Extracellular Matrix of Cultured Bovine Aortic Endothelial Cells Contains Functionally Active Type I Plasminogen Activator Inhibitor

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The extracellular matrix (ECM) of cultured bovine aortic endothelial cells (BAEs) was analyzed by immunoblotting and reverse fibrin autography and shown to contain type 1 plasminogen activator inhibitor (PAI-1). Most PAI-1 in the ECM formed complexes with exogenously added tissue-type plasminogen activator (tPA), demonstrating that this PAI-1 was functionally active. The resulting tPA/PAI-1 complexes were recovered in the reaction solution, indicating that the PAI-1 in such complexes no longer bound to ECM. The PAI-1 could not be removed by incubating ECM in high salt (2 mol/L NaCl), sugars (1 mol/L galactose, 1 mol/L mannose), glycosaminoglycans (10 mmol/L heparin, 10 mmol/L dermatan sulfate), or epsilon-aminocaproic acid (0.1 mol/L). However, PAI-1 could be extracted from ECM by treatment with either arginine (0.5 mol/L) or potassium thiocyanate (2 mol/L), or by incubation under acidic conditions (pH 2.5). ECM depleted of PAI-1 by acid extraction was able to bind both the active and latent forms of PAI-1. In this instance, most of the bound PAI-1 did not form complexes with tPA, indicating that the latent form was not activated as a consequence of binding to ECM. Although the PAI-1 activity in conditioned medium decayed with a half-life (1/2) of <3 hours, the 1/2 of ECM-associated PAI-1 was >24 hours. These data suggest that PAI-1 is produced by cultured BAEs in an active form and is then either released into the medium where it is rapidly inactivated or into the subendothelium where it binds to ECM. The specific binding of PAI-1 to ECM protects it from this inactivation.

THE REGULATION of the fibrinolytic system is of critical importance during hemostasis, wound repair, neoplasia, inflammation, and a variety of other biologic processes. 1,2 It is becoming increasingly apparent that this control is achieved in large part through the action of specific plasminogen activator inhibitors (PAIs). Although four distinct PAIs have been detected, 3-4 the endothelial cell-derived inhibitor (PAI-1) 5 appears to be the physiologic inhibitor of tissue-type plasminogen activator (tPA). PAI-1 is a member of the serine proteinase inhibitor (Serpin) superfamily, 6-8 and differs from other PAIs in its stability to sodium dodecyl sulfate (SDS), 9-10 its electrophoretic mobility, 10 and its ability to inhibit single-chain tPA. 11,12 In addition, it appears to exist in at least two distinct forms in plasma 10 and in media conditioned by cultured cells, 13,14 including both an inherently active form and a latent form. 15-16 Denaturants such as SDS or guanidine hydrochloride convert the latent PAI-1 into its active form. 11 The physiologic mechanism for the activation of latent PAI-1 has not been delineated, but recent reports suggest that it is synthesized as an active molecule that is unstable and that rapidly decays into the latent form. 13,14 In this report, we show that PAI-1 is present in the extracellular matrix (ECM) of cultured bovine aortic endothelial cells (BAEs). The ECM-associated PAI-1 is active and stable, and thus appears to be protected from inactivation.

MATERIALS AND METHODS

Reagents. All chemicals were the best analytic grade commercially available. Tissue culture materials were purchased from the following sources: Plasticware from Corning (Corning, NY); media from Flow Laboratories (McLean, VA); and calf serum, trypsin, penicillin, and streptomycin from GIBCO (Grand Island, NY). Materials were obtained as follows: bovine serum albumin (BSA), Triton X-100, Tris base, casein, a- or (S-)mannose, B-galactosidase, dermatan sulfate, epsilon-aminocaproic acid (EACA), and EDTA from Sigma Chemical (St Louis); lysine-Sepharose and diethylaminoethyl (DEAE)-Sephadex from Pharmacia Fine Chemicals (Piscataway, NJ); L-arginine, glycine, and XRP-1-x-ray film from Eastman Kodak (Rochester, NY); Lysine, Aprotinin, diisopropylfluorophosphate (DFP), and bovine fibrinogen from Calbiochem (La Jolla, CA); LPG-agarose and affinity-purified goat anti-rabbit IgG from Miles Laboratories (Naperville, IL); Tween-80 from J.T. Baker Chemical (Phillipsburg, NJ); rabbit antiserum against bovine factor VIII from Biomedical Technologies (Stoughton, MA); bovine streptavidin-alkaline phosphatase conjugate, p-nitrophenyl phosphate, and 2-aminomethyl-propanediol from Zymed Laboratories (San Francisco); carrier-free Na 125I from Amersham (Arlington Heights, IL); nitrocellulose sheets from Schleicher and Schuell (Keene, NH); 2-mercaptoethanol, solid-state lactoperoxidase-glucose oxidase and reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from BioRad Laboratories (Richmond, CA).

