Elevated Expression of Urokinase-like Plasminogen Activator and Plasminogen Activator Inhibitor Type 1 During the Vascular Remodeling Associated With Pulmonary Thromboembolism

Irene M. Lang, Kenneth M. Moser†, Raymond R. Schleef

Abstract—Information is lacking on the mechanisms involved in the organization, resolution, and repair of the vascular lumen after acute pulmonary thromboembolism. Because recent data suggest that the balance between plasminogen activators (PAs) and type 1 plasminogen activator inhibitor (PAI-1) plays a role in regulating cell migration within the extracellular matrix, we investigated the expression of these molecules by immunohistochemical and in situ hybridization analysis of pulmonary artery specimens from patients suffering fatal pulmonary embolism. The data were compared with the expression of these molecules in both patients’ noninvolved pulmonary arteries and organ donor pulmonary arteries. Regions of initial organization and vascular remodeling were identified by a modified trichrome stain and by the presence of proliferating cell nuclear antigen (PCNA), a cell marker of proliferation. Staining for tissue-type PA antigen was low to undetectable in endothelial cells directly in contact with the fibrin-platelet thromboembolus and in areas in which the endothelial cell lining was replaced by cell growth into the thrombus. Urokinase-like PA (u-PA) expression was detected in mononuclear cells within the thrombus in the initial phase of thromboembolism and within cells migrating into the thrombus during the later stages of organization. PAI-1 expression was elevated in the monolayer of endothelial cells underlying the fresh platelet-fibrin thromboembolus and in a PCNA-positive cell population present between the pulmonary arterial intima and the thromboembolus that represents early organization. Increased expression of PAI-1 may play a role in inhibiting proteolysis and fostering the localization of the acute fibrin-platelet thrombus to the vascular wall, which is followed by the upregulation of u-PA in migrating cells during the reorganization process. (Arterioscler Thromb Vasc Biol. 1998;18:808-815.)

Key Words: thrombus organization ■ plasminogen activators ■ plasminogen activator inhibitor type 1 ■ pulmonary thromboembolism

Recognized venous thromboembolism, comprising both pulmonary embolism and deep venous thrombosis, leads to sudden death in about 10% of patients, accounting for about 300 000 clinical episodes and 50 000 deaths per year in the United States.1,2 The origin of thrombi embolizing to the lungs is in the great majority of cases the deep veins of the legs, particularly iliac, femoral, and popliteal veins.1 The morphological features of a fresh thromboembolus are similar to those of a nondetached thrombus, with alternating layers of platelets, fibrin, and erythrocytes.1 In addition to obstruction of the pulmonary trunk and/or both main pulmonary arteries, pulmonary embolism may progress slowly until the vascular bed has been reduced by multiple small pulmonary thromboemboli. In the latter situation, a single fresh thromboembolus suffices to cause acute right ventricular failure.

Resolution of pulmonary thromboemboli is usually derived by mechanical fragmentation, endogenous thrombolysis, or reparative organization through invasion with fibroblasts and capillary buds, which occurs in the majority of survivors, leaving behind intimal thickenings and bands,4 with restoration of normal pulmonary hemodynamics.5,6 In a small percentage of cases, pulmonary thromboemboli persist and undergo extensive organization, with subsequent obstruction of the pulmonary vascular bed and the development of pulmonary hypertension.7 These nonresolving occlusions are composed of patent, endothelial cell–lined neovascular structures within a smooth muscle cell/fibroblast/collagen-rich matrix.8 Presently, little information exists concerning the proteins synthesized by cells in the pulmonary artery during the early phase of resolution/organization of vascular thrombi.

