

# Association of a Plasminogen Activator Inhibitor (PAI-1) with the Growth Substratum and Membrane of Human Endothelial Cells

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**Abstract.** We have studied the distribution of the plasminogen activator inhibitor type 1 (PAI-1) in cultures of confluent human umbilical vein endothelial cells. Plasminogen activator inhibitor activity measured by the  $^{125}\text{I}$ -fibrin plate assay was detected in the cytosol ( $2.85 \pm 0.16$  U), 100,000 g particulate fraction ( $1.26 \pm 0.30$  U), and in the growth substratum ( $9.82 \pm 1.80$  U). Characterization of the protein responsible for this activity by reverse fibrin autography, immunoprecipitation, and immunoblotting demonstrated that it had an  $M_r$  of 46,000 and was antigenically related to PAI-1. Only the active form of the inhibitor was found in all three fractions. Inhibitor in the cytosol and particulate fraction converted to the latent form during 37°C incubation while the substratum inhibitor remained fully active. Extracellular PAI-1 was detected in the growth

substratum before its appearance in conditioned medium and represented the major protein deposited beneath the cells. The inhibitor was only transiently localized in the substratum, disappearing within 6 h and concomitantly appearing in the culture medium. Incubation of isolated metabolically labeled substratum with tissue plasminogen activator (tPA) resulted in the appearance and release of an immunologically related inactive 44,000  $M_r$  form as well as the tPA-PAI-1 complex (110,000  $M_r$ ). PAI-1 was also converted into its 44,000- $M_r$  form and released by treatment of the substratum with human leukocyte elastase. The rapid deposition and predominance of PAI-1 in the underlying compartment of endothelial cells may explain how the basement membrane is protected from proteolytic degradation by plasmin-generating enzymes.

CULTURED human endothelial cells produce and release an inhibitor of plasminogen activator (PAI-1)<sup>1</sup> that is found in a variety of other cell lines as well as plasma and platelets (1, 4-6, 8, 9, 15, 19, 24, 32). This inhibitor is both immunochemically and biochemically distinct from other known plasminogen activator inhibitors (7, 16, 30, 33-35) and is considered to be a primary inhibitor of both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The inhibitor is found in the conditioned medium of various cell lines in two forms: an active form, which inhibits tPA and uPA, and a latent form, which in its native state has no inhibitor activity but which can be converted to an active form by treatment with protein denaturants such as SDS (1, 12, 20, 31, 32). Greater than 98% of the extracellular PAI-1 of most cells tested is present in the latent form (1, 20, 23), although a melanoma cell line has recently been described in which the active form of this inhibitor predominates in the culture medium (36). In contrast, our studies using human endothelial cells have demonstrated that all of the intracellular inhibitor activity was in the active

form (20). Moreover, during incubation at 37°C the intracellular inhibitor activity declined but could be regenerated by treatment with SDS, suggesting that conversion to the latent form was taking place during incubation (20). Thus it has been suggested that the PAI-1 is synthesized as an active inhibitor but converts to the latent form during or after release from the cell.

It has been reported that the PAI-1 inhibitor is deposited beneath human lung fibroblasts (18) and fibrosarcoma cells (29) as well as secreted into the culture medium. We have investigated whether the PAI-1 is also associated with the growth substratum and/or other cell components of endothelial cells, whether it is active or latent, and whether it is stabilized in the active form. We report here that PAI-1 is associated with the membrane-containing particulate fraction and growth substratum of endothelial cells, as well as the cytosol, and that in all three cases the inhibitor is present in the active form.

## Materials and Methods

### Cell Culture

Endothelial cells were isolated from human umbilical cord veins as previously described (22) and were cultured on 12-well tissue culture dishes or T-75 culture flasks coated with 20 mg/ml calf skin gelatin (Eastman Chemical Products, Rochester, NY). Cells were grown to confluence in RPMI-

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1. *Abbreviations used in this paper:* PAI-1, plasminogen activator inhibitor type 1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

1640 (Whittaker/M.A. Bioproducts, Walkersville, MD) containing 20% FBS (Irvine Scientific, Irvine, CA), 200 U/ml penicillin, and 200 µg/ml streptomycin. Primary or secondary cell cultures were used in this study. Second passaged cells were grown in 50 µg/ml endothelial cell growth factor (Collaborative Research, Waltham, MA) and 90 µg/ml heparin (Sigma Chemical Co., St. Louis, MO). Cell density at confluence was  $\sim 6 \times 10^5/\text{cm}^2$ . All experiments were performed on cultures 1–3 d after the cells reached confluence.

