

# Fibrinolytic Balance of the Arterial Wall: Pulmonary Artery Displays Increased Fibrinolytic Potential Compared with Aorta

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**SUMMARY:** Classic pulmonary thromboembolism research has documented that large pulmonary thromboemboli lyse spontaneously, suggesting potent fibrinolytic activity in human pulmonary artery (Pa). This concept conflicts with published animal studies in which the proximal Pa was reported to be devoid of tissue plasminogen activator (t-PA) expression. The current study used *in situ* hybridization protocols to demonstrate t-PA expression in samples of human main Pa ( $n = 30$ ). Real-time PCR was used to demonstrate quantitatively that the levels of t-PA transcripts were higher than those of its primary regulator [ie, plasminogen activator-inhibitor 1 (PAI-1)] in the Pa samples. Immunologic and functional assays extended these observations by demonstrating that levels of t-PA antigen were higher than PAI-1 antigen, which resulted in the detection of free t-PA activity. This contrasted with the fibrinolytic balance of matched samples of aorta ( $n = 6$ ) in which PAI-1 transcripts and antigen values were higher than the corresponding t-PA values, and only M<sub>r</sub> 110 kDa t-PA-PAI-1 complexes could be detected in functional assays. To assess the relative fibrinolytic contribution of the endothelial cell layer, Pa endothelial cells and aortic endothelial cells were scraped and propagated in culture for  $20 \pm 6$  days. Pa endothelial cell-conditioned media revealed increased t-PA/PAI-1 antigen ratios. Taken together, our data indicate that the balance between t-PA and PAI-1 is shifted in human main Pa to favor net PA activity. (*Lab Invest* 2003, 83:871-876).

**P**ulmonary thromboemboli resolve in the majority of cases, leading to restoration of normal pulmonary hemodynamics (Dalen et al, 1969; Fred et al, 1966; Murphy and Bulloch, 1968; Paraskos et al, 1973; Tow and Wagner, 1967). Resolution may occur by mechanical fragmentation (Wagenvoort, 1995), through organization of the thromboembolus by invasion of capillary buds and fibroblasts leading to recanalization, or through endogenous thrombolysis. Venous thromboemboli fail to lyse in a only small percentage of cases, thereby resulting in chronic thromboembolic pulmonary hypertension (Moser et al, 1990). These data derived from early stages of pulmonary embolism research lead to the conclusion that the pulmonary circulation harbors remarkable capacity with respect to induction of thrombolysis (Dalen et al, 1966; Todd, 1972). Because proteases of the

fibrinolytic system are crucial to the degradation of pulmonary thrombi (Carmeliet et al, 1994), we speculated that, in humans, altered expression of tissue plasminogen activator (t-PA) accounts for this observation. However, data derived from animal studies document that t-PA is not expressed in the main pulmonary artery (Pa) (Levin and del Zoppo, 1994). To define the mechanism by which the pulmonary vascular bed is especially efficient for removal of thromboemboli, fibrinolytic gene expression was investigated in the proximal human Pa and contrasted with that in a systemic human artery of comparable size, the aorta (Ao).