Current data suggest that the plasminogen activation system plays a key role not only in the degradation of
blood clots but also in modulating the extracellular matrix (for review, see Reference 9). Plasminogen circulates as a proenzyme at high concentrations in the vasculature and is proteolytically converted into an active enzyme, plasmin, which is capable not only of degrading matrix components (eg, fibronectin and laminin) but also of activating matrix-degrading enzymes (eg, procollagenases and macrophage elastase). Local dissolution of the basement membrane is achieved by targeted proteolysis and is a prerequisite for cell migration within tissues and subsequent neovascularization. Recent data with transgenic mice have revealed that the physiological role of the two primary PAs are unique with regard to their role in vascular remodeling (for review, see Reference 10). More specifically, t-PA is a key regulator of plasminatic fibrinolysis, whereas u-PA appears to play a role in promoting cell migration required in the remodeling after endothelial cell injury. To prevent widespread activation of plasminogen, a series of serine protease inhibitors (serpins) appear to be utilized by cells to control the activation of plasminogen, with prevailing data suggesting that type 1 plasminogen activator inhibitor (PAI-1) is the primary regulator of t-PA and u-PA (for review, see References 11 and 12). For example, both clinical and experimental studies over the past few years have suggested an important role of PAI-1 in arterial and venous thrombosis and the maintenance of systemic vascular hemostasis (for review, see References 11 through 15). Moreover, experiments with transgenic mice deficient in PAI-1 support a role for this serpin in both vascular remodeling after arterial injury and the formation of pulmonary fibrosis that occurs after inflammatory injury. Sawa et al employed a rabbit model for acute carotid artery thrombosis to document the local upregulation of PAI-1 in endothelial cells juxtaposed to the thrombus and in smooth muscle cells adjacent to the neointima. Our group has observed high levels of this serpin in endothelial cells and smooth muscle cells within chronic nonreversing vascular thrombi. Taken together, these studies indicate a propensity for the upregulation of PAI-1 in endothelial cells and smooth muscle cells after acute arterial injury and thrombus formation, thus raising the possibility that elevated PAI-1 may play a role in the vascular remodeling after deposition of a thromboembolus. Therefore, this study was initiated to investigate the presence of t-PA, u-PA, and PAI-1 in the pulmonary arterial wall immediately adjacent to thromboemboli. For this purpose, we obtained specimens from patients suffering fatal pulmonary embolism and defined the expression of these three proteins using immunohistochemical and in situ hybridization approaches.

Selected Abbreviations and Acronyms
PAI-1 = plasminogen activator inhibitor type 1
PA = plasminogen activator
PCNA = proliferating cell nuclear antigen
t-PA = tissue-type PA
u-PA = urokinase-like PA
vWF = von Willebrand factor

Methods

Subjects
Paraffin-embedded autopsy specimens from six patients suffering acute pulmonary embolism at the University of California Medical Center were entered into the study. The patients were four females and two males (range: 36 to 71 years old; median: 51 years). The premonitory diagnoses were systemic hypertension, gallbladder carcinoma, myasthenia gravis, and postoperative states, eg, cholecystectomy and spinal surgery. In all cases, the patients suffered fatal pulmonary thromboembolism before thrombolytic therapy could be initiated (according to the attending physician or nurse and/or the notes in the patient records, this time span was less than 20 minutes). By special arrangement at autopsy, one of us (Dr Lang) was immediately notified, and samples from both involved and noninvolved regions of the pulmonary arteries were harvested and fixed in buffered formalin within 6 hours after death. Control tissues were obtained from brain-dead individuals at organ harvests. The experimental protocol for this study was approved by the University of California, San Diego Human Subject Investigations Committee (protocol 89-509).

Antibodies
Affinity-purified antibodies to PAI-1 were prepared using human PAI-1 bound to CNBr-activated Sepharose 4B as described previously. Rabbit antisera against human vWF, mouse monoclonal anti–human smooth muscle α-actin (clone 1A4), and anti–human monocyte/macrophage CD68 (clone KP1) were purchased from DAKO. Mouse anti–human PCNA was purchased from Oncogene (Uniondale, NY). Mouse anti–human u-PA (No. 3689) and rabbit anti–t-PA IgG (No. 1120) were purchased from American Diagnostica.

Histochemistry
To differentiate fresh thrombus from organized thrombus and to identify fibrin/fibrinogen and collagen, a trichrome stain was performed as described previously. Fibrin/fibrinogen stains reddish-blue, erythrocytes stain yellow, collagen stains green-blue, and cell nuclei appear dark blue in this technique.

