The Majority of Type 1 Plasminogen Activator Inhibitor Associated with Cultured Human Endothelial Cells Is Located under the Cells and Is Accessible to Solution-phase Tissue-type Plasminogen Activator

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Abstract. The interactions between exogenously added tissue-type plasminogen activator (t-PA) and the active form of type 1 plasminogen activator inhibitor (PAI-1) produced by and present in cultured human umbilical vein endothelial cells (HUVECs) were investigated. Immunoblotting analysis of the conditioned media obtained from monolayers of HUVECs treated with increasing concentrations of t-PA ($\leq$10 μg/ml) revealed a dose-dependent formation of both t-PA/PAI-1 complexes, and of a 42,000-M_r cleaved or modified form of the inhibitor. Immunoradiometric assays indicated that t-PA treatment resulted in a fourfold increase in PAI-1 antigen present in the conditioned media. This increase did not result from the release of PAI-1 from intracellular stores, but rather reflected a t-PA-dependent decrease in the PAI-1 content of the Triton X-100 insoluble extracellular matrix (ECM). Although the rate of t-PA-mediated release of PAI-1 was increased by the removal of the monolayer, similar quantities of PAI-1 were removed in the presence or absence of the cells. These results suggest that the cells only represent a semipermeable barrier between ECM-associated PAI-1 and exogenous t-PA. Treatment of HUVECs with t-PA (1 μg/ml, 2 h) to deplete the ECM of PAI-1 did not affect the subsequent rate of PAI-1 production and deposition into the ECM. Immunogold electron microscopy of HUVECs not only confirmed the location of PAI-1 primarily in the region between the culture substratum and the ventral cell surface but failed to demonstrate significant (<1%) PAI-1 on the cell surface. Thus, the majority of PAI-1 associated with cultured HUVEC monolayers is present under the cells in the ECM and is accessible to solution-phase t-PA.

PLASMINOGEN activators (PAs)[1] play an important role not only during fibrinolysis but also in a variety of other physiological processes, including tissue repair, macrophage function, ovulation, embryo implantation, neovascularization, and malignant transformation (Astrup, 1975; Reich, 1978; Bachmann and Kruithof, 1984). Precise regulation of PA activity thus constitutes a critical feature of many biological systems. Such control may be at the level of the formation and resolution of fibrin itself (Hoylaerts et al., 1982), at the level of the interactions of PAs with cells (Vassalli et al., 1985), or through the action of specific PA inhibitors (PAIs; Erickson et al., 1985; Sprengers and Kluft, 1987; Loskutoff et al., 1988).

Although four molecules with PAI activity have been detected, the endothelial cell-derived inhibitor (PAI-1) (Sprengers and Kluft, 1987; Loskutoff et al., 1988) appears to be the physiologic inhibitor of tissue-type plasminogen inhibitor (t-PA). PAI-1 is a member of the serine proteinase inhibitor (Serpin) superfamily (Carrel and Travis, 1985; Ny et al., 1986, Pannekoek et al., 1986) and differs from other PAIs in its stability to SDS (van Mourik et al., 1984; Hekman and Loskutoff, 1985), its electrophoretic mobility (Erickson et al., 1986), and its ability to inhibit single-chain t-PA (Colucci et al., 1986; Hekman and Loskutoff, 1988). In addition, PAI-1 is synthesized as an active molecule but is unstable in solution and rapidly decays into a latent, inactive form at 37°C (Levin, 1986; Kooistra et al., 1986). Active PAI-1 appears to be stabilized through its binding to other proteins (Loskutoff et al., 1988). For example, PAI-1 has been detected in the extracellular matrix (ECM) of a variety of cultured cells (Laiho et al., 1986; Levin and Santell, 1987; Knudsen et al., 1987; Mimuro et al., 1987; Rheinwald et al., 1987; Pollanen et al., 1987; Knudsen and Nachman, 1988) and shown to be active (Levin and Santell, 1987; Mimuro et al., 1987, Knudsen and Nachman, 1988). The binding of PAI-1 to the ECM protects it from the spontaneous inactivation that occurs in solution, increasing its apparent half-life

1. *Abbreviations used in this paper*: CM, conditioned medium; ECM, extracellular matrix; IRMA, immunoradiometric assay; HUVECs, human umbilical vein endothelial cells; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PAI-1, type 1 plasminogen activator inhibitor; t-PA, tissue-type plasminogen activator.

from <3 h to >24 h (Mimuro et al., 1987). The binding protein in the ECM may be vitronectin (Mimuro and Loskutoff, 1989*a*,*b*). Active PAI-1 in plasma is also detected in a complex with another molecule (Wiman et al., 1988) shown to be vitronectin (DeClerk et al., 1988).

The observation that t-PA is able to bind to and form complexes with the PAI-1 associated with confluent human umbilical vein endothelial cells (HUVECs) has led to the concept that active PAI-1 is also present on the surface of these cells (Barnathan et al., 1988; Sakata et al., 1988). However, accurate interpretation of these results may be hindered by the large amounts of PAI-1 present in the ECM of these cells, especially if it is accessible to t-PA. In this report, we have studied the interaction between exogenous t-PA and the PAI-1 associated with intact cultured HUVECs. We show that the majority of this PAI-1 is present under the cells in the ECM and that it is accessible to solution-phase t-PA. Immunogold electron microscopy revealed that <1% of the PAI-1 was present on the dorsal cell surface of these cells.