## Results and Discussion

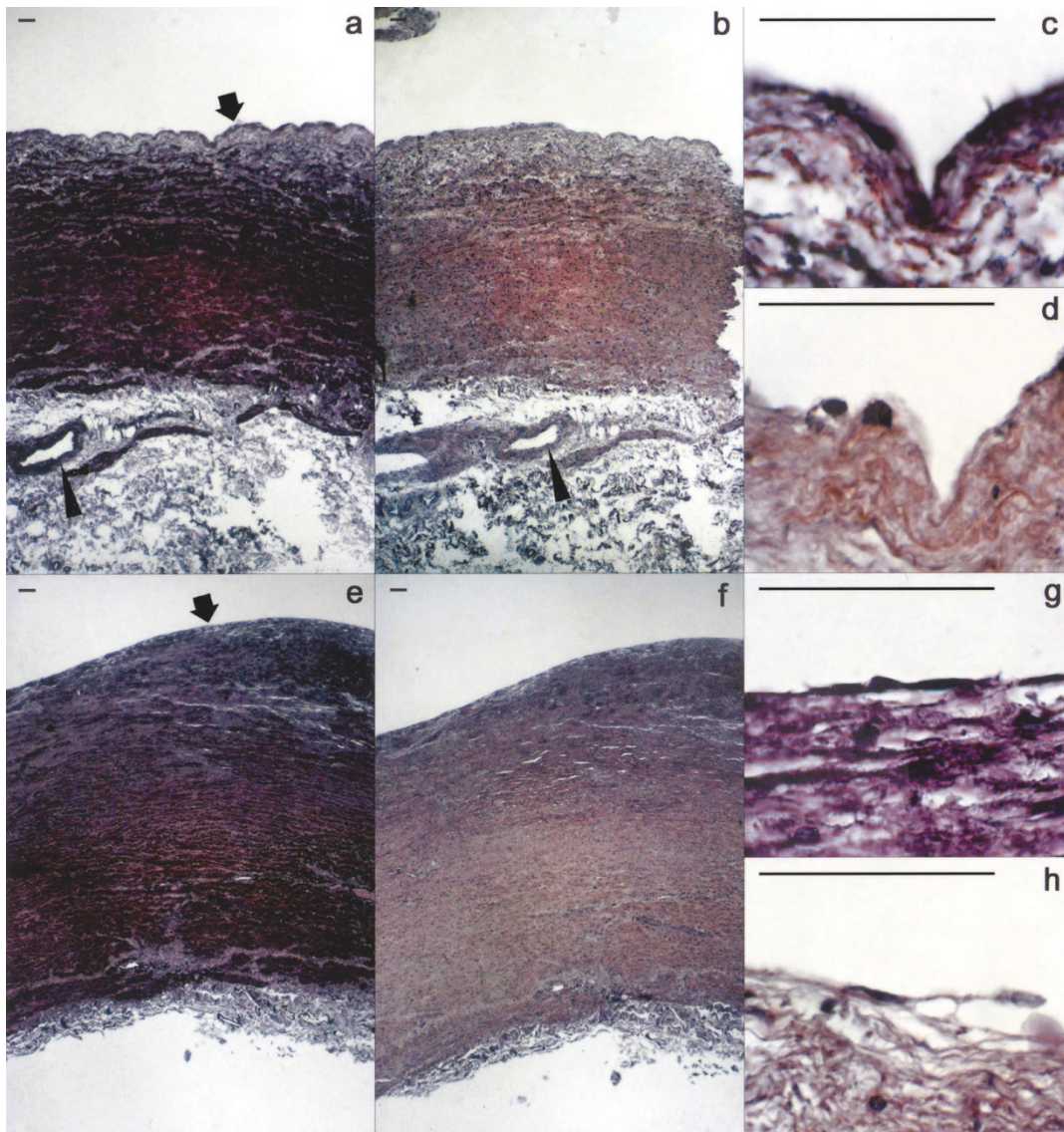
In contrast to animal models in which pulmonary vascular t-PA expression was shown to be confined to the endothelium of distinct vessels measuring 7.5 to 30  $\mu\text{m}$  in diameter (Levin and del Zoppo, 1994; Levin et al, 1997), our analysis revealed t-PA expression in endothelial cells (EC) and smooth muscle cells (SMC) of human main Pa (Fig. 1). In contrast to t-PA, urokinase-type PA is expressed only at low levels in proximal Pa and Ao of normal individuals, whereas it may be up-regulated under pathologic conditions (Lang et al, 1998). More specifically, *in situ* hybridiza-

