Expression of Type 1 Plasminogen Activator Inhibitor in Chronic Pulmonary Thromboemboli

Irene M. Lang, MD; James J. Marsh, PhD; Mitchell A. Olman, MD; Kenneth M. Moser, MD; David J. Loskutoff, PhD; 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 thromboembolism is caused by the local expression of the primary inhibitor of tissue-type plasminogen activator (type 1 plasminogen activator inhibitor, PAI-1), levels of PAI-1 antigen and mRNA were analyzed by immunohistochemistry and in situ hybridization in specimens harvested from a series of patients during pulmonary thromboendarterectomies. Red, fibrin-rich thrombi within the thromboendarterectomy specimens were lined with a single layer of endothelial cells exhibiting high levels of PAI-1 antigen. Quantitation of the in situ hybridization signal revealed that a significant increase in PAI-1 mRNA was present in the endothelial cells lining the fresh thrombi in comparison to the signal present in the endothelial cells from noninvolved areas of patients' pulmonary arteries (n=16, P<.001). In contrast, tissue-type plasminogen activator antigen levels were low in all samples. Yellowish-white thrombi were composed of smooth muscle cells and endothelial cells in numerous vessels that stained prominently for PAI-1 antigen. Both types of cells within the highly organized tissues also exhibited elevated PAI-1 mRNA levels in comparison to patient pulmonary artery specimens that were free of thrombus (n=16, P<.02).

Conclusions The prevalence of PAI-1 expression within pulmonary thromboemboli suggests that this inhibitor may play a role in the stabilization of vascular thrombi. (Circulation. 1994;89:2715-2721.)

Key Words • thrombosis • fibrinolysis • plasminogen 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 or by complex formation with and inactivation by its primary physiological inhibitor, type 1 plasminogen activator inhibitor (PAI-1). The latter molecule is a 50 000-Da glycoprotein that is able to bind to the naturally occurring form of TPA (single-chain TPA) with a second-order rate constant orders of magnitude higher than that for other known protease inhibitors (for reviews, see References 25 and 26). PAI-1 is secreted by a variety of cells including endothelial cells, smooth muscle cells, and fibroblasts, and the synthesis of this inhibitor is regulated by a wide spectrum of physiological compounds (cytokines, growth factors, etc).25,26

Although several previous reports have suggested a causal role of the fibrinolytic system in arterial27 and venous28-30 thrombosis, studies investigating the endogenous plasma 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 after venous occlusion.31 However, data from a number of laboratories indicate that the regulation of plasminogen activation may occur as a localized event within the vascular wall (for reviews, see References 32 and 33). For example, two groups have recently demonstrated elevated PAI-1 expression in several defined areas within atherosclerotic human arteries.34,35 The current study was initiated to analyze the local pulmonary arterial expression of PAI-1 in patients with chronic thromboembolic pulmonary hypertension.
Clinical and Fibrinolytic Parameters of 16 Patients With Chronic Thromboembolic Pulmonary Hypertension

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</table>

PAI-1 indicates type 1 plasminogen activator inhibitor.
*Exposed silver grains were quantitated by direct counting as described in "Methods."
†Cells luminal to internal elastic lamina that replace the endothelial lining of the pulmonary artery.

Methods

Subjects

Patients undergoing pulmonary thromboendarterectomy at the University of California San Diego Medical Center between September 1989 and December 1991 were entered into the study. The diagnosis in each patient was confirmed by careful review of the medical history, measurement of pulmonary vascular resistance, pulmonary angiography, and angioscopy. For quantitative analysis, the signal for PAI-1 mRNA was determined in cells within the thrombi of 16 consecutive patients who had provided informed consent and was compared with the PAI-1 mRNA signal in involved and noninvolved areas of the patients' pulmonary arteries as well as to the PAI-1 antigen levels in the patients' plasmas. The distribution of age, sex, duration of disease, and preoperative and postoperative pulmonary vascular resistance was similar to those in larger series that we have described previously. All patients experienced a dramatic decrease in pulmonary vascular resistance as documented 3 days after the surgical intervention, when all patients were off mechanical ventilation and off vasoactive drugs, just before removal of monitoring catheters (Table). During trimming of the pulmonary artery at the end of the pulmonary thromboendarterectomy, specimens were harvested from pulmonary arteries that were free of thrombus (noninvolved specimens) and from areas that were underlying the excised thrombus (involved specimens). The thrombi and tissues were fixed for 20 hours in paraformaldehyde (4% wt/vol in 0.1 mol/L sodium phosphate) at 4°C, embedded in paraffin blocks, and sectioned at 2-μm thickness using a microtome. The sections were mounted onto poly-l-lysine-coated slides and stored at room temperature until analyzed. For comparative purposes, blood and tissues were also obtained from heart or lung transplant organ donors at the time of exploitation (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 analysis 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 obtained at the time of organ exploitation, mean±SD). In addition, normal plasma levels for TPA and PAI-1 were ascertained 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).