Fibrinolytic proteins. TPA was purified from human melanoma cell conditioned medium (CM), 17 and its activity was determined by the 125I-fibrin plate assay as described previously. 18 The final product had a specific activity of ~500,000 IU/mg of protein according to the tPA International Standard and consisted primarily of the single-chain form. The tPA International standard (83/517) and the urokinase International Standard (66/46) were kindly supplied by the National Institute for Biological Standards and Controls (London). Rabbit antiserum to tPA was prepared by standard procedures, and purified goat antibodies to tPA were obtained from Bio-Pool (Hornelos, Sweden).

PAI-1 was purified from BAE CM by standard chromatographic techniques 12 without using SDS or other denaturing conditions. Analysis of the final preparation by SDS-PAGE and staining with Coomassie brilliant blue or periodic acid-Schiff revealed a single protein of mol wt 50,000. Antiserum to the purified PAI-1 was raised in New Zealand rabbits and was monospecific as determined by immunoblotting and reverse fibrin autography.

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immunoprecipitation experiments and by two-dimensional gel electrophoresis. Monoclonal antibodies against the BAE PAI-1 were prepared as described. Purified latent PAI-1 was activated by incubation with SDS (0.1%) at 37°C for 60 minutes as described and then treated with Triton X-100 (1%) at 4°C for 1 hour to neutralize the SDS. Samples treated in this way were assayed for PAI-1 activity without further treatment or storage. tPA/PAI-1 complexes were prepared by mixing 1 µg tPA with 10 µg SDS-activated PAI-1, and the resulting complexes were purified by chromatography on lysine-Sepharose. Native plasminogen was purified from human plasma by affinity chromatography with lysine-Sepharose followed by DEAE-Sepharose ion-exchange chromatography.

**Isolation of BAEs and preparation of CM and ECM.** BAEs were isolated from bovine aorta and cultured in minimal essential medium (MEM) containing 10% calf serum. The cells used for these studies were cloned from a single factor VIII-positive cell. All cultures were grown to confluency in 60-mm dishes or in 24-well (16 mm each) culture plates and maintained at confluency for 3 days before use. To prepare CM, the monolayers were washed with serum-free MEM to remove the serum and then incubated in serum-free MEM for 4 or 24 hours. This CM was collected, centrifuged at 1,000 g to remove floating cells and cellular debris, and stored in 0.01% Tween-80 at -20°C until used. ECM was prepared from these or similarly treated cultures. The monolayers were washed three times with cold phosphate-buffered saline (PBS: 0.01 mol/L sodium phosphate, 0.14 mol/L NaCl, pH 7.4) and cellular components were extracted by incubation for 10 minutes with PBS containing 0.5% Triton X-100 at 37°C. The plates were washed an additional time with distilled water to remove remaining cellular components and then assessed by light-microscopic examination for the presence of cellular debris. These extraction procedures completely removed visible cellular components from dishes and plates.

**Treatment of ECM.** ECM was treated with various concentrations of tPA (0 to 200 ng/ml) in PBS containing 0.01% Tween-80, and 10 U/ml Aprotinin. After 1-hour incubation at 37°C, the reaction solution was removed and the dishes and plates were incubated for 30 minutes with PBS containing 5 mmol/L DFP to inactivate residual tPA. Dishes and plates were washed with PBS containing 0.05% Tween-80 and then with distilled water, and the ECM was extracted by scraping into 0.1% SDS. The extracts were then centrifuged as described above.