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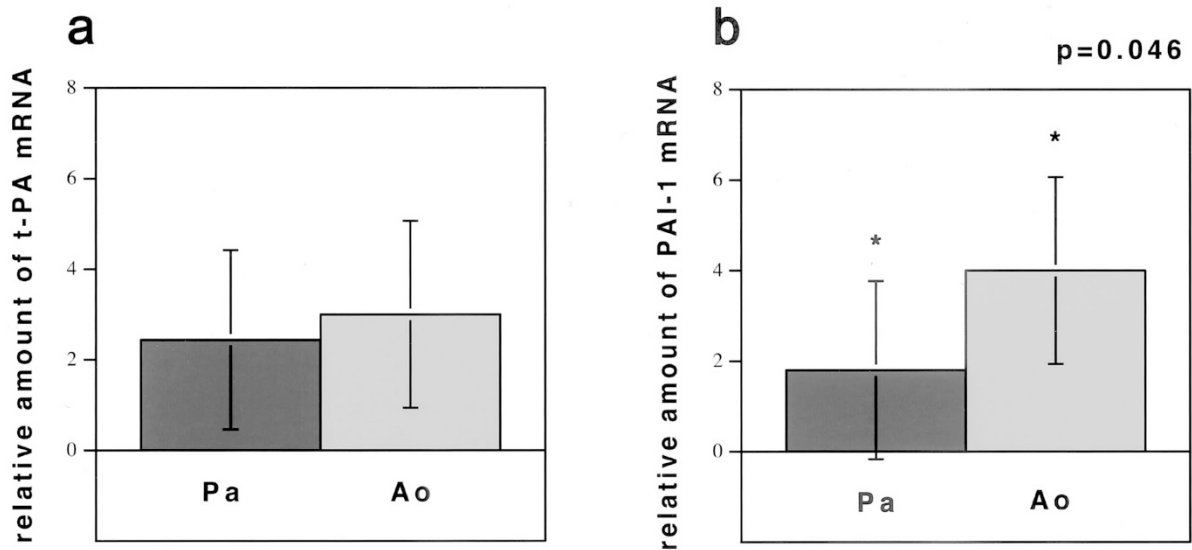


**Figure 1.**

Localization of tissue plasminogen activator (t-PA) mRNA by in situ hybridization analysis of pulmonary artery (Pa) and aorta (Ao) from the same individual. Purple immunoreactivity indicates positive hybridization signal. Sections have been counterstained with hematoxylin. A, t-PA expression in the Pa medial layer and within the adventitia in the muscular layer of the vasa vasorum, and for comparison, in the corresponding ascending Ao (e to h, arrowheads point to vasa vasorum; all  $\times 40$  magnification). b and f, Parallel sections hybridized with the sense control riboprobe. c, t-PA in situ hybridization signal localized within Pa endothelial cells (PaECs; arrow in a); g, a corresponding experiment demonstrating signal within aortic endothelial cells (AoECs; arrow in e). Control experiments using sense probes are shown in d and h ( $\times 1000$  magnification, oil immersion). Scale bars, 10  $\mu\text{m}$ .

tion with an antisense riboprobe for t-PA demonstrated expression in medial SMCs and ECs of Pa (Fig. 1, a and c) and Ao (Fig. 1, e and g). This pattern corresponds to t-PA expression detected by immunohistochemistry, with predominant staining of ECs and some weaker immunoreactivity of medial layer SMCs (data not shown). All these observations contrast with the view that during rat development, t-PA is present in the large arteries of both the thoracic and cranial cavities, including the dorsal Ao and Pa, but gradually disappears from larger vessels (Levin et al, 2000). Our data show that the Pa plasminogen activation system of humans is clearly different from that of nonhuman primates (Levin and del Zoppo, 1994), mice (Levin et al, 1998), and rats (Levin et al, 2000).