Immunohistochemistry
Immunohistochemical staining was carried out using a three-step avidin-biotin-peroxidase method as described previously. In this procedure, sections of paraffin-embedded, paraformaldehyde-fixed tissues were incubated with primary antibodies (ie, affinity-purified rabbit anti–human PAI-1, 10 μg/mL; rabbit anti–human vWF, 5 μg/mL; rabbit anti–human α-actin, 5 μg/mL; mouse anti–human CD68, 5 μg/mL; mouse anti–human PCNA, 5 μg/mL; mouse anti–human u-PA, 10 μg/mL; rabbit anti–t-PA, 10 μg/mL; nonimmune rabbit IgG, 10 μg/mL; and nonimmune mouse IgG, 5 μg/mL), each diluted in 0.1% normal goat serum in Tris-buffered saline. The specimens were washed and incubated with the appropriate biotinylated secondary antibody (ie, goat anti–rabbit IgG or goat anti–mouse IgG (Zymed Laboratories, Inc) for 15 minutes. Subsequent incubations included a streptavidin–peroxidase conjugate, followed by the chromogen aminoethylcarbazole/hydrogen peroxide mixture (Zymed Laboratories) that results in a reddish-brown deposit indicative of positive immunoreactivity.

Riboprobe Preparation and In Situ Hybridization
An EcoR1-HindIII fragment of psP64 human u-PA (gift of Dr Jean-Dominique Vassalli, Geneva, Switzerland) containing nucleotides 1370 to 1985 was cloned into pGEM-3Z. This fragment and a 1085-bp fragment of PAI-1 in pGEM-3Z+ were in vitro transcribed in the presence of digoxigenin-UTP and digoxigenin-labeling mixture (Promega) according to the manufacturer’s instructions. For in situ hybridization, paraffin sections were pretreated as described previously. Hybridizations were performed by incubating (48°C, 16 hours) the sections with 20 μL of
prehybridization buffer containing 2.5 mg/mL t-RNA and 10 ng digoxigenin-labeled riboprobe. The sections were subsequently washed twice with 2× SSC, treated with RNase A (20 μg/mL in 500 mmol/L NaCl/10 mmol/L Tris-HCl, 30 minutes at 22°C), washed twice in 2× SSC, and then washed in 0.1× SSC/50% formaldehyde at 48°C for 2 hours. The sections were washed in 0.5× SSC, blocked, and incubated with 750 U/mL alkaline phosphatase–labeled anti-digoxigenin antibody (Boehringer

Table 1. Application of a Panel of Antibodies for the Analysis of the Endothelial Cell (EC) and Intimal (I) Layer of Nonthrombosed (NT) Patient Vessels in Comparison to the EC Layer and the Remodeling/Organization (R/O) Zone of Thrombosed (T) Vessels

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Regions of vascular remodeling/organization were identified by a modified trichrome stain as areas between the internal lamina and the peripheral thromboembolus (Fig 1I, small arrows). Immunoreactive cells are expressed as a percentage of 100 nucleated cells counted at 400× magnification.
Mannheim). Signal was developed using NBT/BCIP as substrate and hematoxylin as counterstain. Parallel sections were analyzed by using a sense probe as the control for nonspecific hybridization. For quantitative analysis of PAI-1 mRNA at the single cell level, the 1085-bp fragment of PAI-1 in pGEM-3Z was labeled with $^{35}$S-UTP and used for in situ hybridization experiments as described previously. Specimens were analyzed at 10 weeks' exposure using combined light/epiluminescence microscopy to permit simultaneous visualization of the sample and exposed silver grains. The latter appear as green dots and indicate the presence of mRNA. Quantitation of the in situ hybridization signal was done by counting silver grains associated with 100 nucleated cells in a particular region at 400× magnification using oil immersion.

**Results**

**Histological Features of Autopsy Lung Specimens From Patients Suffering Pulmonary Thromboembolism**

Trichrome stain analysis of thromboembolic obstructions in the main, lobar, segmental, and subsegmental pulmonary arteries (n=6) revealed two distinct morphological features. First, portions of occlusive thromboemboli, which were composed of layers of erythrocytes, platelets, and fibrin, were detected loosely attached to the luminal surface of the pulmonary arterial wall (Fig 1A and 1B, arrows facing each other). This technique results in a reddish-blue stain in the presence of fibrin/fibrinogen, whereas collagen is stained green. Second, areas of early thrombus organization and pulmonary artery vascular remodeling were identified in which the endothelial lining of the vessel was replaced by a zone of nucleated cells that appeared to be derived from the vessel's intima. These areas are indicated by the small arrows in Fig 1A and appear purple in the modified trichrome stain due to the abundance of dark-stained cell nuclei. The observation of different stages of thrombus organization suggests that several subclinical episodes of thromboembolism had occurred in the six patients under analysis.