### Fractionation of Cells and Isolation of Growth Substratum

Endothelial cell cultures were washed twice with cold  $\text{Mg}^{++}$ - and  $\text{Ca}^{++}$ -free Dulbecco's PBS and the cells were removed either intact or by detergent extraction. To remove the cells intact (13, 27), cultures were washed twice with 0.1% EDTA and then treated with 0.2 M urea (Bethesda Research Laboratories, Bethesda, MD) –0.1% EDTA in PBS. The cultures were placed on a rotator and incubated for 10 min at room temperature. The flask was hit sharply on a hard surface until the cells detached. The wells or flask were washed two more times with the EDTA–urea–PBS solution and the surface monitored visually to insure that all cells were removed. Cell viability as determined by trypan blue staining was 85% after treatment. Alternatively, cells were removed by extraction with Na deoxycholate as described (11). Briefly, cultures were exposed to 0.5% Na deoxycholate in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl three times for 10 min each at 4°C followed by three washes in 2 mM Tris-HCl, pH 8.0. Extraction solutions contained 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide, 10 mM EDTA, and 100 U/ml aprotinin. Substratum was removed from the plastic dish by adding 0.5% SDS in PBS (0.4 ml in a T-75 flask or 400 µl per well in a 12-well dish) and scraping with a rubber policeman.

Cells to be fractionated into soluble and particulate fractions after release by urea–EDTA–PBS were washed twice in PBS, suspended in 0.01 M Tris-HCl, pH 7.2, 0.015 M NaCl, and allowed to swell for 10 min at 4°C. Cells were homogenized with a Dounce homogenizer until no intact cells could be detected visually. The homogenate was cleared of large membrane fragments and nuclei by centrifugation at 600 g for 10 min twice. The particulate fraction was pelleted by centrifugation at 100,000 g for 15 min in an Airfuge (25 psi; Beckman Instruments, Inc., Palo Alto, CA) or through a 5% sucrose cushion for 60 min in a rotor (SW 50.1; Beckman Instruments, Inc.). The pellet was then washed with PBS and recentrifuged. Samples to be assayed for inhibitor activity were prepared in the absence of protease inhibitors, while samples used in immunoblot experiments contained the inhibitors listed above.

[<sup>35</sup>S]methionine labeling was performed by washing cultures twice with methionine-free DME (Irvine Scientific) and by adding the same medium containing 50 µCi/ml L-[<sup>35</sup>S]methionine (1320 Ci/mmol; Amersham Corp., Arlington Heights, IL) for the times indicated.

### Preparation of Antibodies to PAI-1

Polyclonal antibodies were prepared from purified human PAI-1 isolated from human endothelial cell-conditioned medium by immunoaffinity chromatography on a PAI-1 monoclonal antibody–agarose column. Monoclonal antibody (250 µg; Monozyme, Lyngby, Denmark) to PAI-1 was coupled to 1 ml Affigel-10 (BioRad Laboratories, Richmond, CA), the gel equilibrated in PBS, and 24-h conditioned medium passed through the column twice. The column was washed with 5 column vol of 1.5 M NaCl, 0.1 M sodium phosphate, pH 7.2, reequilibrated in PBS, and the inhibitor eluted with 0.1 M glycine-HCl, pH 2.5. The eluate was immediately neutralized with 1 M Tris-HCl, pH 8.0. The fractions containing PAI-1 activity were pooled, dialyzed against PBS, and concentrated by lyophilization. The sample was further purified by molecular exclusion chromatography on a 1 × 40-cm P30 column (BioRad Laboratories) in 0.05 M sodium phosphate, pH 7.2, 0.25 M NaCl. The product migrated as a single band on 9% SDS polyacrylamide gels and contained inhibitor activity as demonstrated by reverse fibrin autography.

Antisera against purified PAI-1 was raised in rabbits by subcutaneous injection of 50 µg PAI-1 in PBS emulsified in Freund's complete adjuvant. Booster injections of 50 µg were given at 2-wk intervals, and serum collected 1 wk after the third booster. The IgG fractions were isolated by chromatography on a protein A–Sephrose column and dialyzed against PBS.