Results from several independent real-time PCR experiments (eight Pa and Ao whole wall vessel pairs) demonstrated that t-PA expression did not differ between the two vessels (Fig. 2a,  $p = 0.26$ ). However, plasminogen activator inhibitor-1 (PAI-1) expression was significantly lower in Pa compared with Ao (Fig. 2b,  $p = 0.046$ ). To substantiate these data, fibrin autoradiography was performed. Homogenates from Pa displayed a large zone of fibrinolytic activity, indicating free t-PA under these conditions. In contrast, a corresponding Ao sample demonstrated a zone of fibrinolysis known to be t-PA (Lang et al, 1993), as well as a t-PA-PAI-1 complex (representative examples shown in Fig. 3a). Western blot experiments under reducing conditions confirmed that specimen t-PA migrates at a



**Figure 2.**

Real-time PCR analysis of t-PA (a) and plasminogen activator inhibitor 1 (PAI-1) (b) gene expression in Pa and Ao isolated from the same individual, in comparison with glyceraldehyde-3-phosphate dehydrogenase gene expression. Defined dilutions of cDNA were amplified to determine the linear relationship between the threshold cycle (Ct) and the log starting copy number. In all experiments the correlation coefficients were between 0.98 and 0.99. Quantitation relies on the detection of Ct, which represents the cycle when a fluorescence signal exceeds the mean of background fluorescence plus 10 times the standard deviation in cycles 3 to 15. Plotting the log of the starting copy number of template molecules for several standard concentrations versus Ct reveals a linear correlation. The bars show the ratios between t-PA and PAI-1 Cts and glyceraldehyde-3-phosphate dehydrogenase Ct. Arbitrary units of the relative amount of the gene of interest are displayed on the y-axis. The differences illustrated in b were statistically significant. Error bars represent standard deviations.

lower position than t-PA standard (Fig. 3b). As expected, Ao and Pa do not display significant quantities of urokinase (Fig. 3c). Furthermore, differential protein analysis of six vessel pairs revealed an increase of more than 3-fold ( $p = 0.02$ ) in the t-PA/PAI-1 ratio of Pa compared with Ao (Fig. 4a). In view of these data, it was not surprising that there was lower PAI-1 activity in extracts from Pa compared with Ao (Fig. 4b,  $p = 0.04$ ). Within hours after pulmonary thromboembolism, a sequential up-regulation of t-PA and PAI-1 occurs in the wall of mainstem Pa (Lang et al, 1998). The elevated t-PA/PAI-1 ratio reflects decreased steady-state expression of PAI-1 in Pa, at the mRNA and protein level, with free t-PA rapidly accessible for thrombolysis in the Pa vessel wall and minimal or no formation of high  $M_r$  t-PA-PAI-1 complexes.

Because of the immediate impact of a thromboembolus on the EC surface (Lang et al, 1998), the contribution of the EC layer to the fibrinolytic balance is important. Therefore, t-PA and PAI-1 antigens were analyzed in conditioned medium from parallel cultures of the same donor. ECs adhered within the first 8 hours after plating, forming numerous small colonies, and reached confluence after  $19 \pm 5$  days [Pa endothelial cells (PaECs),  $n = 6$ ] and  $20 \pm 5$  days [aortic endothelial cells (AoECs),  $n = 6$ ]. A significant difference in t-PA antigen levels was observed between media conditioned by PaECs ( $7.5 \pm 2.5$  ng/ $10^5$  cells) and media conditioned by AoECs ( $3.9 \pm 2.2$  ng/ $10^5$  cells,  $p = 0.02$ ). In contrast, there was no difference in PAI-1 antigen ( $p = 0.37$ ) or PAI-1 activity levels ( $p = 0.32$ , Fig. 5). The increased secretion of t-PA protein may facilitate rapid fibrinolysis in response to acute pulmonary thromboembolism. In vitro, the fi-