Materials and Methods

Reagents

Reagents were obtained as follows: FCS, trypsin, penicillin, and streptomycin from Gibco Laboratories (Grand Island, NY); medium 199 (M199) from M. A. Bioproducts (Bethesda, MD); tissue culture plasticware from Corning Science Products (Corning, NY); endothelial cell growth factor from Biomedical Technologies, Inc. (Stoughton, MA); methionine-free media from Irvine Scientific (Irvine, CA); BSA, ovalbumin, chymotrypsinogen, phosphorylase b, β-galactosidase, Triton X-100, Tris base, casein, porcine intestinal heparin, purified goat IgG, and EDTA from Sigma Chemical Co. (St. Louis, MO); Con A–Sepharose and lysine-Sepharose from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ); glycine and XAR x-ray film from Eastman Kodak Co. (Rochester, NY); aprotinin (Trasylol) and bovine fibrinogen from Calbiochem-Behring Corp. (La Jolla, CA); affinity-purified goat anti-rabbit IgG from Miles Laboratories Inc. (Naperville, IL); Tween 80 from J. T. Baker Chemical Co. (Phillipsberg, NJ); carrier-free Na ^{125}I from Amersham Corp. (Arlington Heights, IL); nitrocellulose sheets from Schleicher & Schuell, Inc. (Keene, NH); purified fibronectin from Meloy Laboratories Inc. (Springfield, VA); solid-state lactoperoxidase-glucose oxidase and reagents for SDS-PAGE from Bio-Rad Laboratories (Richmond, CA).

Fibrinolytic Proteins

t-PA was purified from human melanoma cell-conditioned medium, and its activity was determined by the ^{125}I-fibrin plate assay as described previously (Schleef et al., 1985). The final product had a specific activity of ~500,000 U/mg of protein when compared with the t-PA International Standard and consisted primarily (>90%) of the single-chain form. The t-PA International Standard (83/517) and the urokinase International Standard (66/46) were kindly supplied by the National Institute for Biological Standards and Controls (London, Great Britain). Goat antiserum to t-PA was obtained from Bio-Pool (Hornefors, Sweden), and the immunoglobulin G fraction was prepared as described previously (Schleef et al., 1985).

Human PAI-1 was purified from the media conditioned by a transformed human lung fibroblast cell line (SV_{40} WI38 VA13 2RA) according to procedures described previously (Hekman and Loskutoff, 1988). PAI-1 was isolated primarily in a latent form and was activated by treatment with 4 M guanidine-hydrochloride (1 h, 37°C) followed by dialysis against PBS at 4°C (Hekman and Loskutoff, 1985, 1988). PAI-1 activity was quantitated by measuring its ability to inhibit t-PA mediated lysis of ^{125}I-labeled fibrin (Schleef et al., 1988*b*). 1 U of PAI-1 is defined as the amount required to inhibit the activity of 2 IU of t-PA by 50%. Antiserum to purified PAI-1 was raised in both New Zealand rabbits and White Lewis rats (Breeding Colony, Research Institute of Scripps Clinic) according to standard procedures. Rabbit antibodies to PAI-1 were affinity purified using human PAI-1 bound to cyanogen bromide-activated Sepharose 4B.

Growth and Treatment of HUVECs

HUVECs were isolated from 2–5 freshly collected umbilical cords, pooled, and grown in culture dishes coated with purified fibronectin (1 μg/cm^2). The growth medium consisted of M199 with 20% FCS, and was supplemented with endothelial cell growth factor (ECGF; 75 μg/ml), porcine intestinal heparin (90 μg/ml), and antibiotics (Schleef et al., 1988*a*). For experimental use, HUVECs were grown to confluency in 24-well Costar culture plates (Corning Science Products) and maintained at confluency for 3 d before use. To prepare conditioned media (CM), the monolayers were washed with 0.5 ml of serum-free M199 and then incubated in the presence or absence of t-PA (0–10 μg/ml) for up to 8 h in M199. The CM was centrifuged (10,000 *g*, 10 min, 4°C) and stored at −20°C until used. Cell lysates were prepared by washing the monolayers with cold PBS (0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4) and extracting the cellular components for 10 min at 37°C with 0.5 ml of PBS containing 0.5% Triton X-100. The lysates were removed and stored at −20°C until used. ECM was prepared by washing Triton X-100 extracted plates three times with distilled water to remove remaining cellular components. Light microscopic examination for the presence of cellular debris indicated that this extraction procedure completely removed visible cellular components from the culture dishes (Mimuro et al., 1987). ECM bound to the culture dishes was solubilized by the addition of 0.5 ml of SDS sample buffer.

Immunoradiometric Assays (IRMAs)

t-PA/PAI-1 complexes were measured in a two-site IRMA as described previously (Mimuro et al., 1987; Hekman and Loskutoff, 1988). Briefly, samples were diluted in IRMA buffer (PBS supplemented with 3% BSA, 5 mM EDTA, 0.1% Tween 80, and 0.02% NaN_3) and incubated for 1.5 h at 37°C in microtiter wells precoated with goat anti-t-PA (50 μl/well, 10 μg/ml). Plates were blocked with 3% BSA and after each incubation step, washed with PBS containing 0.1% BSA, 0.05% NaN_3, and 0.05% Tween 80. Bound PAI-1 was quantitated radiometrically after incubating the washed wells first with rabbit anti-PAI-1, and then with ^{125}I-labeled goat anti-rabbit IgG (10^5 cpm/well). The wells were individually removed, and the radioactivity in each was determined. PAI-1 antigen was quantitated by a similar two-site IRMA. In this case, affinity-purified rabbit antibodies to human PAI-1 (5 μg/ml in PBS) were coated onto microtiter wells (4°C for 16 h). After blocking the wells with 3% BSA and washing, test samples or purified PAI-1, diluted in IRMA buffer, were incubated in the antibody-coated wells at 37°C for 1.5 h. Bound PAI-1 was quantitated radiometrically by incubating the washed wells first with rat antiserum to human PAI-1 and then with ^{125}I-labeled goat anti-rat IgG.

Quantitation of ECM-associated PAI-1

PAI-1 antigen associated with the Triton X-100–insoluble ECM was quantitated immunologically either directly on the plate (Mimuro et al., 1987) or by solubilizing the ECM into SDS sample buffer and analyzing the samples by immunoblotting (described below). To quantitate ECM PAI-1 directly on 96-well microtiter plates, the ECM were incubated (4°C, 1 h) with M199 supplemented with 5 mg/ml normal goat IgG (M199-IgG) to block nonspecific IgG binding sites. The wells were then incubated (1 h, 37°C) with a primary antibody (10 μg/ml of either affinity-purified rabbit anti-PAI-1 or of normal rabbit IgG) diluted in M199-IgG. The plates were washed and the bound antibodies were detected with ^{125}I-goat anti-rabbit IgG (2×10^5 cpm/100 μl). After washing the wells with M199, the ECM was solubilized into SDS sample buffer and the radioactivity in each well was determined in a gamma counter.