The ECM were also incubated with either 2 mol/L NaCl, 0.1 mol/L glycine, pH 2.5, or a variety of other reagents (Table 1). The pH of each of these solutions (except for the 0.1 mol/L glycine hydrochloride) was adjusted to pH 7.4 by adding 1 mol/L Tris, pH 7.4. After 2-hour incubation at 37°C, the plates were washed with solid-phase radioimmunoassay buffer (SPRIA buffer: PBS containing 1% BSA and 0.02% Tween-80), and the ECM was characterized.

**Characterization of proteins in ECM.** The proteins remaining in the ECM after various treatments were characterized by SDS-PAGE followed by immunologic approaches. For the latter, the plates were incubated at 37°C for 2 hours with rabbit antiserum against either bovine PAI-1, bovine fibronectin, or bovine von Willebrand Factor (vWF), each diluted (1:500) in immunoradiometric assay buffer (IRMA buffer: PBS containing 3% BSA, 0.1% Tween 80, 20 U/mL Aprotinin, and 5 mmol/L EDTA). After the plates were washed, the bound antibodies were detected by incubating the plates at 37°C for 2 hours with 125I-labeled goat anti-rabbit IgG in IRMA buffer. The plates were then washed sequentially with SPRIA buffer and distilled water. The bound antibodies were eluted from the ECM by incubating the plates for 30 minutes at 37°C in 1% SDS and were counted in a γ-counter. A standard curve for the quantification of PAI-1 in ECM was constructed using purified PAI-1 coated directly onto empty plates. Purified PAI-1 (100 µL) at various concentrations in distilled water was added to 24-well culture plates, and the plates were incubated at 37°C for 16 hours. The plates were then incubated with 200 µL PBS containing 3% BSA at 37°C for 1 hour, washed extensively with SPRIA buffer, and then incubated with rabbit anti-bovine PAI-1 followed by 125I-labeled goat antibody against rabbit IgG as described above. A similar approach was used to determine whether ECM treated in various ways was able to bind exogenously added PAI-1. Purified PAI-1 (100 µL) in IRMA buffer was added to ECM and after the plates were washed, was quantified as above.

**SDS-PAGE.** SDS-PAGE was performed in slab gels according to the procedures described by Laemmli. The upper stacking gel contained 4% acrylamide, and the lower separating gel contained 10% acrylamide. Samples were subjected to electrophoresis at room temperature for 16 hours at 50 V. After electrophoresis, the gels were stained with silver nitrate or were analyzed for PAI-1 by immunoblotting (described below), or by either fibrin autography or reverse fibrin autography. For fibrin autography, the formation of clear lytic zones in the opaque indicator film after 2 to 4 hours at 37°C reveals PA activity. For reverse fibrin autography, the development of opaque, lysis-resistant zones in the otherwise clear indicator film reveals PAI activity.

**Immunoblotting.** Samples were first subjected to SDS-PAGE, and then the proteins were electrophoretically (30 V, 1.5 hours) transferred to nitrocellulose using a buffer containing 30 mmol/L Tris base, 95 mmol/L glycine, 20% methanol, and 0.01% SDS. The nitrocellulose sheets were soaked in PBS containing 1% casein (PBS/casein) for 1 hour at room temperature to block additional protein binding sites and then were incubated overnight at 4°C in PBS/casein containing the indicated antiserum (1:500 dilution). The nitrocellulose sheet was washed three times (10 minutes each wash) with PBS/casein and then incubated for 2 hours at room temperature with 125I-labeled goat anti-rabbit IgG (250,000 cpm/mL). After being washed, the nitrocellulose sheets were dried and exposed to XRP-1 x-ray film for 48 hours at -70°C. To quantitate results obtained by immunoblotting, regions of the nitrocellulose sheets containing PAI were removed and counted in a γ-counter. These results were then compared directly to a standard curve.
obtained by using known amounts of purified PAI-1, fractionated, and analyzed in parallel.

