Endogenous Fibrinolytic System in Chronic Large-Vessel Thromboembolic Pulmonary Hypertension

Mitchell A. Olman, MD; James J. Marsh, PhD; Irene M. Lang, MD; Kenneth M. Moser, MD; Bernd R. Binder, MD; and Raymond R. Schleef, PhD

**Background.** Chronic thromboembolic pulmonary hypertension (CTEPH) is a disorder characterized by pulmonary arterial hypertension as a consequence of organized thrombotic material in the central pulmonary arteries. Incomplete resolution of acute pulmonary emboli is believed to be pathogenically important; however, the mechanism for poor thrombus dissolution remains to be explained. We undertook this study to assess the major determinants of plasma fibrinolysis in patients with CTEPH (n=32).

**Methods and Results.** Immunological and functional levels of tissue-type plasminogen activator (t-PA) and type 1 plasminogen activator inhibitor (PAI-1) were quantified in platelet-poor plasma (PPP) from patients with CTEPH as well as age-matched controls. Although basal PPP t-PA antigen levels (CTEPH mean, 29.5 ng/ml; control mean, 2.7 ng/ml) and PAI-1 antigen levels (CTEPH mean, 55.8 ng/ml; control mean, 21.0 ng/ml) were higher in the CTEPH group, no between-group differences were detected in the enzymatic activities of these two molecules. The CTEPH group demonstrated a greater rise in t-PA antigen (CTEPH mean rise, 53.0 ng/ml; control mean rise, 5.6 ng/ml) and PAI-1 activity (CTEPH mean rise, 10.5 IU/ml; control mean rise, 1.2 IU/ml) than controls in response to an experimentally induced venous occlusion. Immunoprecipitation and fibrin autography of PPP from two patients with markedly elevated t-PA antigen levels demonstrate that the t-PA antigen was present in PPP primarily in complex with PAI-1.

**Conclusions.** Although abnormalities of the fibrinolytic system were detected, neither a high resting plasma PAI-1 activity nor a blunted response of t-PA to venous occlusion can be invoked as an etiology for CTEPH. (Circulation 1992;86:1241-1248)

**Key Words** • fibrinolysis • plasminogen activator, tissue-type • occlusions

Clinical studies of acute pulmonary embolism have indicated that embolic vascular obstruction resolves rapidly in the majority of cases.1-3 However, the acute thrombus fails to resolve in approximately 0.1% of the patients and initiates a series of events that lead to the development of chronic thromboembolic pulmonary hypertension (CTEPH).4-6 This clinical disorder carries a high morbidity and mortality without surgical intervention.7 Pathologically, CTEPH can be distinguished from primary pulmonary hypertension by the unique presence of thrombi in the segmental and/or larger size pulmonary arteries.8,9 Presently, the mechanisms responsible for the persistence of embolic residua of such magnitude in this patient population remain to be established.

Research over the past 30 years has established that fibrin thrombi are degraded in large part by the proteolytic action of plasmin, a serine protease that circulates in plasma as a proenzyme, plasminogen.10-12 The activation of plasminogen in the vascular space is regulated by a balance between the presence of plasminogen activators (PAs) and their inhibitors.13 Recent studies have demonstrated that tissue-type PA (t-PA), a 72,000-d glycoprotein serine protease, is the major endogenous PA in plasma.14,15 The primary physiological inhibitor of t-PA is type I PA inhibitor (PAI-1),16 a 50,000-d glycoprotein that is able to bind to and inhibit t-PA in its naturally occurring form17-19 (i.e., single-chain t-PA [sc-PA]) with a second order rate constant 10,000-fold higher than that for other known protease inhibitors.20 In light of this observation, it is not unexpected to find that approximately 70% of the total t-PA...
in normal human plasma is in complex with PAI-1. Both t-PA and PAI-1 are synthesized primarily in vascular endothelial cells, and these two molecules and their bimolecular complex are cleared from the plasma by the liver with a half-life measured in minutes.