Immunoblotting

The relative molecular mass species of PAI-1 antigen in the various samples was assessed by immunoblotting as described previously (Schleef et al., 1988*a*). Samples were first subjected to discontinuous SDS-PAGE (Laemmli, 1970), and then the proteins were electrophoretically transferred (50 V, 1.5 h) to nitrocellulose using a buffer containing 50 mM Tris base, 95 mM glycine, 20% methanol, and 0.01% SDS (Towbin et al., 1979). The nitrocellulose sheets were soaked in PBS containing 1% casein (PBS/casein) for 1 h at room temperature to block additional protein binding sites and then were incubated overnight at 4°C in PBS/casein containing rabbit antiserum to human PAI-1 (1:500 dilution). The nitrocellulose sheets were washed three times (10 min each wash) with PBS/casein and then incubated for 2 h at

room temperature with ^{125}I-labeled goat anti-rabbit IgG (250,000 cpm/ml). After washing, the nitrocellulose sheets were dried and exposed to XAR x-ray film for 24–48 h at −70°C.

Immunoelectron Microscopy

HUVEC monolayers in 24-well Costar plates were incubated (4°C, 1 h) with M199-IgG to block nonspecific IgG binding sites. The cells were then incubated (1 h, 4°C) with a primary antibody (10 μg/ml of either affinity-purified rabbit anti-PAI-1 or normal rabbit IgG) diluted in M199-IgG. The cells were washed and incubated (4°C, 1 h) with 10-mm-diam gold-conjugated goat anti-rabbit IgG (Janssen Life Sciences Products, Piscataway, NJ) diluted in M199-IgG. After washing the monolayers with M199, the cells were stored in cold Karnovsky's fixation buffer (1.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) until processed. The cells were briefly rinsed with PBS, postfixed with 1.5% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature, and dehydrated in a graded ethanol series. The wells were flat embedded in Epon 812 and after polymerization, stained en block with uranyl acetate.

To quantify the amount of anti-PAI-1 specific gold particles bound to the cell surface or culture substratum, four strips of 3 × 8 mm from each group were cut out from the Epon sheet containing cell monolayers. Two such strips from the same group were sandwiched together, cells apposed, with fresh Epon. Sections from these sandwiched blocks bearing two layers of cells were cut perpendicular to the culture substratum, double stained with uranyl acetate and lead citrate, and mounted on 100-mesh parallel lined grids (Ted Pella, Inc., Tustin, CA) so that both cell layers ran longitudinally between two grid bars. Quantification of gold particles on nucleated cells was carried out at 15,000× on a Hitachi 12-UA electron microscope. Only one section from each block was used for quantitative analysis. A total of eight blocks containing 16 different areas of cells was examined in each group. The density of gold markers is expressed as the number of particles per 25 nucleated cells from a representative section.

Miscellaneous

Protein concentration was determined by the method of Bradford (1976). Antibodies were enzymatically labeled with ^{125}I by using solid-state lactoperoxidase-glucose oxidase reagents and carrier-free Na ^{125}I. The ^{125}I-labeled goat anti-rabbit IgG had a specific activity of 2–5 × 10^6 cpm/μg protein.

Results

Effect of t-PA on the Distribution of PAI-1 in HUVECs

The interactions between exogenously added t-PA and endogenous PAI-1 associated with intact HUVEC monolayers were initially assessed by immunoblotting analysis. As several other investigators have reported (Kooistra et al., 1986; Barnathan et al., 1988; Sakata et al., 1988), the addition of t-PA to HUVECs in vitro resulted in the formation of t-PA/PAI-1 complexes of 100,000 M_r in the CM, and of a cleaved or modified form of PAI-1 of 42,000 M_r (Fig. 1, *A*). Neither the size nor the relative amount of PAI-1 antigen present in cell lysates (i.e., the Triton X-100–soluble fraction) of HUVECs was affected by t-PA treatment (Fig. 1 *B*). In contrast, t-PA caused a dose-dependent decrease in the PAI-1 content of the Triton X-100–insoluble ECM (Fig. 1 *C*). Approximately 10–20% of the total PAI-1 remained associated with the ECM even at the highest t-PA concentrations. This PAI-1 appeared to be resistant to t-PA since neither modified PAI-1 nor t-PA/PAI-1 complexes could be detected in the ECM by immunoblotting. Phase-contrast microscopic examination indicated that the HUVEC monolayers exhibited a normal cobblestone morphology in serum-free conditions for short periods of incubation. t-PA-treatment (≤10 μg/ml) did not affect HUVEC morphology or viability as indicated by staining with trypan blue. Furthermore, incubation of HUVEC monolayers with t-PA diluted in either serum-free media supplemented with 1% BSA/ECGF/heparin or in 20%