Analysis of TPA and PAI-1 in Plasma Samples

Patient whole blood was collected from an arterial line into polypropylene tubes containing 200 mmol/L ethylenediaminetetraacetate (final concentration, 20 mmol/L) between 7 AM and 9 AM on the day of surgery before induction of anesthesia. Donor blood was collected 1 hour before organ exploitation. Blood was centrifuged (680g, 15 minutes, 4°C), and platelet-poor plasma was harvested and stored at −80°C. Plasma TPA antigen and PAI-1 antigen were quantitated using two-site immunoassays.31,37 PAI-1 activity was quantitated in a functional immunoassay.57,58 Standard curves for this assay were prepared using purified human PAI-1 that was reactivated by treatment with guanidine hydrochloride (37°C) followed by extensive dialysis against phosphate-buffered saline (4°C) and stored at −80°C in 30-μL aliquots. This preparation was calibrated using the International Standard TPA (83/517,
Histochemistry

Immunohistochemical staining was carried out using a three-step avidin-biotin-peroxidase method as described in detail previously. In this procedure, sections of paraffin-embedded, paraformaldehyde-fixed tissues were incubated with either rabbit antibodies (eg, anti-von Willebrand factor [vWF]31, anti–PAI-1,40,41 anti-TPA, American Diagnostica, Inc.) or mouse monoclonal antibodies (eg, anti-human smooth muscle–α actin, clone 1A4; anti-human monocyte/macrophage, CD68, clone KPI, Dako Corp) followed by the appropriate second antibody (ie, biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG, Zymed Lab). Subsequent incubations included a streptavidin-peroxidase conjugate followed with the chromogen aminoethylcarbazole/hydrogen peroxide mixture (Zymed Lab) that results in a reddish-brown deposit indicative of positive immunoreactivity. To differentiate fresh thrombus from organized thrombus and to identify fibrin/fibrinogen and collagen, a trichrome stain was performed according to published procedures.42

In Situ Hybridization

A PAI-1 cDNA fragment containing nucleotides 1 to 1085 in pGEM-3Z (Promega Corp) was used for the preparation of antisense and sense riboprobes by in vitro transcription using SP6 RNA polymerase or T7 RNA polymerase, respectively, in the presence of 35S-UTP (specific activity, 1200 Ci/mmol, Amersham) as described previously.34,39 The riboprobe was purified and used to detect PAI-1 mRNA on paraffin sections using the in situ hybridization protocols described in detail previously.34,39 After in situ hybridization of the 35S-labeled antisense probe with the tissue sections, the slides were coated with Kodak NTB2 emulsion and exposed in the dark at 4°C for 10 weeks. Slides were developed for 2 minutes in Kodak D19 developer, fixed, washed, and counterstained with hematoxylin and eosin, which stains the nuclei blue. Parallel sections were analyzed using a sense probe as the control for nonspecific hybridization, and in no instance was a specific signal detected even after 12 weeks of exposure. Specimens were analyzed using combined light/epliluminescence microscopy to permit a simultaneous visualization of the sample and exposed silver grains. The latter appear as black or green dots, depending on illumination, and indicate the presence of PAI-1 mRNA.