**Immunohistochemical Analysis of the Single Layer of Cells Underlying a Pulmonary Thromboembolus**

Immunohistochemical analysis of the pulmonary arterial wall in contact with the thromboemboli revealed areas composed of a single layer of cells that stained positively for vWF (Fig 1C). Staining for α-actin (a marker for smooth muscle cells, Fig 1D) was detected in intimal cells distinct from those within the layer of vWF-positive cells (Fig 1C) that were immediately adjacent to the fibrin-platelet thrombus, whereas the monocyte/macrophage marker CD68 (Fig 1E) was detected primarily in cells scattered within thromboemboli. These latter cells appeared to stain weakly for u-PA (Fig 1F), whereas t-PA immunoreactivity was weakly detected in endothelial cells (Fig 1G). Quantification of the t-PA–positive endothelial cells in sections of thrombosed vessels with sections of nonthrombosed vessels did not reveal a significant difference (Table 1). In comparison, intense PAI-1 immunoreactivity was detected in the pulmonary arterial endothelial cells underlying the thrombus (Fig 1H).

**Immunohistochemical Analysis of the Patients’ Pulmonary Vasculature in Areas of Thrombus Organization**

Fig 1I through 1P shows representative photomicrographs of parallel sections of a pulmonary thromboembolus that appears to be undergoing organization by cells derived from the pulmonary artery (ie, lower magnification shown in A and highlighted by two small white arrows) and characterized by the replacement of the single layer of endothelial cells with a zone of nucleated cells that have migrated into the thrombus. This organization process is evidenced by the detection of smooth muscle cell α-actin in cells that have migrated into the thromboembolus (Fig 1I and 1K). Quantification of stained sections revealed that 45.7±3.8% of the cells within the regions of remodeling/organization stained positively for this marker (Table 1). Immunohistochemical staining for u-PA revealed a weak signal in stellate-shaped cells (Fig 1L) that represented 26.1±3% of the cells in this area (Table 1), whereas t-PA staining (Fig 1M) was low to undetectable, as only 2.6±2.1% of the cells within this area were positive for this protein (Table 1). In comparison, immunohistochemical analysis for PAI-1 (Fig 1N) revealed the presence of this protein (Table 1). In comparison, immunohistochemical analysis for PCNA,21,22 a marker for cell proliferation, revealed that 25±8.8% of the cells in this region reacted positively for this protein (Table 1, Fig 1O) compared with only 1.3±0.8% of PCNA-positive nuclei in the intimal layers of nonthrombosed patient pulmonary arteries (Table 1) (*P*=.0011, unpaired *t* test). Staining of parallel sections demonstrated that 28.3±4.4% of the cells were immunoreactive for CD68 (Table 1) in the area of remodeling/organization. vWF-positive endothelial cells (24.3±7.8%) were arranged in the form of capillary sprouts extending into the fibrin-platelet thrombus (Fig 1P).

**In Situ Hybridization Analysis for u-PA and PAI-1 mRNA**

Because the immunohistochemical data in Fig 1 and Table 1 suggest that levels for u-PA and PAI-1 antigen are increased in the patients’ thrombosed vessels, we used in situ hybridization to confirm that the steady state mRNA levels for these proteins was comparably elevated in these specimens.