**IRMA for tPA/PAI-1 complexes.** Purified goat anti-tPA (50 μL/well, 10 μg/mL) in PBS was bound to U-bottom microtiter plates by incubation overnight at 4°C. The plates were then incubated for 1 hour in PBS containing 3% BSA to block any remaining reactive sites on the plastic and then were washed four times with SPRIA buffer. This washing step was included after each incubation step. Test samples and a standard curve of purified tPA/PAI-1 complexes were diluted in IRMA buffer and incubated (16 hours, 4°C) in the antibody-coated wells. Bound tPA/PAI-1 complexes were quantitated by incubating each washed well first with rabbit anti-PAI-1 (1:750 dilution in IRMA buffer) and then with 125I-labeled goat anti-rabbit IgG (10^6 cpm/well). The wells were individually removed, and the radioactivity in each well was determined.

**Enzyme-linked immunosorbent assay (ELISA) for bovine PAI-1.** Purified monoclonal antibody (11B2) against BAE PAI-1 (100 μL/well, 5 μg/mL) in PBS was coated onto polystyrene microtiter plates by incubation at 4°C for 16 hours. The plates were then incubated in PBS containing 3% BSA at 37°C for 1 hour and then washed four times with SPRIA buffer. This washing step was included after each incubation step. Samples diluted in IRMA buffer were incubated in the antibody-coated wells at 4°C for 16 hours. Bound PAI-1 was quantitated by incubating each well first with biotin-labeled rabbit anti–PAI-1 antibody and then with streptavidin-alkaline phosphatase conjugate. Each well was incubated with alkaline phosphatase substrate (p-nitrophenyl phosphate) in 0.75 mol/L 2-amino-2-methyl-propanediol, pH 10.3, and the change in absorbance at 405 nm was analyzed using a Titertek multiscan plate reader.

**Miscellaneous.** Protein concentration was determined by the method of Bradford.27 Bovine fibrinogen was treated with DFP (1 mmol/L) before use. Bovine fibronectin was purified from bovine plasma by affinity chromatography with gelatin sepharose as described, and rabbit anti-bovine fibronectin antiserum was provided by Dr C. Birdwell (La Jolla, CA). Purified human α-thrombin was a generous gift from Dr J. Fenton (Albany, NY). Antibodies were enzymatically labeled with 125I by using solid-state lactoperoxidase-glucose oxidase reagents (Enzymobead, BioRad) and carrier-free NA 125I. The 125I-labeled goat anti-rabbit IgG had a specific activity of 2 to 5 × 10^6 cpm/μg protein.

**RESULTS**

**ECM-associated PAI-1 and its interaction with tPA.** BAE ECM was extracted with SDS and consisted of a variety of proteins ranging in size from mol wt ~50,000 to mol wt >200,000 (Fig 1, lane 2). The smallest of these proteins appeared to comigrate with purified PAI-1 (lane 1). Analysis of the gel by reverse fibrin autography (lane 3) revealed that this protein exhibited inhibitory activity. Immunoblotting using monospecific antibodies to PAI-1 indicated that this inhibitor was PAI-1 (lane 4). Quantitative immunoblotting analysis demonstrated that these ECM extracts contained 2.5 to 5 ng/cm² PAI-1, representing ~10% to 20% of the protein present (data not shown). Similar amounts of PAI-1 were detected in the ECM of cultured human umbilical vein endothelial cells (data not shown).

We showed previously that most PAI-1 in CM is inactive but can be converted into an active form by SDS.13 To determine whether the PAI-1 of ECM was inherently active, or only active after exposure to SDS during extraction and electrophoresis, we evaluated the ability of untreated ECM to interact with exogenously added tPA. The ECM of BAEs were incubated for 1 hour at 37°C in the presence of increasing concentrations of tPA. The reaction solutions were removed, the ECM were extracted with SDS, and both solutions were then tested for PAI-1 activity by reverse fibrin autography (Fig 2). The addition of tPA caused a dose-dependent decrease in the PAI-1 activity of ECM with little detectable activity remaining in ECM treated with 100 to 200 ng/mL tPA (Fig 2A). Analysis of the reaction solution indicated that the decrease in PAI-1 activity in the ECM did not reflect a corresponding increase in PAI-1 activity in the medium (Fig 2B). A small amount of PAI-1 was spontaneously released when ECM was incubated in buffer alone (Fig 2B, lane 1). This spontaneously released PAI-1 activity also decreased in the presence of tPA.