### Enzyme Treatment of Isolated Substratum

[<sup>35</sup>S]methionine-labeled substratum isolated by urea–EDTA was treated with 10 µg/ml collagenase (40 U/µg; Calbiochem-Behring Corp., La Jolla,

CA) in 50 mM Tris-HCl, pH 7.2, 10 mM Ca acetate, 10 U heparinase (1,000–2,000 U/mg; Sigma Chemical Co.) in PBS, 0.05 U/ml chondroitinase ABC (Sigma Chemical Co.) in 40 mM Tris-HCl, pH 8.0, 80 mM NaCl, or 0.2 M heparin for 3 h at 37°C. tPA diluted into PBS containing 0.01% Tween 80 was added in increasing concentrations up to 1.25 µg/ml for 15 min. Human leukocyte elastase (10 µg/ml, 1,000 U/mg; provided by Dr. E. Plow, La Jolla, CA) treatment was for 15 min at 37°C, after which 2.5 mM elastatinal (an elastase inhibitor; Calbiochem-Behring Corp.) was added. After treatment the level of PAI-1 in the substratum and supernatant was analyzed by SDS-PAGE and autoradiography, and, when necessary, by laser densitometry.

### Assay of Fibrinolytic Activity and Inhibitor Activity

Fibrinolytic activity was assayed on [<sup>125</sup>I]-fibrin plates as previously described (21). PAI-1 activity was measured by adding increasing volumes of sample to a standard amount of tPA and measuring residual tPA activity on [<sup>125</sup>I]-fibrin plates. Human tPA was a gift from Dr. D. Collen (University of Leuven, Leuven, Belgium) and had a specific activity of 272,000 IU/mg protein. Percent residual activity versus sample volume was plotted on a linear graph and the number of inhibitor units was calculated from the volume of sample giving 50% inhibition of tPA activity. 1 U of inhibitor activity is that neutralizing 1 IU of tPA activity.

Inhibitor samples to be assayed were prepared as follows (20). To measure the effect of SDS on inhibitor activity in membrane and cytosol isolates, the samples were made 0.2% SDS and the mixture incubated at 37°C for 10 min. The samples were made 2% Triton X-100 and tPA added. Samples not treated with SDS were made 2% with Triton X-100 only. Substratum, which was removed from the plastic surface with 0.5% SDS, was also treated with 2% Triton X-100 and diluted five times in the same detergent. The presence of SDS and Triton X-100 had no effect on tPA activity.

### SDS-PAGE and Reverse Fibrin Autography

SDS-PAGE was performed by the method of Laemmli (17) with resolving gels of 5–15% and stacking gels of 4%. Molecular mass standards included myosin (200,000), β-galactosidase (116,000), phosphorylase B (92,500), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). Samples were boiled in 1% SDS in the presence or absence of 5% β-mercaptoethanol before electrophoresis.

Gels to be used for reverse fibrin autography were soaked in 5% Triton X-100 for 90 min, dried with a paper towel, and placed on a reverse fibrin autograph containing 1% agarose, 0.057 NIH U/ml thrombin, 2 mg/ml fibrinogen, 27 mg/ml plasminogen, 1% Triton X-100, and 0.56 IU/ml tPA. The agarose gel was prepared as previously described (10). Complete lysis occurred in  $\sim 3$  h.

Gels to be analyzed by autoradiography were stained with 0.1% Coomassie Brilliant Blue R-250 and 50% TCA, destained in 10% acetic acid, and then treated with autoradiography enhancer (EN<sup>3</sup>HANCE; New England Nuclear, Boston, MA).

### Immunoblotting and Immunoprecipitation

After electrophoresis, the samples in the gel were electrophoretically transferred onto a 10 × 15-cm sheet of nitrocellulose (Trans-Blot; BioRad Laboratories, Richmond, VA). Transfer buffer contained 25 mM Tris-glycine, pH 8.3, and 20% methanol; transfer was for 3 h at 3°C at 0.25 A and 80 V. After transfer the portion of the nitrocellulose sheet containing the standards was removed and stained with 0.1% Amido black in 45% methanol and 10% acetic acid. The remaining portion was exposed to a polyclonal antibody to human PAI-1 (10 µg/ml) as described (3, 14). The presence of antibody to the inhibitor was detected by the addition of affinity-purified goat anti-rabbit IgG (Zymed, South San Francisco, CA) labeled with [<sup>125</sup>I] (Amersham Corp.). Labeling was done with the chloramine T method (25) and 50 µCi of the labeled antibody was used per transfer.

To prepare samples for immunoprecipitation studies, substratum was removed from the flask with 500 µl 0.1% SDS and made 0.5% Triton X-100. A 1:10 dilution of anti-PAI-1 or nonimmune rabbit serum at the same concentration was added to samples containing 1% BSA (fraction V), 0.2 M NaCl, 1 mM PMSF, and 50 mM Tris-HCl, pH 8.0. Samples were incubated for 4 h at room temperature, precipitated with protein A–Sephrose (Sigma Chemical Co.), and analyzed by SDS-PAGE as previously described (19).