**TABLE 1. Continued**

<table>
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<th>Percentage of Immunopositive Cells</th>
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radioactive in situ hybridization techniques revealed the presence of u-PA mRNA in mononuclear/macrophage-like cells at the periphery of the fibrin-rich thrombus adjacent to the single layer of endothelial cells (Fig 2A). Slight brown cytoplasmic staining that was observed in the sense control sections (Fig 2B) resulted from hemosiderin deposits within these cells. In areas of remodeling/organization, u-PA antisense probes revealed a positive signal in cells migrating from the pulmonary arterial margins into the thrombus (Fig 2C, arrowheads) in comparison with the signal detected with the sense probe (Fig 2D). PAI-1 mRNA was observed in both the single layer of cells lining the thrombus (Fig 2E) and in the cells within the remodeling/organization region (Fig 2F). Because this latter observation coupled with the immunohistochemical data in Table 1 suggested that PAI-1 expression was elevated in cells derived from the patients’ pulmonary arterial wall both at early times of thrombus deposition and subsequently during the remodeling process, we attempted to document this observation in a quantitative manner by using radioactive in situ hybridization protocols. Fig 2G indicates
that this technique also permitted the detection of PAI-1 mRNA in the single layer of cells in direct contact with the thromboemboli. Quantification of the exposed silver grains revealed that the steady state level of PAI-1 mRNA was significantly higher (129 ± 31 silver grains per nucleus in patient thrombosed pulmonary arterial endothelial cells, mean ± SD, unpaired t test, \( P = .0003 \)) than the number of exposed silver grains in cells obtained by analyzing a patient’s patent pulmonary artery branch (Table 2, 12 ± 7 silver grains per nucleus). Cells within the remodeling/organization zone also displayed an intense PAI-1 in situ hybridization signal (Fig 2H) using the PAI-1 antisense probe. Quantification of exposed silver grains in the area of remodeling/organization revealed 176 ± 51 silver grains per nucleus (n = 6) versus 0.27 ± 0.13 silver grains per nucleus (mean ± SD, \( P = .005 \), unpaired t test, Table 2) in patient nonthrombosed pulmonary arterial cells luminal to the internal elastic lamina. Background using sense probes for PAI-1 mRNA was low (eg, Fig 2J), as previously reported.8

**Discussion**

The present study demonstrates that the local expression of PAs and PAI-1 follows a distinct pattern after pulmonary thromboembolism. Staining for t-PA antigen was primarily detected in regions containing an intact endothelial lining regardless of the presence of fibrin, whereas u-PA expression was initially restricted to monocyctic cells within thromboemboli and subsequently in cells that appeared to be migrating from the vessel wall. The distribution of u-PA is in accord with the reported presence in monocytes/macrophages (for review, see References 23 and 24) and with the proposed role of u-PA in supporting cell migration (for review, See References 25 and 26). In comparison, PAI-1 was detected at elevated levels in endothelial cells directly in contact with fibrin and in a heterogeneous cell population migrating from the vessel wall into the thrombus.

Although this study is limited by its observational nature, the detection of enhanced PAI-1 expression in endothelial cells underlying pulmonary thromboemboli in contrast to the low expression of this protein in pulmonary endothelial cells at a distance from the thromboembolus raises the possibility that the mechanisms responsible for inducing PAI-1 were restricted to cells in the immediate local environment of the thrombus. Our observations also extend the data obtained from a number of in vitro or in vivo model systems concerning the interaction of endothelial cells with components of thrombi. For example, PAI-1 expression in endothelial cells has been shown to be elevated by thrombin (for review see Reference 27) and platelet lysates.28,29 One of the most potent stimulators of PAI-1 production in platelet lysates is transforming growth factor \( \beta \), a polypeptide growth factor stored in platelet \( \alpha \)-granules and released from platelets after their activation during thrombosis.30 PAI-1 expression induced by picomolar concentrations of TGF-\( \beta \) is rapid, with increases in its transcript being detected within 2 to 4 hours.31 The ability of compounds associated with vascular thrombi to induce PAI-1 expression has been extended by the study of Sawa et al32 in which thrombosis was induced by the insertion of intraluminal surgical silk sutures in the carotid arteries of rabbits. These investigators observed increased PAI-1 expression in all thrombosed vessels, with the earliest samples (ie, those processed 1 week after thrombus formation)33 revealing elevated PAI-1 mRNA within the neoendothelial cells overgrowing the vascular thrombi. Increased PAI-1 expression was also detected in smooth muscle cells adjacent to the neointima and in macrophages surrounding the suture material. In contrast, endothelial cells and smooth muscle cells in the adjacent vessels did not demonstrate an increase in PAI-1 expression. Thus, the observations described in our current study clinically extend the data obtained in this rabbit carotid artery model system. Experimental balloon injury is another model system in which PAI-1 expression has been found to be upregulated.32 Induction of PAI-1 mRNA can be detected within 3 hours after ballooning, reaching a peak at about 24 hours after the injury and persisting for weeks thereafter. A similar mechanism of vascular injury may be brought about by the embolizing thrombus, which, similar to a balloon, impinges on the endothelial cell surface after deposition. Furthermore, direct contact of endothelial cells with fibrin has been shown to modulate a number of phenotypic properties, including loss of organization, with severing of cell-cell contacts and retraction of individual endothelial cells.33–35