Statistical Analysis

Quantitation of the in situ hybridization signal was done by counting silver grains associated with ≥200 nucleated cells at either a ×400 or ×1000 magnification using oil immersion. Data are expressed as exposed silver grains per 100 nuclei. A mean count of <60 silver grains per 100 nuclei was found in control pulmonary artery endothelial cells and <120 silver grains per 100 nuclei in control pulmonary artery smooth muscle cells. Because the data were not random in a Gaussian distribution, differences were assessed with the use of the Mann-Whitney test.43 Furthermore, the mean numbers of grains per 100 nuclei were correlated with levels of PAI-1 in the patients' plasmas using a Spearman’s rank coefficient.43 Differences were considered significant at \( P<.05 \).

Results

The majority of the thrombi were composed of fresh red material and yellowish-white organized tissue (Fig 1a). Fresh thrombus was not only found superimposed on organized clot (Fig 1a), but an abundance of fibrin and red blood cells could also be detected within vascular lumina in the organized tissue by using a trichrome stain (Fig 1b). This technique results in a reddish-blue stain in the presence of fibrin/fibrinogen (Fig 1c), whereas collagen is stained green. Immunohistochemical analyses of parallel sections revealed that the surfaces of these fresh clots were covered by vWF antigen–positive cells (Fig 1d, high magnification of Fig 1b, arrow). Some parts of the thrombus stained diffusely with antibodies against vWF antigen (data not shown), but this staining was at a low intensity compared with the staining of the cells lining the thrombus. In parallel sections, no immunoreactivity for macrophage-related antigen CD68 (Fig 1e) and a weak signal for TPA (Fig 1f) were detected. However, intense staining for PAI-1 antigen (Fig 1g) and signal for PAI-1 mRNA (Fig 1h) were observed in the cells that lined the surfaces of the fresh thrombi.

In comparison, low-power examinations of the organized parts of the thrombi revealed endothelialized vessels of different sizes (Fig 2a). The parenchymal tissue was composed of cells in a collagen matrix rather than a fibrin meshwork, as revealed by the trichrome stain (Fig 2a). The cells lining neovascular structures were identified as endothelial cells, based on their immunological reactivity toward antibodies to vWF (Fig 2b). Although these cells stained weakly for TPA (Fig 2c), intense staining for PAI-1 antigen was observed in nucleated cells (Fig 2d). The spindle-shaped cells within the collagen matrix demonstrated immunoreactivity both for PAI-1 (Fig 2g) and for smooth muscle α-actin (data not shown). Analyses of the organized regions of the vascular thrombi for PAI-1 mRNA by in situ hybridization revealed a strong signal in (1) endothelial cells lining the neovessels in the thrombus (Fig 2f) and (2) the spindle-shaped, smooth muscle α-actin–positive cells that constituted the thrombus parenchyma (Fig 2h).

