Parallel Analysis of Tissue-Type Plasminogen Activator and Type 1 Plasminogen Activator Inhibitor in Plasma and Endothelial Cells Derived From Patients With Chronic Pulmonary Thromboemboli

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

Background Chronic thromboembolic pulmonary hypertension is the result of nonresolving pulmonary emboli that lead to chronic obstruction of the central pulmonary arteries.

Methods and Results To determine if the failure to lyse pulmonary thromboemboli is caused by an abnormality in the endothelial cell (EC)-associated fibrinolytic system, conditions were established to culture ECs from patient main pulmonary arteries during surgical pulmonary thromboendarterectomies and to analyze the conditioned media for levels of tissue-type plasminogen activator (TPA) and type 1 plasminogen activator inhibitor (PAI-1). Our data indicate that the levels of TPA antigen and PAI-1 activity in media conditioned by primary ECs harvested from areas free of thrombus were not significantly different between patients with chronic thromboemboli and organ donors. In 13 consecutive patients, no correlation was obtained in either the TPA antigen or PAI-1 activity level in a patient’s plasma and the respective levels in media conditioned by the patient’s pulmonary ECs. Moreover, patient pulmonary arterial ECs were observed to increase the secretion of TPA and PAI-1 in response to thrombin in a fashion similar to donor pulmonary artery ECs.

Conclusions The data suggest that an inherent EC-mediated fibrinolytic imbalance is not a generalized phenomenon observed in pulmonary arteries of patients with chronic pulmonary thromboemboli. (Circulation. 1994;90:706-712.)

Key Words • thrombosis • fibrinolysis • plasminogen activators

Pulmonary thromboemboli usually resolve, leading to restoration of normal pulmonary hemodynamics. However, embolic vascular obstruction fails to resolve in 0.1% of cases and results in chronic thromboembolic pulmonary hypertension. In contrast to acute pulmonary embolism, chronic thromboembolic pulmonary hypertension is characterized by its clinical quiescence until severe pulmonary hypertension has developed. Moreover, this disease can be distinguished pathologically from primary pulmonary hypertension by the unique presence of highly organized thrombi in the segmental and/or larger elastic pulmonary arteries.

Untreated, chronic thromboembolic pulmonary hypertension results in a high mortality rate. Restoration of pulmonary blood flow by pulmonary thromboendarterectomy has dramatically improved the long-term function and survival of patients with this disorder.

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. Current information indicates that the major endogenous activator of plasma plasminogen is tissue-type plasminogen activator (TPA). The activity of this serine protease within the vasculature is regulated either via endogenous synthesis and release from endothelial cells (ECs) or by complex formation with and inactivation by its primary physiological inhibitor, type 1 plasminogen activator inhibitor (PAI-1). A 50 000-D glycoprotein that is able to bind to and inhibit TPA in its naturally occurring form (ie, single-chain TPA [scTPA]). Both TPA and PAI-1 are synthesized in vascular ECs. These two molecules and their bimolecular complex are bound to cell receptors and are cleared from the plasma by the liver with a half-life that is measured in minutes.

Despite the progress that has been made in understanding the biochemistry of the fibrinolytic system, the mechanisms responsible for the persistence of thromboembolic residua in patients with chronic thromboembolic pulmonary hypertension remain to be elucidated. Data from a number of laboratories indicate that the regulation of plasminogen activation plays an important role in a number of biological processes and may occur as a localized event within the vascular wall. The present study was initiated to determine if the failure to lyse pulmonary emboli is caused by an altered capability of patient pulmonary artery ECs to produce and secrete two key regulators of the fibrinolytic system. In this study, ECs were harvested from pulmonary arteries...
during pulmonary thromboendarterectomies, and the production of TPA and PAI-1 from these cells was compared with the production of these two proteins in media conditioned by ECs collected from organ donors.

