Serum-derived Vitronectin Influences the Pericellular Distribution of Type 1 Plasminogen Activator Inhibitor

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Abstract. Bovine aortic endothelial cells (BAEs) were used as a model system to study the nature and origin of protein(s) in the extracellular matrix that bind to type 1 plasminogen activator inhibitor (PAI-1). Matrix samples were fractionated by SDS-PAGE and analyzed by PAI-1 ligand binding and by immunoblotting using antibodies to vitronectin (Vn). PAI-1 bound primarily to two Vn-related polypeptides of M_r 63,000 and 57,000, and both of these partially degraded polypeptides were present in the culture serum. Radiolabeling experiments failed to detect significant Vn biosynthesis by BAEs (<0.03% of total), or by human umbilical vein endothelial cells and HT 1080 cells. The binding of PAI-1 to Vn was relatively specific since direct binding studies failed to demonstrate significant interactions between PAI-1 and other matrix proteins (e.g., fibronectin, type IV collagen, laminin, or matrigel). Kinetic studies indicate that PAI-1 rapidly accumulates in the matrix when BAEs are plated on Vn, appearing in the conditioned medium only after a significant lag period (1–2 h). However, no PAI-1 was detected in the matrix when the cells were plated on fibronectin-coated dishes, and there was no lag period for PAI-1 accumulation in the medium. These results indicate that PAI-1 binds specifically to serum-derived Vn in the matrix, and suggest that the composition of both the matrix and serum itself may influence the pericellular distribution of this important inhibitor.

THE extracellular matrix (ECM)[1] of cultured cells is composed of a fibrillar network of proteoglycans and glycoproteins such as fibronectin (Fn), vitronectin (Vn), collagen, elastin, and laminin (Alitalo and Vaheri, 1982; Hayman et al., 1983). The ECM mediates the attachment of cells to their substratum and participates in the regulation of cell differentiation, proliferation, and morphogenesis (Kleinman et al., 1981). The fibrinolytic system can proteolytically modify the structure and function of the ECM (Dano et al., 1985). This degradation is mediated largely by plasmin, a proteolytic enzyme capable of degrading most glycoprotein components of the matrix (Dano et al., 1985; Saksela, 1985; Saksela and Rifkin, 1988). Plasmin is formed from the abundant extracellular proenzyme plasminogen through the action of plasminogen activators (PAs). Thus, regulation of PA activity is a key step in controlling biological events occurring in the ECM.

Specific PA inhibitors (PAIs) appear to be the primary regulators of PA activity (Loskutoff et al., 1989). One of these, type 1 PAI (PAI-1), has been detected in the ECM of a number of cultured cells (Laiho et al., 1986; Rheinwald et al., 1987; Knudsen et al., 1987) including bovine (Mimuro et al., 1987) and human (Levin and Santell, 1987) endothelial cells. Although the majority of PAI-1 in conditioned medium (CM) is inactive (Hekman and Loskutoff, 1985), the PAI-1 present in the ECM appears to be fully active since it binds to and inhibits both tissue-type PA (t-PA; Knudsen et al., 1987; Levin and Santell, 1987; Mimuro et al., 1987) and urinary-type PA (u-PA; Knudsen et al., 1987; Laiho et al., 1987). The binding of PAI-1 to ECM protects it from the spontaneous loss of activity that occurs in solution after it is secreted (Mimuro et al., 1987; Mimuro and Loskutoff, 1989*b*). Kinetic data suggest that PAI-1 may be secreted in a polar manner since it is detected in the ECM immediately after secretion but appears in the CM only after a lag period (Rheinwald et al., 1987; Laiho et al., 1987). It has been suggested that active, matrix-associated PAI-1 may protect ECM proteins against cellular proteases and thus play a role in a variety of biological processes (Dano et al., 1985; Saksela and Rifkin, 1988).

Although the identity of the PAI-1 binding protein(s) in ECM is unknown, a PAI-1 binding protein recently was purified from human (Declerk et al., 1988; Wiman et al., 1988) and bovine (Mimuro and Loskutoff, 1989*a*) plasma and shown to be the adhesive glycoprotein Vn. Purified Vn competes with ECM for binding to PAI-1, suggesting that PAI-1 in ECM is bound, at least in part, to Vn (Mimuro et al., 1989*a*). The relative affinities of PAI-1 for other matrix proteins are unknown.

In this report, we investigate the role of Vn and other ECM proteins on the binding and concentration of PAI-1 on the culture substratum. We show that while Vn is indeed the ma-

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial cell; CM, conditioned medium; ECM, extracellular matrix; Fn, fibronectin; HUVEC, human umbilical vein endothelial cell; PA, plasminogen activator; PAI, PA inhibitor; PAI-1, type 1 PAI; t-PA, tissue-type plasminogen activator; Vn, vitronectin; u-PA, urokinase-type plasminogen activator.

jor PAI-1 binding protein in the matrix of cultured bovine aortic endothelial cells (BAEs), it is not synthesized by the cells but rather derived from the tissue culture serum. Moreover, BAEs plated on fibronectin, type IV collagen, laminin, and matrigel deposited little PAI-1 into ECM, suggesting that the composition of the ECM itself affects the actual pericellular distribution of PAI-1.