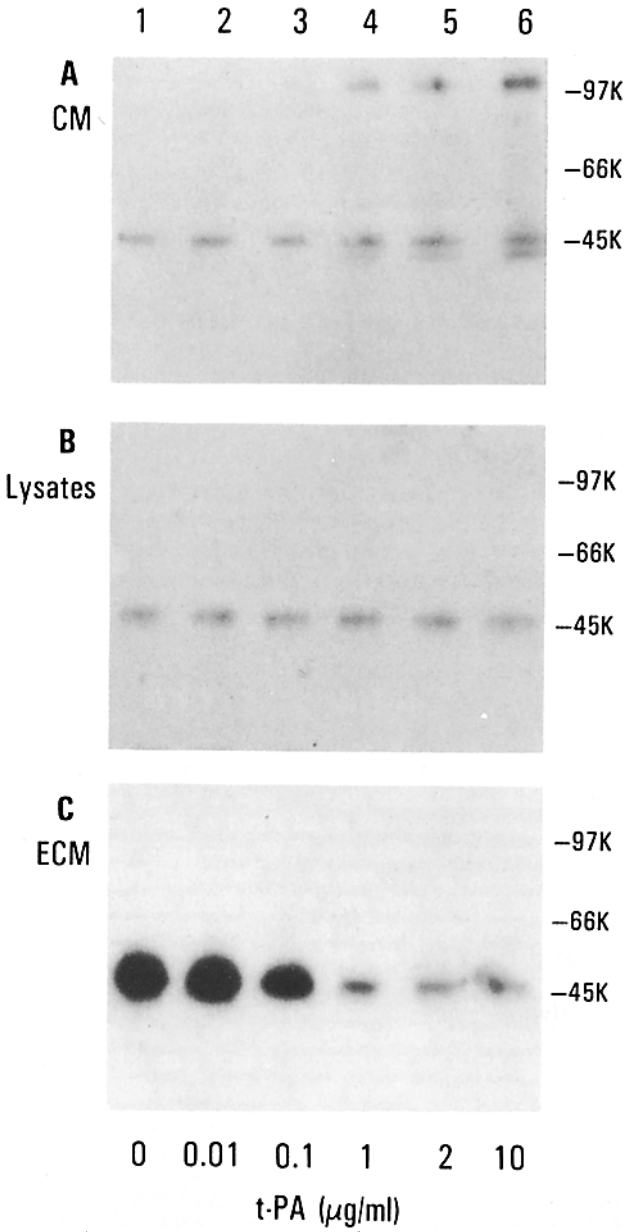

Figure 1. Immunoblotting analysis for PAI-1 in HUVECs treated with t-PA. Confluent monolayers were incubated (1 h, 37°C) in serum-free media supplemented with the indicated concentrations of t-PA (lane *1*, 0; lane *2*, 0.01 μg/ml; lane *3*, 0.1 μg/ml; lane *4*, 1 μg/ml; lane *5*, 2 μg/ml, lane *6*, 10 μg/ml). The CM were removed and the cells were lysed with 0.25% Triton X-100. After washing, the Triton X-100–insoluble ECM remaining on the plate was solubilized in SDS sample buffer. Samples (100 μl aliquots corresponding to 4 × 10^4 cells) of CM (*A*), cell lysates (*B*), and ECM (*C*) were fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and analyzed by immunoblotting using rabbit anti-PAI-1 followed by ^{125}I-goat anti-rabbit IgG. The results show the dried nitrocellulose sheets exposed to XAR x-ray film for 48 h. Positions of relative molecular mass standards are indicated.

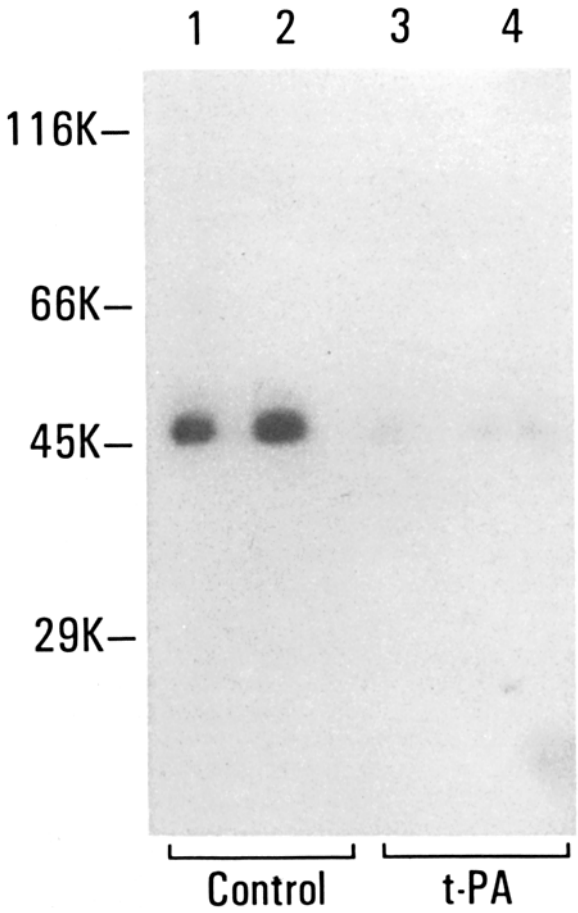

Figure 2. PAI-1 antigen present in ECM prepared by removing HUVECs with Triton X-100 or EDTA. Confluent monolayers were incubated with media alone (lanes *1* and *2*) or with media supplemented with 10 μg/ml t-PA (lanes *3* and *4*). After 1 h at 37°C, the monolayers were washed and ECM were prepared either by removing the cells by lysis with 0.25% Triton X-100 (lanes *1* and *3*) or by detaching the cells with 10 mM EDTA (lanes *2* and *4*). The resulting ECM preparations were washed three times with distilled water and extracted into SDS sample buffer. Samples (100 μl aliquots corresponding to 4×10^4 cells) were fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and analyzed with rabbit anti-PAI-1 followed by ^{125}I-goat anti-rabbit IgG. The dried nitrocellulose was exposed to XAR x-ray film for 24 h. Positions of relative molecular mass standards are indicated.

serum-containing growth media supplemented with 2 U/ml Trasylol resulted in t-PA/PAI-1 complex formation and depletion of PAI-1 from the ECM comparable to the data in Fig. 1 (data not shown).

A number of intracellular proteins are known to remain associated with the ECM after the extraction of cultured cells with detergents (Avnur and Geiger, 1981; Carter and Hakomori, 1981). To determine whether Triton X-100–insoluble intracellular proteins were the source of ECM PAI-1, we also prepared ECM by removing the cells intact with the divalent cation chelator, EDTA. Fig. 2 demonstrates that approximately equivalent amounts of PAI-1 were present in ECM prepared by either Triton X-100 treatment (Fig. 2, lane *1*) or by detaching the cells intact with EDTA (lane *2*). Furthermore, treatment of HUVEC monolayers with t-PA (10 μg/ml) removes the majority of the PAI-1 present in the ECM, independent of whether the cells were subsequently removed by treatment with Triton X-100 (Fig. 2, lane *3*) or with EDTA (lane *4*).