Alterations in plasma fibrinolysis have been detected in several thrombotic disorders, including coronary artery disease and venous thromboembolism. Specifically, in venous thromboembolism, several groups have detected fibrinolytic abnormalities in approximately 40% of the more than 250 cases tested. The reduction in plasma fibrinolysis has been ascribed to either 1) an increased plasma PAI-1 activity level or 2) to a deficient release of t-PA in response to an experimentally induced venous occlusion. To determine if alterations in the fibrinolytic system can be implicated in the pathogenesis of CTEPH, we characterized the plasma t-PA and PAI-1 levels before and after venous occlusion in a series of 32 patients with CTEPH.

Methods

Patient Population and Sample Preparation

Thirty-two consecutive patients with surgically documented CTEPH presenting for thromboendarterectomy from July 1989 through May 1990 and a control group of 10 age-matched otherwise healthy volunteers with no history of thrombotic events were studied. Patients with CTEPH were evaluated at a mean of 6 years (range, 1–29 years) after their embolic event. Informed consent was obtained from all subjects, and the experimental protocol was approved by the University of California San Diego Medical Center Human Subject Investigations Committee (Protocol 89-509). In all subjects, venous blood was collected between 8:00 AM and 10:00 AM after 15 minutes of rest in the supine position (indicated as pre-VO). The blood was immediately anticoagulated with a one-10th volume of 200 mM EDTA acid (4°C) and centrifuged (2,500g, 4°C, 20 minutes). The platelet-poor plasma (PPP) was harvested, aliquoted, and stored at −70°C until assay. EDTA (final concentration, 20 mM) was chosen as the anticoagulant based on its ability to improve the immunological detection of t-PA antigen in plasma. The measurements of plasma antithrombin III (activity), protein C (antigen), protein S (total and free antigen), factor X (antigen), total cholesterol, and triglycerides were made in the clinical laboratories at the University of California San Diego Medical Center by standard techniques.

Experimental venous flow occlusion (VO) was performed by the forearm application of a sphygmomanometer cuff (Hewlett-Packard) that was inflated to a pressure midway between systolic and diastolic pressure for 15 minutes (usually 90 mm Hg). Before cuff deflation, a second venous blood sample (indicated as post-VO) was drawn from a fresh antecubital vein in the occluded arm and processed as described above. In addition, blood samples were obtained from a fresh antecubital vein on postoperative days 1, 2, 4, and 8 from the patients with CTEPH.

t-PA and PAI-1 Antigen Assay

t-PA antigen in PPP was quantified by a modification of the commercially available sandwich ELISA procedure that is able to detect both the free form of t-PA and t-PA complexed to PAIs in plasma. As noted in the manufacturer’s instructions, a monoclonal antibody directed against human t-PA (MoAb 7VPA, Technoclone, Vienna) was used to bind t-PA in the sample, and a peroxidase-labeled monoclonal antibody (MoAb POX-3VPA, Technoclone) was used for detection. The t-PA concentration was determined by comparing the sample’s absorbance at 490 nm in a microplate reader (Molecular Devices, Palo Alto, Calif.) to a standard curve generated with human t-PA purified from the Bowes melanoma cell line. Levels of PAI-1 antigen in PPP were quantified with a commercially available, double–monoclonal antibody ELISA assay kit (Technoclone), and the sample values were compared with a standard curve generated with human PAI-1 purified from human melanoma cell culture supernatant. In preliminary experiments, using purified human sct-PA added to plasma with known amounts of free, active PAI-1, we observed that the PAI-1 antigen level was reduced approximately 30%, suggesting that the assay detects complexed PAI-1 with less efficacy than free PAI-1.