In addition to enhancing the production of PAI-1 in the single layer of endothelial cells lining the vessel wall, the deposition of a thromboembolus within a pulmonary vessel results in the generation of a new interface, which is subsequently penetrated by cells that are involved in the organization process. Our observation of not only elevated PAI-1 but also enhanced u-PA expression in areas of initial thrombus organization and increased cellular proliferation extends the data of Pepper and coworkers,36,37 in which the expression of these two proteins was observed to be upregulated in proliferating and migrating cells. The latter observations led these investigators38 to suggest that the proteolytic balance in the pericellular environment of migrating cells is regulated through the concomitant production of proteases and protease inhibitors. Enhanced PAI-1 expression, as well as accumulation of this serpin in the extracellular matrix, has also been

**TABLE 2. Steady State Levels of PAI-1 mRNA Determined by In Situ Hybridization Analysis of Patient Nonthrombosed (NT) and Thrombosed (T) Pulmonary Arteries**

<table>
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<th>R/O Zone</th>
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EC indicates endothelial cell and R/O, vascular remodeling/organization. R/O regions were identified by a modified trichome stain as areas between the internal and peripheral thromboembolus. Exposed silver grains were quantified by direct counting as described in “Methods.”

Discussion

The present study demonstrates that the local expression of PAs and PAI-1 follows a distinct pattern after pulmonary thromboembolism. Staining for t-PA antigen was primarily detected in regions containing an intact endothelial lining regardless of the presence of fibrin, whereas u-PA expression was initially restricted to monocyctic cells within thromboemboli and subsequently in cells that appeared to be migrating from the vessel wall. The distribution of u-PA is in accord with the reported presence in monocytes/macrophages (for review, see References 23 and 24) and with the proposed role of u-PA in supporting cell migration (for review, See References 25 and 26). In comparison, PAI-1 was detected at elevated levels in endothelial cells directly in contact with fibrin and in a heterogeneous cell population migrating from the vessel wall into the thrombus.

Although this study is limited by its observational nature, the detection of enhanced PAI-1 expression in endothelial cells underlying pulmonary thromboemboli in contrast to the
detected at the interface between the normal and altered tissue in a number of physiological or pathological situations. For example, increased PAI-1 mRNA levels are present in hepatocytes immediately adjacent to a surgical resection line,38 and enhanced deposition of PAI-1 has been detected in the extracellular matrix surrounding atherosclerotic plaques, which is believed to be one mechanism preventing the proteolytic digestion of these structures.39–42 In accord with this concept are observations of intense PAI-1 expression in endothelial cells lining neo-vessels within chronically organizing pulmonary thromboemboli, which are also likely to be in a proliferative stage.43 Furthermore, local hypoxia, which has been shown to be a trigger for PAI-1 upregulation,42,43 could be another factor responsible for the elevation of PAI-1 in the remodeling/organization regions within the thrombi.

Although the clinical scenario of extensive pulmonary embolism is that of rapid deterioration and death, it is known that significant hemodynamic changes in the pulmonary vasculature only occur after 50% of the vessels have been occluded.44 Because pulmonary thromboemboli are usually multiple in nature and are rapidly modified into an organized plaque by ingrowth of cells from the media together with capillary buds,3 the detection of PAI-1 mRNA in areas of remodeling/organization on the periphery of fresh fibrin/platelet-rich thrombi suggests that the majority of these obstructions were lodged into the patients’ vasculature before the event that led to clinical symptoms of pulmonary embolism.

Acknowledgments

This research was supported by fellowships from the Austrian grant FWF P10559-MED and Vermaechtnis Josefine Hirtl Zur Forderung Der Medizinischen Forschung (to Dr Lang) and in part by National Institutes of Health grants HL23584 (to Dr Lang) and in part by National Institutes of Health grants HL23584 (to Dr Lang) and in part by National Institutes of Health grants HL23584 (to Dr Lang). The technical support of Ms Terry Thines and Gertraud Lang, MD, is gratefully acknowledged. The EcoR-HindIII fragment of psp64 human u-PA was a gift of Dr Jean-Dominique Vassalli, Geneva, Switzerland. The authors would like to dedicate this article to the memory of Kenneth M. Moser, a great enthusiast of the field of pulmonary thromboembolism.

References


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Arterioscler Thromb Vasc Biol. 1998;18:808-815
doi: 10.1161/01.ATV.18.5.808

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