**Table I. Quantitation of PAI-1 Activity in Endothelial Cell Cultures**

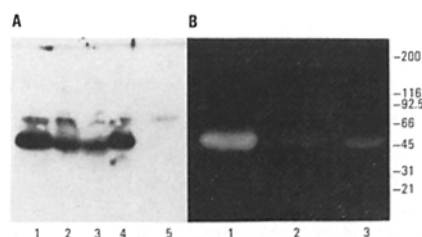
Compartment	Inhibitor activity
	<i>U</i> *
Cytosol	2.85 ± 0.16
100,000 g pellet	1.26 ± 0.30
Matrix (urea)	9.82 ± 1.80

Cell fractions were prepared by removing the cells from a T-75 flask with the EDTA-urea-PBS treatment described in Materials and Methods, followed by homogenization in 0.01 M Tris-HCl, pH 7.2, 0.015 M NaCl and centrifugation at 100,000 g. The substratum was removed with 0.5% SDS, made 2% Triton X-100, and diluted 1:5 in 2% Triton X-100-PBS. Inhibitor activity was determined by the <sup>125</sup>I-fibrin plate method described in Materials and Methods. \* Total inhibitor units in each fraction from one T-75 flask (5 × 10<sup>6</sup> cells).

## Results

### PAI-1 Activity in Endothelial Cells

The cytosol, membrane-containing particulate fraction, and growth substratum of endothelial cell cultures were assayed for PAI-1 activity by the <sup>125</sup>I-fibrin plate method (Table I). Cells released from the substratum by urea-EDTA treatment contained ~70% of the inhibitor activity in the soluble portion of the cell homogenate; the remaining activity was present in the membrane-containing fraction. The substratum from these cultures deposited by 24-h after confluence contained more than twice as much activity as the total cell-associated material (Table I). To insure that the substratum-associated inhibitor activity was not a contaminant from residual plasma membrane fragments remaining on the substratum after urea-EDTA treatment, substratum was also prepared using the Na deoxycholate-hypotonic buffer procedure (11) and inhibitor activity measured. [<sup>35</sup>S]methionine-labeled substratum isolated by either procedure showed band patterns that were identical with respect to both band number and intensity as determined by laser densitometry after SDS-PAGE and autoradiography (data not shown). Inhibitor



**Figure 1.** Reverse fibrin autoradiography and immunoblotting of endothelial cell cytosol, 100,000 g pellet, and substratum. Endothelial cell cultures were fractionated as described in Materials and Methods and reduced samples of cytosol, 100,000 g pellet (prepared from 4 × 10<sup>6</sup> cells), and substratum (one-fifth of the matrix produced by 4 × 10<sup>6</sup> cells 24 h postconfluence) were fractionated by SDS-PAGE on 5–15% gels. (A) PAI-1 was localized by immunoblotting with polyclonal antibody to PAI-1 isolated from human endothelial cells. (Lane 1) substratum; (lane 2) cytosol; (lane 3) 100,000 g pellet; (lane 4) conditioned medium (200 μl 24 h conditioned medium); (lane 5) substratum immunoblotted with nonimmune IgG. (B) Gels were analyzed for inhibitor activity by reverse fibrin autoradiography. (Lane 1) substratum; (lane 2) 100,000 g pellet; (lane 3) cytosol. *M<sub>r</sub>* markers (×10<sup>-3</sup>) are shown at right.

**Table II. Release of PAI-1 from Endothelial Cell Membrane**

Treatment	Pellet	Supernatant
	%	%
None	100	—*
3 M KCl	97	—*
10 mM EDTA	107	—*
6 M guanidine HCl	—*	91
5 mM Mg <sup>2+</sup> -Ca <sup>2+</sup>	104	—*
0.5% Triton X-100	—*	79
0.5% Na deoxycholate	—*	77
0.1 M Na carbonate, pH 11.5		55

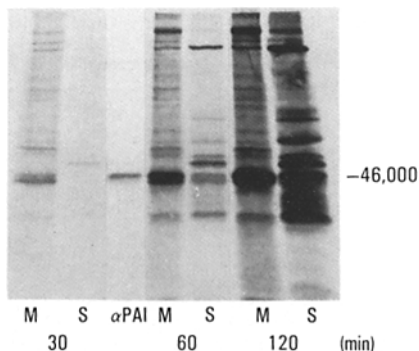
Cells were released from the flask by urea-EDTA-PBS and the membrane fraction prepared by homogenization as described in Materials and Methods. The membrane prepared from one flask of cells was treated with one of the reagents for 30 min at 23°C (KCl, EDTA, guanidine-HCl, and divalent cations) or 15 min at 4°C (Triton X-100, Na deoxycholate, Na carbonate). After treatment the membrane was pelleted by centrifugation at 100,000 g. Supernatants were dialyzed against PBS and the pellets extracted into 0.5% Triton X-100 in PBS. Inhibitor activity was determined by the <sup>125</sup>I-fibrin plate method and the number of inhibitor units in each case compared with control samples treated with PBS alone.