Methods

Subjects and Collection of Specimens

Patients undergoing pulmonary thromboendarterectomy at the University of California San Diego Medical Center between September 1989 and December 1991 were entered into the study. In each patient, the diagnosis was confirmed by documentation of proximal pulmonary arterial thromboemboli through the use of pulmonary angiography and angiography and by measurement of pulmonary vascular resistance. Patients with protein C and/or protein S deficiency or lupus anticoagulant were not included in the study. The distributions of age, sex, duration of disease, and preoperative and postoperative pulmonary vascular resistance was similar to those described previously. Blood samples and tissues were collected from subjects, who had provided informed consent (n=95). Samples from an initial cohort of patients (n=60) were used solely for the optimization of EC harvest and culture conditions. Tissue samples from a subsequent series of patients (n=35) were used for the analysis of TPA and PAI-1 in the media conditioned by ECs cultured under specific experimental conditions. Media conditioned by primary endothelial cells derived from the last 13 patients and cultured under identical conditions were analyzed for TPA and PAI-1 in parallel with plasma samples, which were obtained from each patient immediately before pulmonary thromboendarterectomy. At the time of the study, none of the patients were clinically suffering from an acute pulmonary embolic event. For comparison, blood and ECs were obtained from heart or lung donors at the time of explantation (n=15). Individuals from this latter group had been selected as organ donors based on criteria that are currently used in our institution and include a noneventful previous medical history, normal findings on cardiac echocardiography, normal chemical analyses of blood and urine before the transplantation, and normal hemodynamics. More specifically, blood pressure of the 15 donors was 123.4±22.8/71±12.1 mm Hg; heart rate, 84.7±15.5 beats per minute; central venous pressure, 6.9±3.1 mm Hg; systolic pulmonary pressure, 25.4±4.1 mm Hg; and mean pulmonary pressure, 14.1±2.8 mm Hg (all data were obtained at the time of organ explantation, mean±SD). In addition, normal plasma levels for TPA and PAI-121 were determined in these subjects. The experimental protocol for this study was approved by the University of California San Diego Human Subject Investigations Committee (protocol 89-509).

Whole blood from patients was collected from an arterial line into polypropylene tubes containing 200 mmol/L EDTA (final concentration, 20 mmol/L) between 7:00 AM and 9:00 AM on the day of surgery before induction of anesthesia. Donor blood was collected 1 hour before organ explantation. Blood was centrifuged (600g for 15 minutes at 4°C), and the platelet-poor plasma was collected and stored at −70°C.

Specimens from patients with chronic thromboembolic pulmonary hypertension were obtained at pulmonary thromboendarterectomy. In the patients entered into the study, the main pulmonary artery and the lobar and segmental pulmonary arteries were filled with chronic thromboemboli that had become incorporated into the vessel wall, which prevented the harvest of ECs from those sites. Fortunately, in the main pulmonary artery, ECs were available immediately adjacent to the thromboemboli, and these areas were harvested to determine the phenotype of ECs in the immediate proximity of the thromboemboli. Briefly, after pulmonary arteriography, ECs were harvested by scraping approximately 1 cm² of the luminal surface with a surgical blade. The blades were rinsed with 2 mL of complete tissue culture medium, visible EC sheets were carefully broken up by repeated pipetting with a 5-mL pipette, and the resulting mixture was placed onto fibronectin-coated (Sigma Chemical Co) 33-mm tissue culture plates. The thrombus subsequently was removed from the artery by endarterectomy. Control ECs from pulmonary arteries and aortas were obtained at organ harvests by using this technique after removal of the whole vessel. Human synovial fibroblasts were generously provided by the laboratory of Dr Nathan J. Zvaifler (Division of Rheumatology, UCSD Medical Center).