To quantitate the amount of complexes that are formed during the interaction of t-PA with HUVECs, a two-site IRMA was used. Control HUVECs secrete t-PA at a constant rate of ~0.25 ng/h per 10^5 cells and this t-PA forms complexes with endogenously released PAI-1 (Fig. 3 *A*). In contrast to these results, when exogenous t-PA is added to the cultures, there is an initial rapid rate of complex formation which is then followed by a slower more linear second phase (Fig. 3 *A*). Analysis of these samples in a two-site IRMA for PAI-1 antigen (Fig. 3 *B*) revealed a corresponding rapid, t-PA mediated increase in the inhibitor, followed by a second slower rate of PAI-1 accumulation. The initial rapid increase is again in contrast to the slower, relatively constant linear release of PAI-1 into the CM of untreated HUVECs which occurs at ~8 ng/h per 10^5 cells.

Ultrastructural Localization of PAI-1

Immunoelectron microscopy was used to more precisely localize the PAI-1 that is associated with intact HUVEC monolayers and is accessible to exogenous t-PA. Analysis of cross sections of nonpermeabilized HUVECs stained first with rabbit antibodies to PAI-1 and then with gold-conjugated goat anti-rabbit IgG revealed the presence of PAI-1 antigen primarily between the culture substratum and the underlying

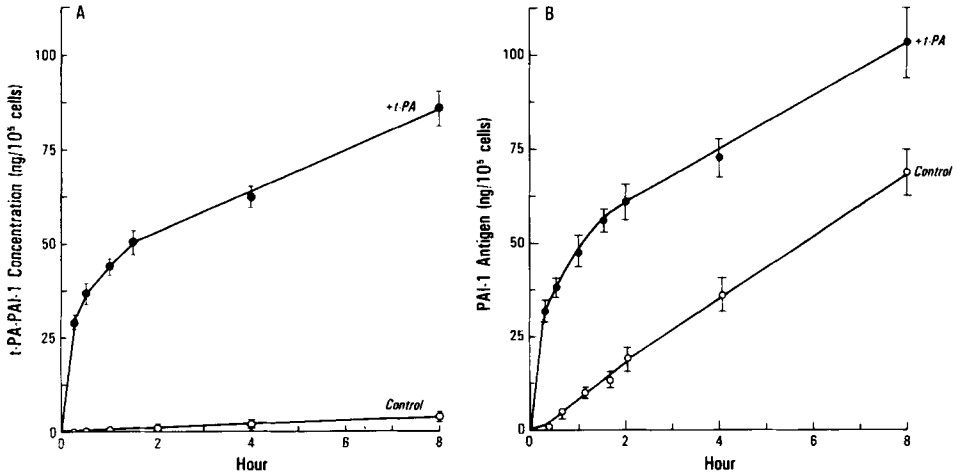

Figure 3. Influence of exogenous t-PA on the rate of t-PA/PAI-1 complex formation and PAI-1 antigen accumulation in the media conditioned by HUVECs. Confluent HUVECs were incubated at 37°C in media alone (○) or in media supplemented with 1 μg/ml t-PA (●). The resulting CM were collected at the indicated times and analyzed for t-PA/PAI-1 complexes (*A*) and PAI-1 antigen (*B*) by two-site IRMAs as described in Materials and Methods.

Table I. Quantitation of PAI-1-specific Immunogold Particles Associated with HUVEC Monolayers

Pretreatment	Primary antibody	Immunogold particles per 25 cell lengths: Dorsal surface	Immunogold particles per 25 cell lengths: Ventral surface/ culture substratum
M199	Rabbit anti-PAI-1	13	1,706
t-PA in M199	Rabbit anti-PAI-1	7	485
M199	Nonimmune rabbit IgG	6	19

Confluent HUVEC monolayers were incubated (1 h, 37°C) in M199 in the presence or absence of 5 μg/ml t-PA. The cells were washed and incubated (1 h, 4°C) with 10 μg/ml of affinity-purified rabbit antibodies to PAI-1 or nonimmune rabbit IgG followed by gold-conjugated goat anti-rabbit IgG. The cells were fixed and processed for transmission electron microscopy as described in Materials and Methods. Data shown are from a representative section and expressed as the total number of immunogold particles detected on the dorsal and ventral cell surfaces of 25 cells displaying nuclei, representing 800–1,000 μM of linear culture substratum surface.

ventral cell membrane (Fig. 4 *A*). Quantitation of the total gold particles distributed over 25 nucleated cell lengths (~800-1,000 μM linear length) confirmed the presence of PAI-1 beneath the ventral cell surface (Table I), and demonstrated that <1% of the total gold particles were present on the dorsal cell surface membrane. Incubation of HUVECs with t-PA (5 μg/ml; 1 h, 37°C) before immunoelectron microscopic localization of PAI-1 significantly decreased the number of gold particles detected both beneath the monolayers (Fig. 4 *B*; Table I), and on the dorsal cell surface (Table I). In control experiments, few gold particles were detected in cross sections of HUVECs that were incubated with normal rabbit IgG followed by gold-conjugated goat anti-rabbit IgG (Fig. 4 *C*; Table I).

Kinetics of the Interaction between t-PA and ECM-associated PAI-1

The above observations indicate that the majority of HUVEC-associated PAI-1 is present in the ECM. Moreover, the PAI-1 in the ECM beneath HUVEC monolayers appears to be accessible to solution-phase t-PA. Kinetic experiments were performed in the presence or absence of the cell monolayer to further investigate the influence of the cells on the accessibility of this population of PAI-1. If the monolayers were first removed, the t-PA-mediated decreases in ECM PAI-1 were quite rapid (Fig. 5), with >70% of the PAI-1 in the ECM being removed within 15 min with 1 μg/ml of t-PA. Immunoblotting analysis of the supernatants from the t-PA-treated ECM revealed the presence of t-PA/PAI-1 complexes and of degraded PAI-1 (data not shown) similar to the PAI-1 profile obtained by t-PA treatment of intact HUVEC monolayers (Fig. 1 *A*). The rate of PAI-1 removal from the ECM was considerably slower in the presence of cells. For example, 1 μg/ml t-PA removed only 25% of the PAI-1 from the ECM beneath HUVEC monolayers within 15 min, and took ~75 min to remove 70% of it. Treatment of HUVEC monolayers with low concentrations of t-PA (0.25 μg/ml) only partially depleted PAI-1 from beneath the monolayers.