\* Undetectable by the assay used.

activity was also detected in Na deoxycholate-prepared substratum and was at similar levels in both samples. Inhibitor activity was also deposited beneath cells cultured directly onto plastic, as opposed to the gelatin layer normally used.

SDS-PAGE and reverse fibrin autoradiography of the substratum, cytosol, and particulate samples showed all inhibitor activities were present at an identical position on the gel corresponding to 46,000 *M<sub>r</sub>* (Fig. 1). Immunoblotting of these samples and conditioned medium with a polyclonal antibody against PAI-1 demonstrated the presence of the PAI-1 at the same position as the lysis-resistant zones. A second lighter band appearing at 60,000 *M<sub>r</sub>* also appeared in the nonimmune IgG controls.

The effect of various chemical treatments on the association of the inhibitor with the membrane-containing fraction and isolated substratum was determined (Table II). The association between inhibitor and cell membrane was not affected by 3 M KCl, 10 mM EDTA, or divalent cations. However, Triton X-100, Na deoxycholate, guanidine-HCl, or 0.1 M sodium carbonate, pH 11.5, did remove all detectable activity. To determine whether these same treatments affected substratum PAI-1, cultures were labeled with [<sup>35</sup>S]methionine, the substratum isolated, treated, and the substratum analyzed with the corresponding supernatant samples for the level of PAI-1 by SDS-PAGE and autoradiography. Except for guanidine HCl, which at the concentration used removed all of the proteins from the substratum, none of the treatments had a significant effect on the association between the inhibitor and the substratum (data not shown). We did find that acid treatment with 0.1 M glycine-HCl, pH 2.5, removed all of the labeled inhibitor within 1 min without detectably affecting the other proteins, although only 50% of the inhibitor activity remained after dissociation from the substratum by this procedure. Isolated substratum was also treated with collagenase, chondroitinase ABC, heparin, or heparinase to determine whether the inhibitor was associated with a specific component of the substratum. None of the enzymes tested altered the relative molecular mass or the amount of PAI-1 in the substratum.

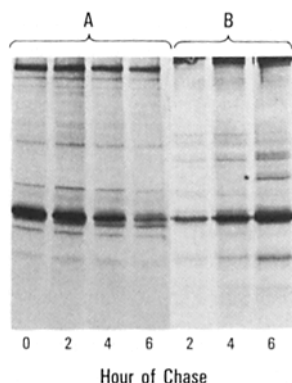


**Figure 2.** Accumulation and immunoprecipitation of PAI-1 in the substratum and conditioned medium. Endothelial cell cultures were grown to confluence and labeled with 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine for increasing periods of time. The cells were removed by urea-EDTA treatment as described in Materials and Methods and the substratum extracted with 400  $\mu\text{l}$  0.5% SDS. Samples to be used for immunoprecipitation were extracted with 0.1% SDS, made 1% Triton X-100, and treated with rabbit antiserum against human PAI-1 as described in Materials and Methods. All samples were fractionated under reducing conditions by SDS-PAGE on 5–15% gels followed by autoradiography,  $\alpha\text{PAI}$ , immune serum; lanes *M*, substratum; lanes *S*, conditioned medium.

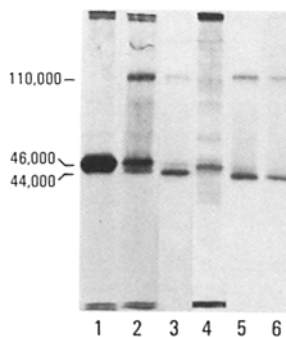
### Deposition of Turnover of Substratum-associated Inhibitor

Endothelial cell cultures were labeled with [ $^{35}\text{S}$ ]methionine for increasing amounts of time and the appearance of PAI-1 in the substratum and conditioned medium proteins was followed by SDS-PAGE and autoradiography (Fig. 2). Within 30 min after label addition two proteins having an apparent  $M_r$  of 46,000 had been deposited into the substratum (lanes *M*). Both of these proteins were first detected in the conditioned medium (lanes *S*) at 60 min. Immunoprecipitation of a 30-min substratum sample with anti-PAI-1 indicated that the slower migrating band was PAI-1. Immunoprecipitation of 60-min conditioned medium gave identical results. PAI-1 was increased rapidly and by 2 h composed the major component of the growth substratum and were also prominent in the conditioned medium.