Tissue Culture

ECs were grown to confluency and subcultured on fibronectin-coated 33-mm culture dishes (Corning) in 0.2 mL/cm² M199 media supplemented with either 20% pooled human plasma-derived serum (HPDS) or 20% fetal bovine serum (FBS; lot C029401, GR Scientific) as well as with heparin (20 U/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), and 0.4% endothelial growth factor prepared from bovine hypothalami.22 HPDS was obtained by recalcifying outdated pooled plasmas that was purchased from the San Diego Blood Bank. All sera were heat inactivated by incubation at 57°C for 30 minutes. For the preparation of conditioned media, confluent EC monolayers were washed twice with phosphate-buffered saline (PBS) and incubated for 24 hours in complete media. The resulting conditioned media were collected, centrifuged (700g) to remove floating cells and cellular debris, and stored in 0.1% Tween 80 at −70°C until used. After the harvest of the conditioned media, the primary pulmonary arterial or primary aortic ECs were counted and analyzed for purity by flow cytometry. In brief, monolayers were washed twice in warm PBS and incubated (4 hours at 37°C) with 10 µg/mL Dil-acetylated low-density lipoprotein (LDL) (Biomedical Technologies Inc). After the incubation period, cells were washed twice, trypsinized, counted using a hemocytometer, resuspended in complete media to a final concentration of 2×10⁵ cells/mL, and analyzed with an Ortho Cytofluorograph model 50H equipped with a Data General 2151 computer (Ortho Diagnostic Systems). Fluorochrome was excited with 200 mW of 514-nm light using a 5-W argon laser. Light emission was detected on a photomultiplier tube using a filter at 560 nm.

Thrombin Stimulation Experiments

Passaged ECs were used to obtain a sufficient quantity of cells for a detailed dose-response curve using thrombin as a stimulator of both TPA and PAI-1 production. Cells were subcultured on fibronectin-coated 12-well cluster tissue culture plates (Costar). The confluent EC monolayers were rinsed twice with warm PBS and incubated (24 hours at 37°C) in 1 mL serum-free media containing various concentrations of human α-thrombin (cell culture grade, Sigma). After incubation, the conditioned media were collected, centrifuged to remove cellular debris, and stored at −70°C in the presence of 0.01% Tween 80 until assayed. In control experiments, thrombin (10 U/mL in M199) was incubated (30 minutes at room temperature) with 50 U/mL hirudin (Sigma) before addition of the mixture to the cell cultures. Viability of adherent cells at the end of the incubation period was >90% (based on Trypan blue exclusion) and did not vary significantly between different experiments.

Assays for TPA Antigen, PAI-1 Activity, and PAI-1 Antigen

For the measurement of TPA, a two-site immunoassay was used as previously described21 with monoclonal anti-human TPA antibody (2 µg/mL; 7VPA, Technoclone) as the immobilized antibody and a peroxidase-labeled monoclonal anti-human TPA antibody (1 µg/mL; 3VPA, Technoclone) as the detecting antibody. PAI-1 activity was quantitated by using immobilized TPA to bind active PAI-1 present in various
Scrape-harvested patient pulmonary arterial ECs in culture for 3 days (A, magnification ×400) or after 15 days (B, magnification ×200). C, Fluorescent activated cell-sorting analysis of the latter EC culture labeled with Dil-acetylated low-density lipoprotein (LDL). For comparative purposes, flow cytometry of human synovial fibroblasts labeled with Dil-acetylated LDL is shown (D). In C and D, the relative number of cells per 10⁶ plotted cells on the y axis is plotted over a logarithmic scale of fluorescent intensity displayed on a 1-to-200 scale on the x axis.

**Statistical Analysis**

Levels of TPA and PAI-1 in conditioned media were compared using ANOVA. The EC-conditioned media time course studies were analyzed by an ANOVA with repeated measures. Correlation analyses between conditioned media and plasma data were based on the Spearman rank correlation coefficient because the values of plasma samples were not normally distributed. A three-factor ANOVA was used in the interpretation of the thrombin stimulation experiments. The degrees of freedom for the F values in this analysis were corrected according to Greenhouse and Geisser. Significance was accepted at the P<.05 level for all analyses.