To determine if the ECM-associated PAI-1 formed a complex with the exogenously added tPA, we examined the ECM extracts and reaction solutions by immunoblotting (Fig 3). The PAI-1 content of untreated ECM is shown in lane 1, and the positions of purified PAI-1 and of purified tPA/PAI-1 complexes are shown in lanes 4 and 5, respectively. Little detectable PAI-1 antigen remained in ECM treated with 200 ng/mL tPA (lane 2), most of it being recovered in the reaction mixture in complex with tPA (lane 3). Variable amounts of immunoreactive material (mol wt 43,000) also accumulated in the reaction solution and may
Fig 2. Effect of tPA on the PAI activity of ECM. ECM were prepared from 60-mm dishes incubated in the presence of increasing concentrations of tPA (1 mL) as described in the Materials and Methods section. The reaction solutions were removed, and the ECM were washed and extracted into SDS (1 mL). Aliquots (50 µL) of the ECM extract (A) or reaction solution (B) were removed, treated with 0.5% 2-mercaptoethanol, and subjected to SDS-PAGE and reverse fibrin autography. Lane 1, 0 ng/mL tPA; lane 2, 10 ng/mL; lane 3, 50 ng/mL; lane 4, 100 ng/mL; and lane 5, 200 ng/mL.

Fig 3. Effect of tPA on the PAI-1 antigen content of ECM. ECM prepared from 60-mm dishes were incubated in 1 mL PBS alone (lane 1) or in PBS containing 200 ng/mL tPA and 10 U/mL aprotinin (lanes 2 and 3). The reaction solutions were removed, and the ECM were washed and extracted into 1 mL 0.1% SDS. Equal aliquots (850 µL) of the ECM extract (lanes 1 and 2) or reaction solution (lane 3) were removed, lyophilized, and subjected to fractionation by SDS-PAGE and analysis by immunoblotting using rabbit anti-PAI-1 as described in the Materials and Methods section. Lane 4 contains purified PAI-1 (100 ng) and lane 5 contains purified tPA/PAI-1 complex (100 ng).

Fig 4. Quantitation of tPA/PAI-1 complex formation by IRMA assay. ECM prepared in 16-mm culture wells was incubated with 200 µL of IRMA buffer in the presence of increasing concentrations of tPA in duplicates. After 1-hour incubation at 37°C, the reaction solution was harvested and treated with DFP and the ECM was washed and extracted into 200 µL SDS (0.1%). Triton X-100 (1% final concentration) was added to the ECM extract to neutralize SDS. The concentration of tPA/PAI-1 complex in the reaction solutions (●) and ECM extracts (○) was measured by the IRMA and was expressed as a tPA equivalent value. Inset shows complexes (upper arrow) revealed by fibrin autography; position of free tPA (lower arrow). Lane 1, purified tPA/PAI-1 complexes (10 ng); lane 2, extract from ECM treated with 100 ng/mL tPA; lane 3, 100 µL reaction supernatant from ECM treated with 100 ng/mL tPA.

represent cleaved PAI-1. No tPA/PAI-1 complexes were detected in the ECM (lane 2).