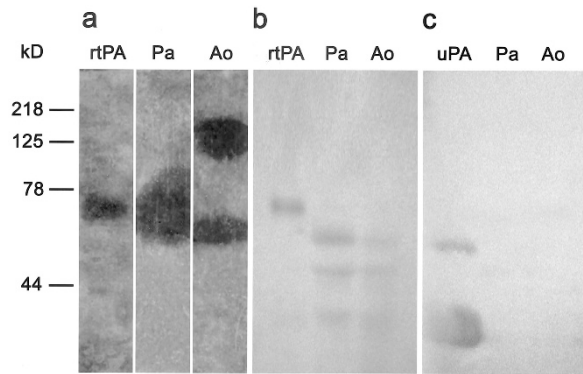
brinolytic system of ECs adopts unphysiologic expression patterns, eg, up-regulation of t-PA and down-regulation of PAI-1, paralleling the length of time in culture (Lang et al, 1994b). However, a comparison between two vascular compartments using primary cells with an identical genetic background during a short period of time in culture allows the assessment of differences in *in vivo* fibrinolytic gene expression (Lang et al, 1994b).

The data demonstrate that the main Pa wall harbors a unique profile of fibrinolytic gene expression. Enhanced fibrinolytic activity as a result of higher t-PA antigen concentration compared with PAI-1 antigen concentration and the presence of free t-PA suggests a basis for spontaneous lysis of emboli in the pulmonary trunk.

## Materials and Methods

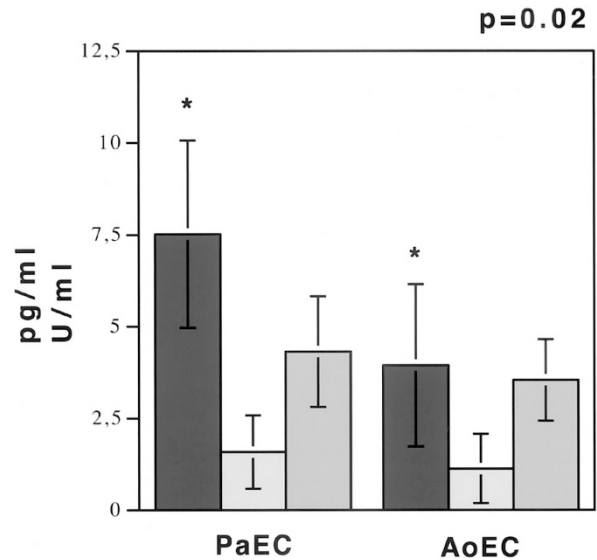
### Subjects and Collection of Specimens

Circumferential cuts of Pa trunk and ascending Ao, 1 to 2 cm above the respective semilunar valves, were harvested from donors for heart and lung transplantation. Before further experimental workup, the luminal sides of the vessels were inspected for signs of atherosclerosis, and if present, sample pairs were not included into the analysis. ECs were harvested by a gentle scrape with a no. 10 blade (Lang et al, 1994b). Patient age was  $51.1 \pm 17.7$  years (mean  $\pm$  sd). Mean pulmonary vascular resistance was  $216 \pm 90.4$  dynes. $\text{sec.cm}^{-5}$ . Confounding factors within the patients' clinical backgrounds were accounted for by comparing paired values of a single individual. A



**Figure 3.**  
 a, SDS-PAGE and fibrin autography of whole vessel extracts of a representative pair of Pa and Ao. The Biorad Kaleidoscope Prestained Standards 61–0234 were used for sizing of proteins. Recombinant t-PA (50 ng) was loaded in *lane rt-PA*. Ao protein extracts (5  $\mu$ g) (*lane Ao*) and Pa protein extracts (5  $\mu$ g) (*lane Pa*) were loaded. Photographs were taken after prolonged exposure at 37° C to favor visualization of t-PA–PAI-1 complexes. b, Western blot experiment using anti-t-PA to visualize 50 ng of recombinant human t-PA, 30  $\mu$ g of Ao protein extract (*lane Ao*), and 30  $\mu$ g of Pa protein extract (*lane Pa*). The additional lower bands are thought to represent cleaved t-PA. Under the given conditions, t-PA–PAI-1 complexes are not visualized. Negative controls in the absence of primary antibody did not show any bands. c, Western blot experiment using anti-urokinase-type PA to visualize 30,000 IU of human urokinase-type PA, 30  $\mu$ g of Ao protein extract (*lane Ao*), and 30  $\mu$ g of Pa protein extract (*lane Pa*).