Pulse-chase experiments were performed to determine the turnover rate of the inhibitor in relation to the other substratum-associated proteins. Cultures were labeled for



**Figure 3.** Pulse-chase analysis of substratum-associated PAI-1. Confluent cultures of endothelial cells were labeled with 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine for 2 h, washed free of label, and then incubated in label-free medium for 2, 4, or 6 h. Cells were removed by urea-EDTA treatment and the substratum (*A*) extracted with 400  $\mu\text{l}$  0.5% SDS, of which 200  $\mu\text{l}$  was analyzed under reducing conditions with the corresponding chase medium (*B*) on 5–15% SDS gels and autoradiography.



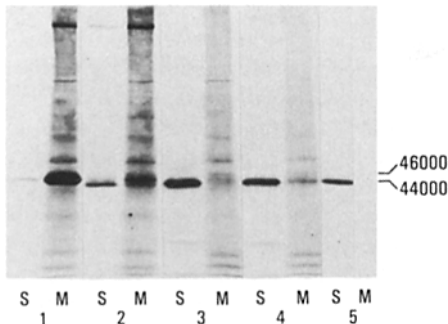
**Figure 4.** Effect of tPA on isolated substratum. [ $^{35}\text{S}$ ]methionine-labeled substratum prepared as described in Materials and Methods was incubated with 1.25  $\mu\text{g/ml}$  tPA for 5 or 15 min and the substratum and supernatant samples were analyzed by 5–15% SDS-PAGE under nonreducing conditions. (Lane 1) control substratum; (lane 2) substratum treated with tPA for 5 min; (lane 3) supernatant from lane 2; (lane 4) substratum treated with tPA for 15 min; (lane 5) supernatant from lane 4; (lane 6) supernatant immunoprecipitated with PAI-1 antibodies as described in Materials and Methods.

2 h, washed, and incubated with medium free of radiolabeled methionine for 2, 4, and 6 h (Fig. 3). Analysis of the substratum (Fig. 3 *A*) and chase medium (Fig. 3 *B*) by SDS-PAGE showed a decline in the PAI-1 associated with the culture vessel surface and a simultaneous increase of the inhibitor in the conditioned medium. The transfer of inhibitor to the medium was almost complete by 6 h. No other bands were significantly effected during the chase period.

### Effect of tPA and Elastase on Isolated ECM

To determine whether the substratum-bound inhibitor was susceptible to complex formation with tPA, increasing concentrations of tPA were added to radiolabeled substratum and the results analyzed by SDS-PAGE (Fig. 4). No change in the inhibitor band was observed when up to 500 ng/ml tPA was added (data not shown). However, after a 5-min incubation with 1.25  $\mu\text{g/ml}$  tPA the 46,000- $M_r$  inhibitor band was reduced and two additional bands at 110,000 and 44,000  $M_r$  appeared on the substratum (lane 2) and in the supernatant (lane 3). After 15 min only a fraction of the 46,000- $M_r$  inhibitor band remained on the substratum (lane 4) while the 110,000- and 44,000- $M_r$  bands were found only in the supernatant (lane 5). Immunoprecipitation of PAI-1 from the supernatant of tPA-treated substratum demonstrated that the 44,000- and the 110,000- $M_r$  bands contained the inhibitor. The inhibitor was not affected by tPA treated with diisopropylfluorophosphate, indicating that the changes were active site dependent. The addition of trasylol did not alter the results, thereby excluding the possibility that plasmin generated from contaminating serum plasminogen was responsible for the changes. Thus, the addition of tPA to the isolated substratum resulted in tPA-PAI-1 complex formation or conversion of the inhibitor to a faster migrating form with an apparent  $M_r$  of 44,000 followed by release into the supernatant.

Elastase, which is a major fibrinolytic protease of leukocytes (28), also had a significant effect on the inhibitor (Fig. 5). Within 30 min after elastase addition ( $4 \times 10^{-7}$  M) several of the bands present in control samples (lane 1 *M*) had disappeared. Most obvious was the disappearance of the band at the top of the gel migrating at the position of fibronectin and the PAI-1 46,000- $M_r$  band (lane 3 *M*). Concomitant with these changes was the appearance of two bands in the supernatant, one at  $\sim 30,000$   $M_r$  (the single band be-