An IRMA specific for tPA/PAI-1 complexes was used to quantitate the accumulation of these complexes in the reaction mixture (Fig 4). A dose-dependent increase of complexes was observed as the concentration of exogenously added tPA was increased. Approximately 30 ng/mL tPA/PAI-1 complexes were detected in the reaction solution when ECM was treated with 200 ng/mL tPA. The tPA in such complexes is resistant to DFP and can be detected after SDS-PAGE by fibrin autography. The inset in Fig 4 (lane 1) shows purified tPA/PAI-1 complexes assayed for tPA activity in this way. Again, no complexes were detected in the ECM (lane 2), but they were present in the reaction mixture (lane 3).
**Nature of the association between PAI-1 and ECM.** Experiments were performed to determine whether the association between PAI-1 and ECM represents a specific interaction (Table 1). BAE ECM were incubated with various reagents at 37°C for 2 hours. After washing, the PAI-1 remaining in the ECM was quantified immunologically as described in the Materials and Methods section. The PAI-1 content was not reduced when ECM was incubated in the presence of high concentrations of NaCl, mannose, galactose, heparin, dermatan sulfate, EACA, or lysine. However, the PAI-1 content was significantly reduced by incubation of ECM with arginine, potassium thiocyanate, and glycine (pH 2.5). These latter treatments only slightly decreased the concentration of fibronectin and vWF in ECM.

Analysis of the control and acid extracted ECM by SDS-PAGE and either reverse fibrin autography (Fig 5A, lanes 1 and 2), or immunoblotting (Fig 5B, lanes 1 and 2) confirmed the data shown in Table 1 and showed that acid treatment removed both PAI-1 activity and PAI-1 antigen. Experiments were performed to determine whether this treatment also removed the PAI-1 binding component of ECM. Acid-treated and washed ECM were incubated with purified PAI-1 for 24 hours at 37°C, washed, and extracted into SDS. The extracts were then tested for the presence of PAI-1. Both PAI-1 activity (Fig 5A, lane 3) and PAI-1 antigen (Fig 5B, lane 3) were present and could be detected after SDS-PAGE. Thus, the acid-treated ECM appears to contain the PAI-1 binding component.

The purified PAI-1 used in these experiments consisted of both active PAI-1 (<5% of the total) and inactive PAI-1.12,13 The experiments shown in Fig 6 were conducted to determine whether both forms could bind to ECM and, if so, whether binding converted the inactive form into an active inhibitor. In these experiments, purified, non-SDS-treated PAI-1 was incubated with acid-treated ECM, and the ability of the bound PAI-1 to interact with exogenously added tPA was assessed. The added tPA did not decrease the amount of exogenous PAI-1 bound to acid-treated ECM (Fig 6, lanes 2 and 4), or result in the formation of detectable tPA/PAI-1 complexes (Fig 6, lane 5). These results are thus in contrast to the results with endogenous PAI-1 (Fig 2) and suggest that the latent PAI-1 was not converted into its active form on binding to ECM. The PAI activity detected in the ECM (Fig 6, lanes 1 and 2) results from the conversion of this latent PAI into its active form on exposure to SDS during extraction and/or SDS-PAGE.

**Comparison of the stability of active PAI-1 in ECM and in CM.** We showed previously that most PAI-1 in 24-hour CM is inactive,13 most likely because it is unstable and rapidly inactivated upon secretion.15,16 The demonstration that most PAI-1 in ECM was active (Figs 2 through 4) raised the possibility that PAI-1 bound to ECM had a longer half life (t1/2) than that in CM. To test this possibility, the

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**Fig 5.** Binding of purified PAI-1 to acid-extracted ECM. ECM prepared on 60-mm dishes were incubated with 0.1 mol/L glycine, pH 2.5, at 37°C for 2 hours. This acid-treated ECM was then incubated with IRMA buffer in the presence of PAI-1 (1 µg/mL) for 24 hours at 37°C. The ECM was then extensively washed, extracted into SDS, and subjected to SDS-PAGE. After electrophoresis, the gels were analyzed for PAI-1 activity by reverse fibrin autography (panel A) and for PAI-1 antigen by immunoblotting (panel B). Lane 1, extract of control, non-acid-treated ECM; lane 2, extract of acid-treated ECM; lane 3, extract of acid-treated ECM subsequently incubated with purified PAI-1.