PA and PAI Activity Assays

PA and PAI activity in PPP were determined with a commercially available, two-stage plasminogen-based chromogenic assay (American Diagnostica Inc., New Haven, Conn.). In preliminary experiments using a highly sensitive PA-activity assay, we compared the PA activity in PPP collected in EDTA (final concentration, 20 mM) with that from PPP collected in citrate buffer and immediately acidified with sodium acetate (pH 4.0). We found no difference in the PA activity from PPP in EDTA (0.37±0.38 IU/ml, mean±SD) relative to acidified PPP (0.35±0.28 IU/ml, mean±SD). Samples were either directly diluted in buffer (0.15 M Tris, pH 8.0) for measurement of PA activity or incubated with 40 IU/ml sct-PA (National Institute for Biological Standards and Controls [NIBSC], London; Ref. 83/517) for the measurement of PA activity. A standard curve was generated by the addition of increasing amounts of sct-PA to PAI-1–depleted plasma (American Diagnostica Inc.). After acidification, the samples were incubated in buffer containing substrate S-2251 (0.3 mM; Kabi-Vitrum, Franklin, Ohio), Glu-plasminogen, and cyanogen bromide digest fragments of fibrinogen (American Diagnostica Inc.). The values for PA activity in sample PPP were obtained by direct comparison with the measured absorbance (405 nm) detected using the purified sct-PA standards. The values for PAI-1 activity were obtained by measuring the residual t-PA activity present in the sample PPP after the addition of 40 IU/ml sct-PA. This assay measures only free, active PAI-1. One inhibitory unit (INU) of PAI-1 activity is defined as the amount of PAI-1 that inhibits 1 IU of human sct-PA.

Fibrin Autography

Selected patients’ plasma were analyzed by fibrin autography with techniques previously described. Briefly, 5 μl of PPP was subjected to SDS-PAGE (4% stacking gel, 9% separating gel) under nonreducing conditions. The polyacrylamide gel was then placed on a 1% agarose indicator film containing bovine fibrinogen (final concentration, 2.5 mg/ml; Calbiochem), 10
µg/ml purified human plasminogen,42 and 0.4 NIH units of human α-thrombin (kindly provided by Dr. Fenton, Albany, N.Y.). Incubation of the film at 37°C for 4–8 hours will result in the development of transparent lytic zones in the opaque indicator film, which reflect PA or protease activity in a location corresponding to their apparent molecular radius (Mr). Molecular weight markers included prestained myosin (Mr, 200,000), phosphorylase b (Mr, 97,400), bovine serum albumin (Mr, 69,000), ovalbumin (Mr, 46,000), and carbonic anhydrase (Mr, 33,000) (Amersham, Arlington Heights, Ill.) as well as the scf-PA international standard (NIBSC, Ref. 83/517) or the urokinase international standard (NIBSC, Ref. 66/46) kindly provided by the World Health Organization. Plasminogen independent protease action was detected using indicator films that were 1) prepared without plasminogen and 2) pre-heated (80°C, 15 minutes) to inactivate any residual protease activity that may contaminate the fibrinogen preparation.43

As previously reported,44 either preimmune New Zealand White rabbit serum, purified rabbit anti-human rt-PA immunoglobulin G35 or monospecific rabbit antiserum against purified human PAI-1,44 were absorbed to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Antigen-coupled beads (100 µl) and PPP samples (35 µl) were then gently rocked overnight at 4°C to deplete PPP of specific proteins.44,45 The immunodepleted plasma supernatants were then subjected to SDS-PAGE and fibrin autography as described above.

Statistical Analysis
The distribution of values for t-PA antigen, t-PA activity and PAI-1 activity, and PAI-1 antigen were found to be non-Gaussian. Therefore, all analyses were done with nonparametric methods.46 The Mann-Whitney test47 was used to compare basal levels of t-PA antigen or activity and PAI-1 antigen or activity between groups. The responses of the control and CTEPH group to venous occlusion were compared with the Mann-Whitney test performed on the change scores. Selected variables, as indicated, were correlated using Spearman’s rank coefficient.46 Significance was accepted at the p<0.05 level for all analyses. Values are individually plotted as indicated and/or reported as the mean with full range.