**Results**

**Characteristics of Cultured Patient Pulmonary Artery ECs**

Experiments were initially performed to optimize the conditions for the growth of ECs scrape-harvested from patient pulmonary arteries during surgical thromboendarterectomies. In brief, one harvest was performed when the right pulmonary artery was opened, just before the endarterectomy was started. To collect a sufficient amount of cells, this procedure was repeated on the left side. Cells adhered within the first 8 hours after plating, formed numerous small colonies (Fig 1A), and reached confluency after 14 to 39 days in culture (29.1±6.1 days using HPDS, 29.3±8.7 days using FBS [mean±SD]; overview of a 15-day culture [Fig 1B]). Flow-cytometry experiments using Dil-acetylated LDL were used to confirm the purity of the EC cultures (Fig 1C), which appear as a single population of bright cells compared with the pattern obtained when human fibroblasts were similarly analyzed (Fig 1D). Initially, HPDS (20%) was used to grow ECs to achieve an in vitro system that used homologous reagents. Fig 2 demonstrates comparable levels of TPA antigen (A) and PAI-1 activity (B) in the 24-hour conditioned media harvested from primary donor pulmonary artery ECs (open bar 1), primary donor aorta ECs (open bar 2), and primary chronic thromboembolic pulmonary hypertension patient pulmonary artery ECs (open bar 3; P=.4 and .8, respectively). Because in a number of laboratories ECs are routinely cultured in FBS, we expanded our study and analyzed the conditioned media levels of TPA and PAI-1 by using patient pulmonary artery ECs that were grown in the presence of FBS. No differences were obtained in the TPA antigen levels in 24-hour media conditioned by patient ECs that were cultured in 20% FBS (Fig 2A, closed bar 3) compared with the patients' cells that were cultured in 20% human serum (Fig 2A,
fibrinolytic system in patients with chronic thromboembolic pulmonary hypertension, we performed a parallel analysis of the levels of TPA and PAI-1 in a patient’s plasma and the respective levels in conditioned media. No significant correlation was observed between the TPA antigen level in a patient’s plasma and the level for this protein in the media conditioned by a patient’s pulmonary artery ECs (Table; Spearman r = –.03, P = .9). A similar analysis for PAI-1 activity (Table; Spearman r = –.3, P = .2) and antigen (Table; Spearman r = –.03, P = .9) also indicated a lack of correlation.

Response of Patient Pulmonary Artery ECs to Thrombin

To investigate a possible abnormality in the ability of patient pulmonary artery ECs to respond to stimuli and produce appropriate amounts of either TPA or PAI-1, we compared the levels of these two components in the conditioned media of thrombin-stimulated patient pulmonary artery ECs with those of thrombin-stimulated donor pulmonary artery ECs. Fig 3 shows the data obtained using third-passage ECs cultured in FBS from patients with chronic thromboembolic pulmonary hypertension (n = 12) and from donors (n = 5). Although levels for TPA antigen in EC-conditioned media increased with each subsequent passage (see inset in Fig 2A), thrombin stimulated the production of TPA antigen similarly in ECs isolated from patient pulmonary artery and donor pulmonary artery (Fig 3A; P = .6). Moreover, the changes in PAI-1 activity in the conditioned media of patient pulmonary artery ECs treated with thrombin were comparable to the values obtained by stimulating donor pulmonary artery ECs with this agent (Fig 3B; P = .5). To demonstrate that the observed changes were due to the proteolytic activity of thrombin, parallel experiments were performed using thrombin that had been preincubated with hirudin. Under these conditions, no increase in TPA and PAI-1 levels in the conditioned media was observed (data not shown).

Discussion

A causal role of the fibrinolytic system in arterial and venous thrombosis has been suggested by data from a number of laboratories. Our previous study of the endogenous fibrinolytic system in patients with chronic thromboembolic pulmonary hypertension revealed neither an excess of PAI-1 activity in peripheral blood nor a blunted rise in TPA following venous occlusion. However, the presence of elevated plasma levels of TPA/PAI-1 raised the possibility that a local abnormality in the fibrinolytic system existed within the pulmonary vasculature. Therefore, we initiated a study to investigate the production and secretion of these proteins in pulmonary arterial ECs cultured from a series of patients.

Initial experiments examined the levels of fibrinolytic proteins in primary pulmonary arterial EC cultures. Our data show no statistical differences in the basal levels of TPA antigen and PAI-1 activity in the media conditioned by ECs cultured from patients with chronic thromboembolic pulmonary hypertension and from organ donors (Fig 2). A number of laboratories have compared the production of plasminogen activators derived from ECs of different vascular compartments and described differences in their fibrinolytic
Parallel Analysis of PAI-1 and TPA in a Patient's Plasma and Levels in Media Conditioned by Patient's Endothelial Cells

<table>
<thead>
<tr>
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<th>Sex/Age, y</th>
<th>Plasma</th>
<th>Conditioned Medium</th>
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<tr>
<td></td>
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<td>PAI-1 Antigen, ng/mL</td>
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<tr>
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<td>13</td>
<td>F/51</td>
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PAI-1 indicates type 1 plasminogen activator inhibitor; TPA, tissue-type plasminogen activator.