total of 74 vessels were studied, including 6 matched pairs of Pa and Ao for protein analysis, 8 matched pairs of Pa and Ao for real-time PCR analysis, and 8 pairs of Pa and Ao from the same donor for parallel in situ hybridization experiments. In addition, 30 unmatched Pa specimens were used for in situ hybridization studies. Samples were obtained according to a protocol approved by the Institutional Review Board of the University of Vienna, Austria (EKZ 96/294). Both Pa and Ao

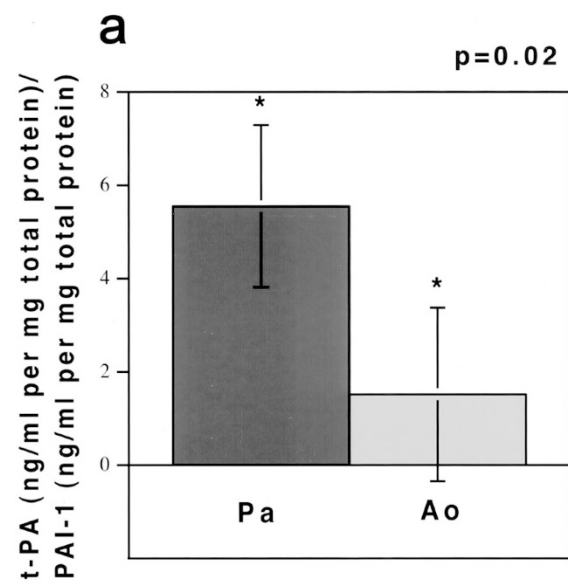


**Figure 5.**  
 Levels of t-PA, PAI-1 antigen, and PAI-1 activity in medium conditioned by unpassaged PaECs and AoECs from the same individual ( $n = 6$ ). ECs were grown to confluence in 20% fetal bovine serum, and 24-hour serum-containing conditioned media was prepared. t-PA and PAI-1 levels were measured by ELISA as described in “Materials and Methods” in conditioned media of primary ECs cultured under basal conditions. *Black bars* represent t-PA antigen levels, *light gray bars* represent PAI-1 antigen levels/100, and *gray bars* represent PAI-1 activity values.

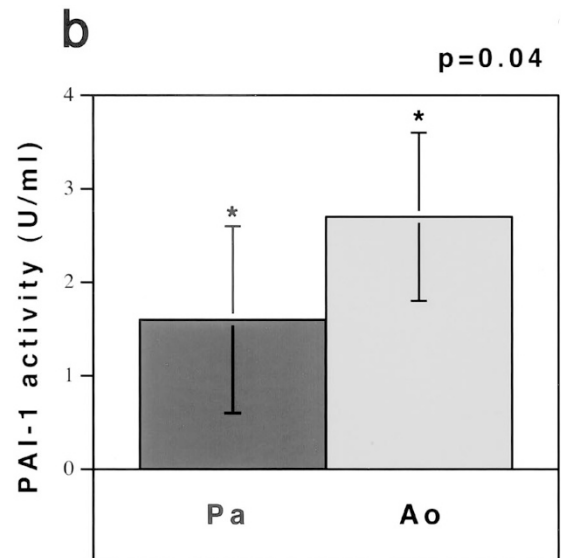
were analyzed from each patient using paired *t* tests. A *p* value < 0.05 was considered significant.