Results

Patient Characteristics
Previous reports from our group and others have described the general characteristics of patients with CTEPH and the criteria for surgical selection.45,46 The 32 patients described in this study have severe pulmonary arterial hypertension (pulmonary arterial pressure [PAP], 47±12.6 mm Hg, mean±SD), and a markedly elevated calculated pulmonary vascular resistance (PVR) (preoperative, 832±320 dyne·sec/cm², mean±SD). Pulmonary arterial thromboendarterectomy was performed for relief of thrombotic obstruction in all patients and resulted in a reduction in PAP (21±14.2 mm Hg, mean±SD) and PVR (3 days postoperative, 370±250 dyne·sec/cm², mean±SD) and an improvement in New York Heart Association functional class. Total protein S antigen was abnormally low in two patients from this study group, and an additional two patients had a demonstrable lupus-type circulating anticoagulant.49

Plasma Fibrinolytic Measurements

Figure 1 illustrates the results from immunological and amidolytic assays performed on PPP obtained from patients with CTEPH and from an age-matched control group. The resting (pre-VO) plasma t-PA antigen levels were higher (p=0.008) in the patients with CTEPH (mean, 29.5 ng/ml; range, 1.1-607.3 ng/ml) in compar-
ison to the controls (mean, 2.7 ng/ml; range, 0.6–7.8 ng/ml) (Figure 1A). However, the PA activity in both groups was below the lower limit of detection (1.2 IU/ml) of our assay system (Figure 1B). Two subjects from the CTEPH group had plasma t-PA antigen values that were markedly elevated (i.e., 158.4 ng/ml and 607.3 ng/ml; open symbols, Figure 1) in comparison to the remaining patient group. Studies will be described below that will focus on the characteristics of t-PA in these two patients (referred to subsequently as “outliers”). The difference in basal PAI-1 activity (Figure 1C) between the CTEPH group (mean, 13.2 IU/ml; range, 1.9–42.7 IU/ml) and the control group (mean, 10.0 IU/ml; range, 4.2–16.2 IU/ml) was not found to be significant. With regard to the PAI-1 antigen in pre-VO PPP, the CTEPH group exhibited higher (p = 0.004) levels (mean, 55.8 ng/ml; range, 5.7–170.8 ng/ml) than the control group (mean, 21.0 ng/ml; range, 4.1–65.8 ng/ml). The pre-VO t-PA antigen level correlated strongly (Spearman ρ = 0.73, p = 0.003) with the pre-VO PAI-1 antigen values; however, a divergence between the t-PA antigen and the PAI-1 antigen was observed in the PPP samples that contained high levels of t-PA antigen (t-PA antigen, >15 ng/ml), suggesting that the antibodies in the PAI-1 antigen assay may react against t-PA–PAI-1 complexes with less avidity.

To characterize the ability of patients with CTEPH to release fibrinolytic proteins from the vessel wall in response to known stimuli, blood was drawn from an arm vein after 15 minutes of experimentally induced venous occlusion, and the resultant plasma samples were analyzed immunologically and functionally. Plasma t-PA antigen was found to increase to a greater extent (p = 0.03) in the CTEPH group (mean rise, 53.0 ng/ml; range, 1.7–908.2 ng/ml) than the control group (mean rise, 5.6 ng/ml; range, 2.3–13.3 ng/ml) following venous occlusion (Figure 1A). Concordantly, the rise in plasma PAI-1 activity was greater (p = 0.029) in the CTEPH group (mean rise, 10.5 IU/ml; range, <0.6–57.5 IU/ml) than in the control group (mean rise, 1.2 IU/ml; range, <0.6–7.6 IU/ml) after venous occlusion (Figure 1B). The increase in plasma t-PA antigen levels was found to directly relate to the rise in plasma PAI-1 activity (Spearman ρ = 0.82, p = 0.00001). A significant correlation was also observed between the venous occlusion–induced decrease in plasma PAI-1 activity and the corresponding increase in plasma PAI-1 activity (Spearman ρ = 0.72, p = 0.00001).

The venous plasma levels of t-PA activity and antigen and PAI-1 activity were measured on postoperative days 1–8 in patients undergoing thromboendarterectomy and compared with the preoperative values in each patient. The t-PA antigen level in plasma rose on average to 160% of the preoperative value on postoperative days 1–8 (Figure 2, upper panel). Plasma PAI-1 activity was induced an average of eightfold on postoperative day 1 but fell toward preoperative values by day 4 (Figure 2, lower panel). Plasma PAI-1 activity remained below the limits of detection postoperatively (data not shown).