Effect of t-PA on the Production of PAI-1 by HUVECs

The production of PAI-1 is regulated by a wide variety of biologically important compounds and conditions (Loskutoff et al., 1988). Experiments were therefore performed to determine if the t-PA treatment itself and/or the depletion of PAI-1 from the ECM alters the rate of PAI-1 production. HUVECs were treated with t-PA for 2 h to remove PAI-1 from the ECM and then were incubated in the presence of [^{35}S]methionine for various times. Analysis of the resulting samples by SDS-PAGE and autoradiography failed to demonstrate significant differences in the amount of PAI-1 deposited into the ECM of control and t-PA-treated HUVECs (Fig. 6). Immunoprecipitation of the ^{35}S-labeled PAI-1 present in the cell lysates or accumulating in the CM over this time period, confirmed these results (data not shown). Thus, t-PA treatment of HUVECs did not affect the subsequent rate of PAI-1 production.

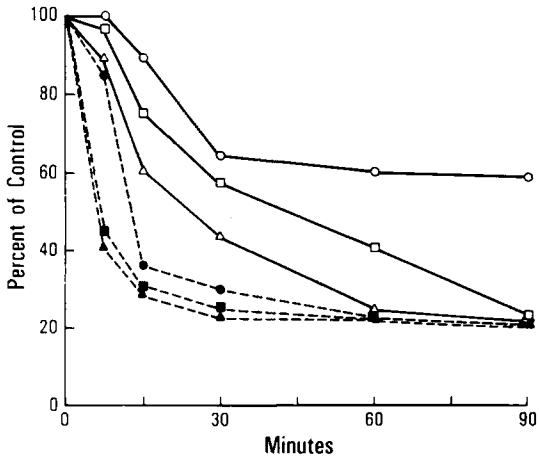

Figure 5. Influence of the cell monolayer on the rate of interaction between exogenous t-PA and PAI-1 in the ECM. Intact HUVEC monolayers (*open symbols*) or ECM preparations (*solid symbols*) were incubated with 0.25 (○, ●), 1 (□, ■), or 5 (△, ▲) μg/ml t-PA in serum-free media. At the indicated times, the plates were washed and the monolayers were lysed with 0.5% Triton X-100. PAI-1 associated with the ECM was then quantitated by incubation (1 h, 37°C) with affinity-purified rabbit antibodies to PAI-1 followed by ^{125}I-goat anti-rabbit IgG. Data points represent the average of duplicates and are expressed as a percentage of control samples incubated in serum-free media in the absence of t-PA.

Discussion

Biochemical studies of ECM prepared by standard techniques using nonionic or zwitterionic detergents demonstrate that PAI-1 is associated with the ECM from a variety of cultured cells. However, the extraction of cells with detergents results in an ECM preparation that may also contain a number of insoluble cellular components (Avnur and Geiger, 1981; Carter and Hakomori, 1981). These considerations raise the possibility that PAI-1 is not an integral component of the ECM, but rather a contaminating cellular component associated with insoluble surface or cytoskeletal elements. In this report, we have used procedures that do not suffer from this problem to study PAI-1 in the ECM of HUVECs. For example, the cells were removed intact from the underlying ECM by incubation with the divalent cation chelator, EDTA. Similar quantities of PAI-1 were detected in these ECM preparations and in ECM prepared by removing HUVECs by lysis with Triton X-100 (Fig. 2), suggesting that PAI-1 is actu-