**Fig 6.** Effect of tPA on the PAI-1 bound to acid-extracted ECM. ECM prepared on 60-mm dishes were incubated with 0.1 mol/L glycine, pH 2.5, for 2 hours at 37°C. The resulting PAI-1-depleted ECM were then incubated for 24 hours at 37°C in IRMA buffer containing purified PAI-1 (1 µg/mL). After extensive washing, the ECM were incubated in PBS (lanes 1 and 3) or tPA (200 ng/mL; lanes 2, 4, and 5) as described in the legend to Fig 2 and extracted into SDS. The extracts (lanes 1 through 4) and reaction supernatant (lane 5) were then analyzed for PAI activity by reverse fibrin autography (lanes 1 and 2) and for PAI-1 antigen by immunoblotting (lanes 3 through 5).
functional t/2 of ECM-bound and fluid-phase PAI-1 were compared (Fig 7). CM and ECM were incubated at 37°C for various times, and the remaining PAI-1 activity was determined. The PAI-1 activity of CM rapidly decayed, with an estimated half life of 3 hours (Fig 7A). The PAI-1 activity of ECM also decreased with time, but its apparent t/2 was 19 hours. PAI-1 antigen was spontaneously released from ECM on incubation (Fig 7B) and thus was not available for binding to tPA in these experiments. When this spontaneous loss of PAI-1 from ECM was taken into consideration (Fig 7A, solid circles), the ECM-associated PAI-1 appeared to be completely stable over a 24-hour period.

DISCUSSION

The ECM of cultured endothelial cells not only influences cell morphology, growth, and differentiation, but also binds and functionally alters components of the plasma fibrinolytic systems. The ECM consists of collagens, proteoglycans, and a variety of glycoproteins including fibronectin, thrombospondin, vWF, laminin, and a 47-kd protein. The n-terminal sequence of the 47-kd protein is identical to that of human PAI-1. The data in this report demonstrate directly that PAI-1 is a component of the ECM of endothelial cells (Figs 1 and 2), and as such may also influence endothelial cell physiology.

The PAI-1 is a specific ECM constituent and not a nonspecifically adsorbed cellular protein since the addition of 35S-labeled PAI-1 to the cells during the isolation of ECM did not result in the binding of appreciable amounts of labeled PAI-1 to the ECM preparation (data not shown). More important, relatively harsh dissociating conditions, such as those that dissociate antibody/antigen interactions, were required to elute the PAI-1 from the ECM. For example, PAI-1 could not be removed from ECM by extraction with 2 mol/L NaCl (Table I), suggesting that binding was not mediated by simple ionic interactions. The inability of heparin, dermatan sulfate, galactose, or mannose to elute PAI-1 suggests that neither glycosaminoglycans nor carbohydrate side chains are involved in the interaction between PAI-1 and ECM. Lysine residues may modulate the interaction between plasminogen and ECM. However, these residues do not appear to be involved in PAI-1/ECM interactions since neither lysine itself nor EACA removes ECM-associated PAI-1. PAI-1 was only removed when ECM were extracted with acidic glycine, chaotropic agents, or arginine. The elution of PAI-1 from ECM by the basic amino acid arginine, but not lysine, suggests a specific role for arginine residues in this process. All treatments that removed PAI-1 from ECM did so without significantly decreasing ECM-associated fibronectin or vWF (Table 1). Thus, the binding of PAI-1 to BAE ECM, while strong and specific, differs from the binding of other matrix proteins.

PAI-1 is present in media conditioned by cells, in platelet releasates, and in blood in both an active and a latent form. The ratio of active to latent PAI-1 varies with cell type and culture conditions. The observation that these two forms are immunologically and biochemically similar and that there is a single gene for PAI-1 in the human genome suggests that the active and latent form are indeed two forms of the same molecule. Very little is known about which form is actually synthesized by cells or about the mechanism by which one form is converted into the other. The finding that the cell-associated form is active and rapidly converted into the latent form on release from the cell supports the hypothesis that PAI-1 is synthesized in the active, not the latent form. The data presented in this report are consistent with this hypothesis and may also provide clues about why PAI-1 is primarily active in some cells and latent in others. For example, the ECM-associated PAI-1 from BAEs binds to, and forms specific, SDS-resistant complexes with tPA (Figs 2 through 4), indicating that it is primarily active. Latent PAI-1 does not bind to or interact with tPA. These results suggest that the interaction between PAI-1 and ECM influences PAI-1 activity. The observation that SDS and other protein denaturants convert the latent form into the active form initially suggested that the latent form was converted into the active form on binding. However, our results using purified PAI-1 do not support this hypothesis. For example, although exogenously added latent PAI-1 binds to ECM (Fig 6), the bound form still does not interact with tPA and thus does not appear

