knobs*), which protrudes from the central "E" regions. If fibrinolysis is delayed, endothelial cells and fibroblasts may migrate in response to the presence of the N-termini of the β-chains, remodeling the fibrin polymer into a vascular scar.

The resistance to lysis we observed in the fibrin from patients with CTEPH, especially in the connective tissue cell–stimulating N-terminus of the β-chain, may set the stage for remodeling, rather than resolution, of thromboemboli. In this regard, the natural history of pulmonary embolism within the vascular lumen may be thought of as a contest between fibrinolysis and cellular remodeling of the thrombi, as illustrated in Figure 4. Under normal circumstances, plasmin should eventually solubilize the entire fibrin network of the clot. However, if the fibrin is not completely solubilized, it could act as a stimulus for proliferation of neighboring cells into the clot residua and thereby contribute to the remodeling of the fibrin network into organized tissue.

Fibrinolytic resistance that causes persistence of the N-terminus of the β-chain of fibrin, as we observed in patients with CTEPH, is particularly interesting to us because this segment has been implicated in a variety of physiologic events, including cell signaling (16) and angiogenesis (17). In addition, platelet spreading and the growth of fibroblasts and endothelial cells onto fibrin polymers are stimulated by peptides found at the N-terminus of the β-chain (18, 19). Thus, delayed fibrin degradation in this area could be an important step in the remodeling of an acute pulmonary embolus into chronic organized scar tissue.

Although these experiments are the first to systematically study fibrinolytic resistance in a series of patients with CTEPH, numerous genetic variants of human fibrinogen have been implicated in thrombotic diseases (20). Notably, fibrinogen Bellingham, which involves a γ_{275} Arg → Cys substitution, was reported in a patient with CTEPH and conferred a relative resistance to plasmin-mediated fibrinolysis (21). Family studies of this patient showed that the mutation alone was not sufficient to cause CTEPH but required the combination of acute pulmonary embolism and resistance to fibrinolysis. Numerous post-translational modifications of fibrinogen have also been reported (22), which may affect susceptibility to fibrinolysis (22).

These experiments do have limitations, which we plan to address in future studies. First, the comparison group was composed of healthy volunteers, rather than patients with acute pulmonary embolism who have completely resolved their thrombi. However, complete anatomic thrombus resolution after acute pulmonary embolism is not universal (23), so a valid comparison would need to take into account statistically the degree of perfusion recovery, as measured objectively after the acute pulmonary embolism. That study will be performed as a follow-up to the current one, which simply establishes that fibrin polymers from patients with CTEPH are abnormally resistant to lysis.

Second, although our experiments demonstrate that fibrin derived from patients with CTEPH is resistant to lysis, they do not define the structural basis of the resistance. Although fibrinogen genetic polymorphisms could be responsible, the resistance could also be ascribed to variances in post-translational modifications. It is also possible that circulating fibrinogen is subjected to alteration in patients with CTEPH. These and other possibilities would result in predictable changes in the molecular structure of patient fibrinogen. We are performing detailed analyses of fibrinogen structures in patients with CTEPH to clarify the mechanisms responsible for it, and develop strategies to prevent or at least detect CTEPH at an early stage.

Conflict of Interest Statement**:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

***Acknowledgment*:** The authors thank Dr. Russell Doolittle for his insight and suggestions during this work. They would also like to take the opportunity to thank him for being a constant inspiration and example.

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