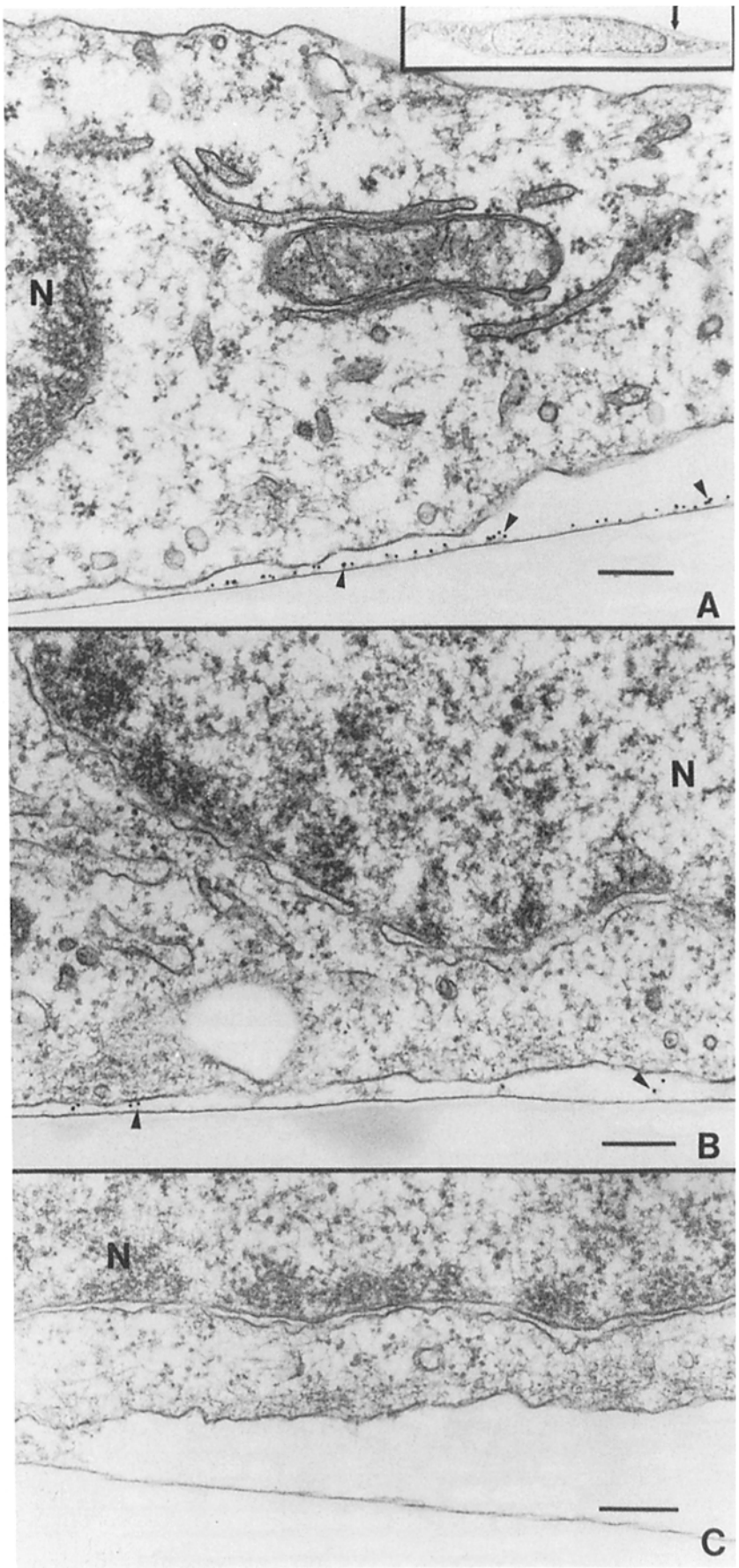

Figure 4. Immunoelectron microscopic localization of PAI-1 in intact nonpermeabilized HUVECs. HUVEC monolayers in 24-well Costar plates were incubated (1 h, 37°C) in serum-free media either in the absence (*A* and *C*) or presence (*B*) of 10 μg/ml t-PA. The cultures were washed, blocked with M199-IgG, and incubated (1 h, 37°C) with 10 μg/ml of either affinity-purified rabbit antibodies to PAI-1 (*A* and *B*) or normal rabbit immunoglobulins (*C*) in M199-IgG. Washed cells were incubated (4°C, 1 h) with gold-conjugated goat anti-rabbit IgG and then processed for electron microscopy as described in Materials and Methods. *N*, nucleus; *arrowheads*, immunogold particles. (*A*, *inset*) Low magnification (2,000×) of HUVEC with arrow indicating approximate area enlarged in *A*. Bars, 250 nm.

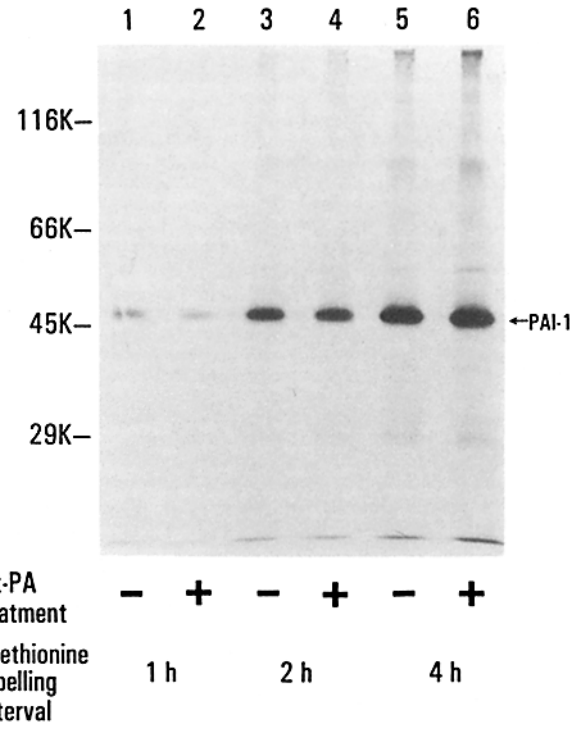

Figure 6. Effect of t-PA-pretreatment on the deposition of [^{35}S]-methionine-labeled proteins into HUVEC ECM. HUVECs were incubated (2 h, 37°C) in the absence (lanes *1*, *3*, and *5*) or presence (lanes *2*, *4*, and *6*) of 1 μg/ml t-PA. The monolayers were washed and incubated with [^{35}S]methionine for 1 (lanes *1* and *2*), 2 (lanes *3* and *4*), or 4 h (lanes *5* and *6*) at 37°C. The cells were lysed with 0.25% Triton X-100 and the insoluble ECM extracted into SDS sample buffer. Aliquots (100 μl) of the ECM were subjected to SDS-PAGE followed by autoradiography. Positions of relative molecular mass standards are indicated.

ally present in the ECM and not a contaminating cellular component. This conclusion is supported by additional studies in which immunoelectron microscopy was used to localize PAI-1 in endothelial cells (Fig. 4; Table I). These studies demonstrate the presence of considerable PAI-1-specific immunogold particles on the cell substratum underlying the ventral cell surface of intact (nonpermeabilized) monolayers. Staining was prominent at the cell periphery suggesting that the primary and gold-conjugated secondary antibodies were able to penetrate between the regions of cell–cell contact and then bind to PAI-1 present in the ECM beneath HUVECs. Taken together, these results demonstrate that PAI-1 is present in the ECM of these cells. Recent observations suggest that it is bound, at least in part, to vitronectin also present in the ECM (Mimuro and Loskutoff, 1989*a*,*b*). The significance of this association remains to be determined.