Fig 7. Stability of active PAI-1 in ECM and in CM. ECM were incubated in distilled water at 37°C for various times (panel B). The supernatents were removed, and their PAI-1 content ( ) was determined by ELISA as described in the Materials and Methods section (panel A). The amount of active PAI-1 remaining in the ECM ( ), or in 4-hour CM ( ), incubated in parallel with ECM was also determined by measuring their ability to form complexes with exogenously added tPA (100 ng/mL) using the IRMA. The amount of active PAI-1 in ECM ( ) after correction for the spontaneous loss of PAI-1 antigen shown in panel B.
to have been converted into its active form. Our results do not exclude the possibility that acid-extracted ECM, although still able to bind latent PAI-1, has lost the ability to convert it into its active form. Despite this, the data in Fig 7 imply a quite different role for ECM in this interaction. Thus, although the PAI-1 activity of CM decays with an estimated functional t½ of 3 hours, the PAI-1 activity of ECM decreases with an apparent t½ of 19 hours. In addition, when the data are corrected for the loss of PAI activity due to the spontaneous release of PAI-1 from the ECM (Fig 7B), the functional t½ of the ECM-associated PAI-1 is considerably longer than 24 hours. These data suggest that the binding of active PAI-1 to ECM stabilizes it against the rapid decay that generally occurs when PAI-1 is released directly into the medium. Thus, PAI-1 is probably released from the cell in an active form and remains active when bound to ECM but rapidly decays in CM. The possibility that the active form detected in the cell15 and in CM from some cells45 also reflects the presence of PAI-1 binding protein(s) that stabilize it remains to be explored. The recent demonstration of an active, but very high mol wt form of PAI-1 in plasma and cells10,46 is consistent with this idea.

The accumulation of tPA/PAI-1 complexes in the supernatant following exposure of the ECM to tPA suggests that the PAI-1 in such complexes has lost its ECM binding site. Little information is available concerning the nature of the ECM binding region of PAI-1. That inactive PAI-1 binds to ECM suggests that the reactive center of the molecule is not required. tPA cleaves the P1-P2 amino acids (Arg357 Met355) of the reactive center of PAI-1, resulting in the formation of a C-terminal fragment (leaving group) of PAI-1 of mol wt 3,000. The ECM binding site of PAI-1 may itself be contained within this C-terminal fragment. Alternatively, cleavage of PAI-1 by tPA and the subsequent formation of tPA/PAI-1 complexes may induce a conformational change in PAI-1, resulting in its decreased binding affinity for ECM. The nature of the binding component in ECM also remains to be determined.

The extracellular matrix of endothelial cells binds several proteins involved in blood coagulation and fibrinolysis, including vWF,38,39 plasminogen,31 fibronectin34 and, as detailed in this study, PAI-1. When the endothelial cell lining is disrupted, blood coagulation is initiated and a fibrin clot is formed in association with the subendothelial matrix. In view of the results we present, active PAI-1 bound to the matrix may be expected to play a critical role in protecting the ECM and the ECM-associated fibrin from PA-mediated degradation through its specific inactivation of PAs. Furthermore, active PAI-1 released from platelets on blood coagulation3,44 may also bind to the ECM, thus providing additional protection from fibrinolysis. Plasminogen activation provides an important source of localized proteolytic activity not only during fibrinolysis but also during ovulation, cell migration, epithelial differentiation, tumor invasion, and a variety of other physiologic processes.50,51 Many of these events involve movement of cells through ECM. The presence of PAI-1 in the ECM may thus influence a variety of biologic events.

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