**Functional Characterization of Fibrinolysis in Selected Patients With CTEPH**

The above data indicate that as a group, patients with CTEPH have normal PA and PAI-1 activity but elevated immunological levels of t-PA and PAI-1 in pre-VO plasma. To characterize the fibrinolytic profile of this unique patient group, we analyzed the patient and control PPP by fibrin autography, as well as by a specific immunological assay for free set-PA. Figure 3A illustrates a representative experiment in which four controls (lanes 3–6) and three patients (lanes 7–9) were analyzed by fibrin autography. Major lysis zones were noted at an Mr of 115 and 90 kd, and a minor zone of lytic activity was seen at approximately Mr of 95 kd in both patients’ and controls’ PPP. In addition, a smear of lytic activity from Mr of 90–60 kd in PPP was routinely recorded with prolonged incubation of the indicator gel (Figure 3A; lanes 3–9). Plasminogen-dependent PA was localized to Mr of 115 kd, whereas the lytic activity zone at Mr of 90 kd was shown to be independent of the presence of plasminogen (Figure 3B). The plasminogen-independent band at 90 kd was specifically removed with antibodies to human plasminogen (data not shown). To identify the nature of the plasminogen-dependent enzymatic activity, fibrin autography was also performed on PPP from a representative control (Figure 3C; lanes 1–4) and an outlier (Figure 3C; lanes 1–4’) using PPP immunodepleted of either t-PA or PAI-1. Figure 3C illustrates the selective removal of the plasminogen dependent lytic activity at Mr of 115 kd and Mr of 90–60 kd as a consequence of immunodepletion with antibodies to human PAI-1 (lanes 3, 3’) or to human t-PA (lanes 4, 4’). Lytic zones indicative of urokinase-like fibrinolytic activity were not seen in any of the control or patient PPP samples tested. Because dissociation of t-PA–PAI-1 complexes during SDS-PAGE is known to occur and antibodies to PAI-1 removed all PA activity on fibrin autography, we conclude that the majority of t-PA in control, patient, and outlier PPP is in complex with PAI-1. These studies were complemented by the quantitation of free set-PA in the PPP samples by using a specific immunoassay for this molecule. Our data indicate that free set-PA antigen levels rose equally in all groups in response to venous occlusion, from 7.1±4% (mean±SD) of the total t-PA antigen in pre-VO PPP to 51.6±10% (mean±SD) of the total measured t-PA antigen in post-VO PPP.

**Discussion**

We have studied the fibrinolytic system in a group of patients with pulmonary arterial hypertension as a consequence of unresolved pulmonary embolism. To our knowledge, this is the first study of the fibrinolytic system in CTEPH. Compared with the control group, the major fibrinolytic findings in the CTEPH patients are 1) elevated levels of t-PA and PAI-1 antigen in resting plasma and 2) a greater plasma rise in t-PA antigen and activity in response to venous occlusion. The observed eightfold rise of plasma PAI-1 activity in the CTEPH patients after thromboendarterectomy is a typical response to surgical procedures that require cardiopulmonary bypass and may be mediated by the release of PAI-1 from platelets.

Based on the large number of reports indicating that elevated PAI-1 activity may play a role in the pathogenesis of venous thromboembolism, we were quite surprised to find no difference in the basal PAI-1 activity level in the CTEPH group despite increases in t-PA and PAI-1 antigen. Because our analysis of the hepatic enzyme profile of these patients did not indicate a
defect in the clearance t-PA–PAI-1 complexes (data not shown), the increased antigen levels for t-PA and PAI-1 detected in the CTEPH patients may be a result of an overproduction of t-PA and/or PAI-1 by a hyperactive vascular endothelium. Specifically, the possibility exists that rheologically mediated thrombin generation in proximity to thrombotic pulmonary vascular stenoses may induce vascular endothelial cell release of t-PA and PAI-1, as has been observed in vitro.21,54,55 Our observed increment in t-PA and PAI-1 antigen levels with no apparent change in enzymatic activities is consistent with this proposed scenario. Moreover, the fibrinolytic profile noted on the autographs is compatible with chronic oversecretion of t-PA rather than an acute release of t-PA into the plasma, where t-PA would be expected to bind to other circulating plasma protease inhibitors.56,57 Furthermore, if systemically circulating high levels of t-PA interact with high levels of PAI-1 locally produced in the pulmonary vasculature, this could account for our findings as well as support the existence of a local inhibition of fibrinolytic capacity. In addition to the previously proposed induction of PAI-1 by locally generated thrombin, high levels of t-PA have also been found to induce the expression of PAI-1 in cultured endothelial cells.58 In fact, preliminary data from our group reveal large amounts of PAI-1 messenger RNA and PAI-1 antigen in the pulmonary arterial tree in patients with CTEPH.59