**Figure 5.** Elastase treatment of isolated growth substratum. Confluent cultures of endothelial cells were labeled with [<sup>35</sup>S]methionine for 4 h and the substratum isolated by urea-EDTA. Elastase (10 µg/ml) or tPA (1.25 µg/ml) were added for 15 min at 37°C, and the substratum removed with 0.4 ml 0.5% SDS and analyzed under nonreducing conditions on SDS gels. Immunoprecipitation experiments were performed as described in Materials and Methods. (Lanes 1) control substratum; (lanes 2) tPA treated; (lanes 3) elastase treated; (lanes 4) elastase treated in the presence of 5 µg/ml anti-tPA antibodies; (lanes 5) immunoprecipitation with anti-PAI-1 antibodies. S, supernatant; M, substratum.

tween the tracker dye and PAI-1) and a second at 44,000 *M<sub>r</sub>* (lane 3 S). The latter band represented PAI-1 as demonstrated by immunoprecipitation with anti-PAI-1 antibodies (lane 5 S). Elastase treated with affinity-purified antibodies to tPA had an identical effect, eliminating the possibility that contaminating tPA was responsible (lane 4).

Previous reports have demonstrated that the decline in the apparent relative molecular mass of PAI-1 that occurs during incubation with tPA also results in an irreversible loss of inhibitor activity, i.e., PAI-1 that has no anti-tPA activity even after treatment with denaturants (26). To determine whether tPA and elastase-modified PAI-1 were also affected in this manner, isolated substratum was treated under the conditions described above and the resulting substratum and supernatant analyzed for activity by reverse fibrin autography. In both cases no lysis-resistant zones were observed in the lanes containing treated samples, indicating that the products of tPA and elastase treatment were inactive (data not shown).

#### Activity State and Stabilization of the Inhibitor

The effect of SDS treatment on the 100,000 *g* pellet and cytosol-associated inhibitor activity was determined (Table III). No significant change in the amount of inhibitor activity was observed after SDS treatment in either case, indicating that neither the particulate nor cytosol fractions contained detectable latent inhibitor. Incubation of the membrane-containing fraction and cytosol at 37°C resulted in similar loss of activity after 3 h to ~40% of that observed in control samples. In both cases, <90% of the original activity was restored by SDS treatment. Thus, the association of the inhibitor with particulate components did not alter the decline in inhibitor activity (20).

In contrast, no effect on substratum-bound inhibitor activity was observed after long-term incubation at 37°C. Endothelial cells were removed from [<sup>35</sup>S]methionine-labeled substratum by Na deoxycholate-hypotonic buffer extraction, the substratum washed, and the tissue culture dish incubated

**Table III.** Effect of 37°C Incubation and SDS on PAI-1

Treatment	Inhibitor activity			
	-SDS	+SDS	-SDS	+SDS
	<i>U</i>		% control	
Cytosol	2.45	2.60	100	100
Cytosol at 37°C (3 h)	0.96	2.58	39	99
Particulate fraction	0.30	0.27	100	100
Particulate fraction at 37°C (3 h)	0.12	0.25	42	93

Cytosol and membrane were prepared from cells released by EDTA-urea and homogenized as described in Materials and Methods. Samples were assayed for inhibitor activity by the <sup>125</sup>I-fibrin plate method after incubation at 37°C for 3 h and compared with samples left at 4°C. SDS treatment was with 0.2% SDS for 10 min followed by the addition of Triton X-100 to a final concentration of 2%. Cytosol was diluted threefold in 2% Triton X-100 and membrane samples were assayed neat. Values are the average of at least two determinations.

at 37°C for up to 6 h. tPA was then added to the substratum and the shift of the inhibitor to complex or to the smaller relative molecular mass species was followed. This treatment resulted in the complete removal of PAI-1 from the substratum and the appearance of both 110,000- and 44,000-*M<sub>r</sub>* bands in the supernatant in a manner identical to that observed after tPA addition immediately following cell removal (Fig. 4). Because tPA has no effect on the latent form of PAI-1, the inhibitor must have remained in the active form during the incubation period even though cytosol or membrane-bound inhibitor reverted to the latent form during the same time period.

#### Discussion

We have found that plasminogen activator inhibitor activity is associated with three compartments of endothelial cell cultures: the cytosol, the membrane-containing particulate fraction, and the growth substratum. All three fractions showed tPA inhibitor activity on reverse fibrin autographs, and immunoblotting analysis and immunoprecipitation studies indicated that this inhibitor activity was PAI-1 (endothelial cell type inhibitor).