Previous studies have suggested that PAI-1 is also present on the surface of the plasma membrane of HUVECs (Sakata et al., 1988; Barnathan et al., 1988). This conclusion was based on the observations that the binding of t-PA to a high-affinity binding site on HUVECs (*a*) required the catalytic site of t-PA, (*b*) was inhibited by antibodies to PAI-1, and (*c*) formed immunoprecipitable complexes with PAI-1 associated with radioiodinated HUVECs (Barnathan et al., 1988). Our data suggest that it is the PAI-1 located in the ECM beneath cultured HUVECs, and not on the cell surface, that may actually comprise this high-affinity binding site for t-PA (Hajjar et al., 1987; Barnathan et al., 1988). For example, the PAI-1 in the ECM of confluent monolayers is clearly accessible to exogenously added tPA (Figs. 1, 2, and 4; Table I). Moreover, the immunoelectron microscope studies (Fig. 4; Table I) revealed that very little PAI-1 was actually present on the dorsal cell surface (<1% of the total detectable PAI-1). The ECM-associated PAI-1 readily forms complexes with exogenous t-PA (Fig. 1) and these complexes are rapidly released into the medium when the cells are incubated at 37°C (Fig. 1). This behavior is similar to the behavior of the t-PA/PAI-1 complexes described in the previous studies (Barnathan et al., 1988). Thus, based on the available information, it would seem that the majority of exogenously added tPA binds to PAI-1 in the ECM and not on the cell surface. Additional studies are needed to delineate the significance of the small amount of PAI-1 actually detected on the surface (Table I).

Kinetic analysis of the t-PA-mediated decreases in ECM-associated PAI-1 suggest that cultured HUVEC monolayers retard, but do not completely exclude, the access of various exogenous proteins to the culture substratum. The almost complete accessibility of solution-phase t-PA for PAI-1 in the ECM is not surprising, since the junctional relationships of in situ endothelium may not be preserved in vitro. In fact, these relationships may change dramatically in cultured cells since the passage of macromolecules tracers between cells is dependent on a variety of factors (Furie et al., 1984; Del Vicchio et al., 1987; Huang et al., 1988) including the culture substratum used for the growth of the cells. Thus, bovine microvascular endothelial cells grown on gelatin do not form intracellular junctions impenetrable to macromolecular tracers (i.e., tight junctions). However, when grown on a complex basement membrane (e.g., human amnion) relatively impenetrable intracellular junctions are formed similar to those present in intact blood vessels (Furie et al., 1984). Thus, our results suggest that HUVECs grown on fibronectin-coated plastic may also lack tight junctions, a possibility that may contribute to the current confusion in the literature concerning the cellular location of PAI-1 (i.e., surface vs. ECM). It should be noted that other pathways of transport also may exist in endothelial cells (e.g., vesicles) and contribute to the accessibility of solution-phase t-PA for ECM PAI-1. In addition, the inability of low concentrations of t-PA (e.g., 0.25 μg/ml; Fig. 5) to efficiently deplete PAI-1 from beneath the monolayers may result from the ongoing deposition of newly synthesized PAI-1 by the HUVECs.

Although the majority of ECM PAI-1 interacts with solution-phase t-PA, a variable proportion (e.g., 10–20%) was found to be non-reactive and remained associated with the ECM. Similar quantities of t-PA-resistant PAI-1 also were detected in isolated ECM preparations (Fig. 5) suggesting that the cells themselves do not interfere with this interaction. Latent PAI-1 is not cleaved by t-PA (Hekman and Loskutoff, 1985; Erickson et al., 1986), nor does it form complexes with it (Hekman and Loskutoff, 1985; Erickson et al., 1986), suggesting that the resistant PAI-1 may represent a small amount of the inactive inhibitor. Alternatively, the resistant inhibitor may be sterically inaccessible to solution-phase t-PA.

Endothelial cells in vivo may be exposed to a wide range of t-PA concentrations. For example, the level of t-PA in hu-

man plasma is usually 1–12 ng/ml under normal conditions (Juhan-Vague et al., 1988; Prowse and MacGregor, 1988), but may increase threefold after physical exercise, venous occlusion, or infusion of 1-deamino-8-D-arginine (Juhan-Vague et al., 1988; Prowse and MacGregor, 1988). Furthermore, the recent use of recombinant t-PA for thrombolytic therapy can result in a transient increase in plasma t-PA levels to >1 μg/ml (Loscalzo and Braunwald, 1988; Lucore and Sobel, 1988; Sobel, 1988). Whether PAI-1 is present in the ECM of endothelium in vivo and is affected by such alterations in the plasma level of t-PA remains to be determined.

Infusion of t-PA during thrombolytic therapy frequently results in a rapid increase in circulating t-PA/PAI-1 complexes (Lucore and Sorbel, 1988), and a small number of patients demonstrate increased plasma PAI-1 activity within 8–24 h after infusion with therapeutic doses of t-PA (Lucore and Sorbel, 1988). The inability to demonstrate increased PAI-1 synthesis by cultured HUVECs after t-PA treatment (Fig. 6), suggests that t-PA infusion does not directly stimulate the production of PAI-1 by endothelial cells. It should be noted, however, that PAI-1 gene expression is induced by a wide variety of substances (e.g., cytokines released by activated leukocytes, transforming growth factor β released by platelets, etc.) (Loskutoff et al., 1988). Thus, increased PAI-1 production in these patients may be mediated by compounds released from cellular elements of the thrombus itself during its degradation.

In addition to PAI-1, the ECM of endothelial cell binds several other proteins involved in blood coagulation and fibrinolysis, including plasminogen (Knudsen et al., 1986), fibronectin (Jaffe et al., 1976), and von Willebrand factor (Hormia et al., 1983). When the endothelial cell lining is disrupted, blood coagulation is initiated and a fibrin clot is formed in association with the subendothelial matrix. The location of active PAI-1 in the ECM may play a critical role in protecting the ECM and the ECM-associated fibrin from PA-mediated degradation through its specific inactivation of PAs. In this regard, Knudsen and Nachman (1988) demonstrated that PAI-1 associated with HUVEC ECM prevents plasmin formation and inhibits degradation of ECM preparations that are incubated with plasminogen and the t-PA secreting Bowes melanoma cell line. Thus, PAI-1 in the ECM may influence a variety of biologic events, including hemostasis, tumor invasion, and cell migration.

This research was supported in part by National Institutes of Health grants HL40376 (R. R. Schleef), HL16411 and HL22289 (D. J. Loskutoff), and in part by Eli Lilly and Company, Indianapolis, IN. J. Mimuro is a recipient of an American Heart Association Fellowship, California Affiliate.

Received for publication 8 February 1989 and in revised form 30 May 1989.

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