To obtain quantitative data on the steady-state levels of PAI-1 mRNA, we determined the mean number of exposed silver grains per 100 nuclei (Table). No statistical difference was detected in the level of PAI-1 mRNA associated with either endothelial cells \( (P=.385) \) or smooth muscle cells \( (P=.5) \) of patients' noninvolved pulmonary arteries \( (n=16) \) in comparison to the signal in the pulmonary arteries of organ donors \( (n=5) \). The analysis of pulmonary arterial vessel specimens in areas underlying the excised thrombus revealed broad luminal sheets of residual thrombus material that were composed of smooth muscle cells and replaced the endothelial cell lining in 5 of 16 patients (patients 7, 8, 11, 12, and 16; Table). PAI-1 mRNA levels were elevated in the smooth muscle cells of these patients when compared with the levels in smooth muscle cells in the medial layer of the patients' noninvolved pulmonary arteries \( (P=.023) \). Thrombus smooth muscle cells exhibited a significantly higher level of PAI-1 gene expression when similarly compared with the medial layer of the patients’ noninvolved pulmonary arteries \( (P=.017) \). Furthermore, an elevated in situ hybridization signal for PAI-1 mRNA in thrombus endothelial cells was observed in all 16 patients in comparison to the signal in endothelial cells within specimens harvested from noninvolved regions of the pulmonary artery or from organ donors \( (P<.001) \). Although the cells within the thrombus exhibited elevated PAI-1 mRNA levels, no correlation was observed between the number of silver grains per 100 nuclei and either the plasma PAI-1 antigen or activity levels \( (P>.22; \) Table).
Figure 1. Photomicrographs: Analysis of the surface of a fibrin deposit within an organized chronic pulmonary thromboembolus. A representative chronic thromboembolus (a) was fixed, sectioned, and processed as described in "Methods" by (1) histochemical analysis using a trichrome stain (b, ×100 magnification; c, ×1000 magnification of area indicated by arrow in b; fibrin/fibrinogen stains red or reddish-blue, collagen stains green, red blood cells stain yellow; single cell layer lining the thrombus is marked by arrowheads); (2) immunohistochemical procedure for von Willebrand factor (d), the monocyte/macrophage antibody CD68 (e), tissue-type plasminogen activator (f), and type 1 plasminogen activator inhibitor (PAI-1) (g) (reddish-brown deposits in blue nucleated cells are indicative of positive immunoreactivity, ×1000 magnification for panels d through g); and (3) in situ hybridization for PAI-1 mRNA (h, plane of focus on black exposed silver grains that indicate positive hybridization signal; ×1000 magnification).
Fig 2. Analysis of an endothelialized blood channel within an organized chronic pulmonary thromboembolus. Representative photomicrographs of an organized region within a chronic thromboembolic pulmonary hypertension thrombus as analyzed by (1) histochemical procedures using a trichrome stain (a); (2) immunohistochemical procedures for von Willebrand factor (b), tissue-type plasminogen activator (c), type 1 plasminogen activator inhibitor (PAI-1) (d and g), and nonimmune rabbit IgG (e) (reddish-brown deposits in nucleated cells as emphasized by arrowheads indicate positive staining; ×400 magnification); and (3) in situ hybridization procedures for PAI-1 mRNA (f and h) (black or green exposed silver grains represent positive hybridization signal; ×400 magnification).
Discussion

Our data indicate that high levels of PAI-1 are present within pulmonary thromboendarterectomy specimens in chronic thromboembolic pulmonary hypertension. The organization process of thrombi has been reported by other laboratories using specimens derived from patients as well as from animal models. Pulmonary thromboemboli in humans have been found to become organized and recanalized over periods of several months to more than a year. Subsequently, the thromboemboli are degraded to fibrous intimal thickenings and bands. In contrast to this type of resolution, we found massive, highly organized pulmonary arterial thromboemboli that contain areas of fresh thrombus, indicating an ongoing thrombotic process. The morphology of these pulmonary thromboemboli resembles the histopathological characteristics reported for canalized deep venous thrombi but is distinct from arterial thrombi seen with atherosclerosis.

Several groups have investigated local gene expression in human atherosclerotic lesions using in situ hybridization. For example, platelet-derived growth factor, tissue factor, and PAI-1 have been detected in defined regions of atherosclerotic tissue, suggesting an involvement of these molecules in atherogenesis. Research involving a patient defective in PAI-1 antigen as well as animal model systems have substantiated the essential role of PAI-1 in vascular clot dissolution in vivo. However, little is known about the source and localization of PAI-1 within thrombi or the factors that regulate PAI-1 expression in the local environment within a thrombus. We define the PAI-1-expressing cells within pulmonary arterial thrombus as primary endothelial cells in unorganized regions and as both endothelial cells and smooth muscle cells in more organized areas. Because deep venous thrombi are the source for pulmonary thromboemboli, it is possible that partly organized venous thrombi that contain cells expressing significant amounts of PAI-1 could play a role in the resistance of the thrombi to lysis after their embolization. In a similar manner, the cellular expression of PAI-1 could foster the persistence of thrombi residing in the deep venous system.

Our observation that PAI-1 mRNA levels within the thrombi are not correlated with plasma PAI-1 antigen levels presents another situation in which tissue expression is not reflected by measurements in the vascular compartment. Factors generated during ongoing coagulation could stimulate PAI-1 expression in endothelial cells and smooth muscle cells and thus promote thrombus formation in a pathological cycle. Experiments directed at defining the factors and conditions that mediate the high level of PAI-1 expression in the cells that constitute chronic thromboemboli should contribute to our understanding of the biological basis for this disease.

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References


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