Our observation that the PAI-1 inhibitor is found in the substratum is in agreement with reported studies using human lung fibroblasts (18) and fibrosarcoma cells (29). In both cases a radioactive band with an apparent *M<sub>r</sub>* of 46,000 was shown to contain PAI activity and PAI-1 antigen and immunofluorescent studies showed PAI-1 as a smooth, evenly distributed protein within the growth substratum. We have observed that PAI-1 is rapidly deposited into the substratum of human endothelial cells, preceding its entry into the culture medium, and represents a major component of the substratum. Most of the inhibitor is released from the substratum within 6 h after deposition, indicating that the inhibitor reaches the medium via the substratum and not directly from the cells. It is not apparent from the data whether all of the inhibitor released into the medium originates from the substratum or whether the endothelial cells secrete PAI-1 in a bidirectional manner. The release of the inhibitor is dependent on the presence of the cells, since incubation of substratum from which cells have been extracted does not result in a significant decline in PAI-1 levels (Levin, E. G., unpublished

observations). The rapid deposition of the inhibitor may play a role in protecting other matrix proteins from proteolysis by plasminogen activator-generated proteases.

Incubation of isolated substratum with tPA results in a depletion of the 46,000- $M_r$  band and the appearance of two major inhibitor-containing bands of 110,000 and 44,000  $M_r$ . After a 5-min incubation the two new inhibitor-containing bands are associated mostly with the substratum but also appear in the supernatant. After 10 min more both bands are found exclusively in the supernatant, suggesting that tPA modifies the inhibitor on the surface of the growth substratum and is followed by dissociation. It is possible that cleavage of the reactive center of PAI-1 by tPA reduces the affinity of the inhibitor for the component of the substratum to which it binds. In addition to modifying the inhibitor, incubation of the substratum with tPA appeared to reduce the total amount of the [ $^{35}$ S]methionine-labeled PAI-1 that could be recovered on SDS gels (Fig. 4). Most of this apparent loss could be explained by the separation of the COOH-terminal end of the inhibitor (residues 347-379), which contains 30% of the methionines (26). Aside from this, it is not obvious what is responsible for the rest of the discrepancy. We do not detect any low molecular weight (10,000 or greater) degradation products of PAI-1 after tPA treatment on 5-15% gels, nor do we detect a significant decrease in the total number of  $^{35}$ S counts per minute during any step of the experiment, thereby eliminating sample loss as the reason. We are now investigating whether further degradation of PAI-1 occurs during treatment with tPA.

The reduction in apparent inhibitor relative molecular mass and its irreversible inactivation after tPA addition is similar to that reported by Nielsen et al. (26) after addition of tPA or uPA to soluble SDS-treated PAI-1. However, in contrast to our observations, no tPA-inhibitor complexes were observed and all of the inhibitor was converted to the faster migrating species. Whether the difference in these results represents different fractions of the inhibitor, the effect of SDS, or different intermediate reaction products is not known.

Incubation of the substratum with elastase had a profound effect on the band pattern observed on acrylamide gels. Most obvious was the disappearance of the band representing fibronectin and the 46,000- $M_r$  PAI-1. The PAI-1 was converted to a form that was identical in mobility on SDS gels to that produced by tPA, and, like the latter, had no activity. Thus elastase, like tPA, has multiple effects on the substratum-associated inhibitor: modification to a faster migrating protein, irreversible inactivation, and release. Interestingly, attempts to modify the latent form of the inhibitor with elastase have failed; neither a change in relative molecular mass or irreversible inactivation are observed. Previous reports have demonstrated that incubation of elastase with  $\alpha_2$ -antiplasmin and C1 inactivator (2) results in a loss of functional inhibitor activity that corresponds to a limited proteolytic cleavage of each inhibitor. Such a cleavage would explain the reduction in the PAI-1 relative molecular mass seen after elastase addition and could be responsible for its release from the matrix. The inactivation of the inhibitor by elastase may contribute to the regulation of fibrinolysis and may also play a role in vivo in leukocyte invasion of the vascular wall.

With regard to the form and stability of the PAI-1 activity

we found that inhibitor associated with the membrane-containing particulate fraction was fully active, although association with the components of this fraction did not inhibit its conversion to the latent form, which occurred at a rate comparable to the cytosol inhibitor. Therefore, simple association with the insoluble cellular components is not capable of stabilizing PAI-1 activity unless it requires an intact cell environment. However, the association of the inhibitor with the substratum appeared to stabilize the inhibitor in a form that was susceptible to cleavage and complex formation with tPA. Because PAI-1 cannot be cleaved by, or form complexes with tPA in the latent form (26), it is reasonable to conclude that the substratum-associated inhibitor is fully active and that even after 6 h of incubation at 37°C no conversion to the latent form occurs. Thus the inhibitor is stabilized by its association with a component of the substratum. More work will be needed to identify the relationship of this inhibitor to the various components of the substratum and the mechanism by which conversion is inhibited.

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