Levels for PAI-1 antigen, PAI-1 activity, and TPA antigen were determined in the plasma collected before surgery and in the media conditioned for 24 hours by primary endothelial cell monolayers in 20% fetal bovine serum cultured from patient pulmonary arteries.

potential. For example, human umbilical vein ECs have been found to produce only about one fourth of the TPA antigen secreted into media conditioned by fat microvascular cells.\textsuperscript{31} We also found that umbilical vein EC-conditioned media contained significantly less TPA antigen and more PAI-1 activity per 10^6 cells than media conditioned by pulmonary artery and aortic ECs (data not shown). Although slightly higher mean TPA antigen levels were detected in media conditioned by donor pulmonary arterial ECs in comparison to donor aortic ECs (column 1 versus column 2 in Fig 2A and 2B), there were no significant differences when the values were analyzed by ANOVA. We did observe a significant increase in TPA antigen and a decrease in PAI-1 activity within the first three passages of patient ECs, which agree with data that describe progressive changes of the EC fibrinolytic phenotype in vitro.\textsuperscript{35} Our results emphasize the importance of standardizing data derived from cultured ECs to the days in culture and the number of passages. When experiments were performed using FBS as the serum source, patient EC-conditioned media were found to contain significantly more PAI-1 activity as well as PAI-1 antigen (data not shown). These data are in accord with the report of Sawdey et al\textsuperscript{36} indicating that growth conditions play an important role in the levels of TPA and PAI-1 secreted from cultured vascular ECs.

To gain information about the ability of patient ECs to respond to a secretory stimulus, we added thrombin to the culture media in increasing concentrations. Thrombin, which has been reported to stimulate the production of both TPA\textsuperscript{37-39} and PAI-1,\textsuperscript{39,40} was selected because it can be anticipated that this enzyme will be present in the local environment of a thrombus. Our results indicate that TPA antigen and PAI-1 activity were increased by thrombin treatment of both patient and donor ECs. The decrease in PAI-1 activity in Fig 3 (B) observed above 0.4 U/mL thrombin is likely mediated by either complex formation or proteolytic cleavage of this inhibitor at high thrombin concentrations,\textsuperscript{41} as control experiments using a two-site immunoassay\textsuperscript{25} revealed that PAI-1 antigen levels remained elevated above 0.4 U/mL thrombin (data not shown). Although our data indicate that thrombin increased TPA and PAI-1 levels similarly in both patients and donor ECs, other stimulatory molecules are likely to be present in the vicinity of the thrombus, and it remains to be determined if the TPA/PAI-1 response of patient ECs is normal in response to all stimuli.

Presently, most published studies analyzing the secretion of proteins from cultured human ECs have been performed on material derived from either umbilical cords, autopsies, or organ donors.\textsuperscript{32-34} This is the first study containing information about ECs grown from the main pulmonary arteries of a well-defined series of patients with chronic proximal pulmonary arterial thromboemboli. Our data suggest that a basic abnormality in the production of either TPA or PAI-1 does not exist in the pulmonary ECs of these patients. Furthermore, we have directly compared two key fibrinolytic proteins in patient plasma and EC-conditioned media. The lack of correlation between the TPA/PAI-1 levels in a patient's plasma and the EC-conditioned media is in accord with current information suggesting that plasma TPA/PAI-1 levels result from a balance between the production by ECs, the formation of complexes in a plasma milieu and on cell surfaces, and the clearance of these components in the systemic and hepatic system.\textsuperscript{16,42} Therefore, current experiments by our group are directed at determining if a fibrinolytic imbalance occurs in the local environment of the thromboemboli in vivo. Preliminary immunohistochemical and in situ hybridization studies\textsuperscript{43} indicate that PAI-1 expression is elevated in specific cells within the throm-
boemboli compared with the expression of PAI-1 in noninvolved areas of a patient’s pulmonary artery.

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References


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