The finding of an increased response of t-PA to venous occlusion in CTEPH was also unexpected in light of prior reports that demonstrate a blunted t-PA response in patients with venous thromboembolism.27–30 Vascular fibrinolytic capacity has been shown to vary as a function of the anatomic location60,61; hence, factors that govern the peripheral venous response to experimental occlusion may be different from those that control the fibrinolytic response of the pulmonary arterial tree to an embolic episode. Thus, it is still possible to propose the existence of a local pulmonary artery fibrinolytic defect in association with an enhanced t-PA response to venous occlusion. In addition, parenteral heparin has been suggested to influence the t-PA response to venous occlusion in a number of reports,30,62 and the administration of this agent to the CTEPH patients may have influenced our results. Unfortunately, methodological differences between this study and those from other laboratories preclude direct comparisons. Finally, our data from the CTEPH group were collected many months after the initial embolic event. It is possible that we have observed a response to chronic
pulmonary thrombembolism rather than identified a de novo alteration in fibrinolysis that predated the embolic event and inhibited its timely dissolution.

Patients with CTEPH are clearly different from the typical patient with pulmonary embolism. The vast majority with acute pulmonary embolism resolve their emboli with minimal vascular obstruction. Although we did not systematically rule out rare congenital defects such as an abnormal fibrinogen molecule, relatively common deficiencies classically associated with thrombosis (i.e., antithrombin III, protein C, protein S(0-79)) were identified in only two patients in this study group. We therefore generated the hypothesis that defect(s) in thrombotic dissolution might account for pulmonary embolic persistence and lead to the development of CTEPH. Data from this study suggest that neither a high resting plasma PAI-1 activity nor a blunted t-PA response to venous occlusion can be invoked as an etiology for CTEPH. Mechanisms responsible for poor thrombus dissolution may involve local abnormalities in the regulation, activity, inhibition, or release of t-PA, as well as intrinsic properties of the embolus itself that may render it resistant to lysis (i.e., size, platelets, age). Studies that focus on these parameters should provide a further understanding of the pathogenesis of chronic thromboembolic pulmonary hypertension.

Acknowledgments

The authors wish to acknowledge the help of Carol Archibald and Drs. Peter Fedullo, William Auger, and Stuart Jamieson in the tireless care of the patients with chronic thromboembolic pulmonary hypertension. In addition, the authors would like to thank Ms. Karen Roegner for instruction in the technique of fibrin autoradiography and Dr. Candee Gladson for many helpful discussions while preparing this manuscript.

References

17. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiafactor) synthesized by endothelial cells. J Biol Chem 1984;259:14914–14921
57. Lang I, Marsh J, Olman MA, Moser KM, Loskutoff DJ, Schleef RR: Type I plasminogen activator inhibitor (PAI-1) expression in pulmonary arterial thrombi of patients with chronic thromboembolic pulmonary hypertension. Fibrinolysis 1992;6(suppl 2):96–103


Endogenous fibrinolytic system in chronic large-vessel thromboembolic pulmonary hypertension.
M A Olman, J J Marsh, I M Lang, K M Moser, B R Binder and R R Schleef

Circulation. 1992;86:1241-1248
doi: 10.1161/01.CIR.86.4.1241

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/86/4/1241

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/