**Protein Extraction, ELISA Assays, SDS-PAGE, Western Blots, Fibrin Autography, and Tissue Culture**

Protein extraction was performed using a standard procedure. Tissues were harvested fresh and 1 gm of



**Figure 4.**  
 a, T-PA and PAI-1 antigen analyses of extracts of whole Pa and Ao wall isolated from the same individual ( $n = 6$  pairs). The bars represent t-PA–PAI-1 ratios. b, PAI-1 activity analysis of extracts of Pa and Ao isolated from the same individual ( $n = 6$  pairs).



whole vessel wall was homogenized in PBS (pH = 7.4) containing 0.1% Triton X-100 using a rotating blade (TissueMizer, IKA Laborortechnik, Germany). Homogenates were incubated at 4° C for 8 hours with gentle agitation. The extracts were centrifuged at 10,000 ×g (4° C, 20 minutes) and the supernatants subjected to a protein assay (BCA assay; Pierce, Rockford, Illinois). Equal amounts of protein were assayed by ELISA and fibrin autography. Samples were analyzed pairwise per patient and not pooled. For the measurement of t-PA, a two-site immunoassay was used as previously described (Lang et al, 1994b). PAI-1 activity was measured using immobilized t-PA to bind active PAI-1 as described (Christ et al, 1993). SDS-PAGE, Western blots, and fibrin autography were performed according to published protocols (Lang et al, 1993; Lang and Schleaf, 1996). Goat anti-human melanoma t-PA IgG (product no. 387) and human affinity-purified urokinase mAb against B chain (product no. 3689) were from American Diagnostica (Greenwich, Connecticut). ECs were grown to confluence and cultured as described in detail (Lang et al, 1994b). Medial SMC were not systematically analyzed in culture because of the difficulty in obtaining a representative cell population (Stenmark and Frid, 1998).

### Molecular Techniques

Total RNA was extracted from specimens stored in liquid nitrogen immediately after harvest in the operating room (Chomczynski and Sacchi, 1987). For reverse transcription, the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV; Boehringer Mannheim, Mannheim, Germany) was used.

To investigate the relative expression of t-PA mRNA in Pa, we used an RT-PCR protocol with Pa and Ao whole vessel sample pairs ( $n = 8$ ). Because quantitation of differential expression is only valid in the logarithmic phase of amplification, a real-time PCR assay was optimized. This RNA quantitation assay is based on the 5'-3' endonuclease activity of TaqDNA polymerase to cleave a TaqMan probe during PCR. PCR reactions are performed in the presence of three sequence-specific oligonucleotides. Two of them serve as conventional PCR primers for the amplification of target molecules. The third oligonucleotide, the TaqMan probe, contains a reporter dye at its 5' end and a quencher dye at its 3' end and hybridizes to the generated template. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter dye. The fluorescence emitted is directly proportional to the number of template molecules in the tube. The accuracy of this method is based on the determination of the threshold cycle (Ct) within the logarithmic phase of the amplification as opposed to the end point determination of conventional PCR. All PCR assays were performed (Heid et al, 1996) in duplicates and run on an ABI PRISM 5700 Sequence Detector System (Perkin Elmer, Foster City, California). Effective primer and probe combinations were as follows: PAI-1-740F (position 740-757), tgc-

ccatgatggctcaga; PAI-1-824R (803-824), gccagttc-caggatgctgtagt; PAI-1 probe (775-797), actgagttcac-cacgcccgatgg; t-PAF (933-955), tgacgtgggagtact-gttagtg; t-PAR (1019-1036), ggcatgctcggcgaaga; t-PA probe (961-982), ctgtccacactgcggcctgaga (all sequences 5' to 3').

In situ hybridization was performed as described (Lang et al, 1994a). An *EcoRI-HindIII* fragment of pGEM3Z human t-PA (a gift from Dr. Tor Ny, Uppsala, Sweden) containing nucleotides 1370-1985 was used (Edlund et al, 1983).

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