Materials and Methods

All chemicals were the highest analytical grade commercially available. Tissue culture materials were purchased from the following sources: Plasticware from Corning Glass Works, Corning Scientific Products (Corning, NY); MEM, and methionine-free MEM from Irvine Scientific (Santa Ana, CA); Medium 199 from M. A. Bioproducts (Bethesda, MD); F12 and DME from Whittaker Bioproducts (Walkersville, MD); calf serum, FCS, trypsin, penicillin, and streptomycin from Gibco Laboratories (Grand Island, NY); endothelial cell growth factor from Biomedical Technologies, Inc. (Stoughton, MA). Other biologicals were obtained as follows: BSA, Triton X-100, Tris-base, heparin-agarose, bovine fibronectin, soybean trypsin inhibitor, porcine intestinal heparin, and casein from Sigma Chemical Co. (St. Louis, MO); CNBr-activated Sepharose 4B and protein A Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, NJ); Tween-80 from J. T. Baker Chemical Co. (Phillipsburg, NJ); L-^{35}S methionine (1,100 Ci/mmol), ^{125}I-labeled donkey anti-rabbit IgG, and carrier-free ^{125}I (12.5 mCi/μg) from Amersham Corp. (Arlington Heights, IL); nitrocellulose sheets from Schleicher & Schuell, Inc. (Keene, NH); iodobeads from Pierce Chemical Co. (Rockford, IL); reagents for SDS-PAGE from Bio-Rad Laboratories (Richmond, CA); XAR-5 x-ray film from Eastman Kodak Co. (Rochester, NY); prestained high molecular weight standard protein mixture from Bethesda Research Laboratories (Gaithersburg, MD). Bovine PAI-1 was purified from BAE CM as described (Hekman and Loskutoff, 1988) using standard chromatographic techniques in the absence of SDS. Analysis of the final preparation by SDS-PAGE and staining with silver (Morrissey, 1981) revealed a single protein of M_r 50,000. Activation of purified PAI-1 with 4 M guanidine hydrochloride was performed as described (Hekman and Loskutoff, 1985). Bovine Vn was purified from pooled bovine plasma as described (Yatohgo et al., 1988). Analysis of the final preparation by SDS-PAGE under reducing conditions and staining with silver revealed two polypeptides of M_r 80,000 and M_r 65,000. Radioiodination of Vn was performed with ^{125}-iodine and iodobeads according to the manufacturer (Pierce Chemical Co.). Antiserum to bovine Vn was raised in rabbits, and the IgG fraction was further purified by affinity chromatography on immobilized Vn using standard procedures (Harlow and Lane, 1988). Laminin, type IV collagen, and matrigel were generous gifts from Dr. J. Madri (Yale University, New Haven, CT). Rabbit antihuman Vn was kindly provided by Drs. J. Smith and D. Cheresh (Research Institute of Scripps Clinic, La Jolla, CA).

Cell Culture

BAEs were isolated from bovine aortae and cloned from a single factor VIII-positive cell as described (van Mourik et al., 1984). Cells were grown to confluence in MEM containing 10% calf serum on either 150-mm culture plates or 75 cm^2 flasks and maintained at confluence (10^5 cells/cm^2) for 3 d before use unless otherwise specified. Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords as described (Schleef et al., 1988). HT 1080 cells were grown in DME with 10% FCS, while HEP 3B cells were cultured in DME; F12 with 10% FCS. HUVEC, HT 1080, and HEP 3B were grown to confluency in 6-well culture plates (9.4 cm^2).

Preparation of ECM

The term ECM as employed here refers to the ECM-substratum-attached proteins. It thus consists of cell-derived ECM proteins and absorbed serum proteins. It was prepared as described (Mimuro et al., 1987). Briefly, the cells were removed from the culture plate by extraction into 0.5% Triton X-100. The extracts were discarded, and the ECM remaining on the culture dish was washed three times with PBS and then extracted into 0.1% SDS. A serum-derived matrix fraction was obtained by incubating empty 150-mm culture plates with MEM containing 10% calf serum at 37°C for similar times as the cells grown for ECM preparation. The plates were then washed three times with PBS, and the serum-derived matrix fraction was prepared as for ECM by extraction with 0.5% Triton X-100, and then with 0.1% SDS. In other experiments, culture dishes were coated with purified Vn, Fn, laminin, type IV collagen, matrigel (Kleinman et al., 1986), or serum. Briefly, 6-well dishes (9.4 cm^2) were incubated overnight at 4°C in 1 ml of PBS containing 25 μg of the purified components, or matrigel, or 10% serum in MEM. The dishes were washed with PBS, incubated with 3% BSA in PBS (4°C, 16 h) to block remaining protein binding sites, and washed again. This procedure has been reported to promote the saturable and reproducible binding of matrix components to tissue culture dishes (Form et al., 1986).

Metabolic Labeling of Cells

BAE monolayers and trypsin released cells were washed two times with serum-free MEM and one time with methionine-deficient MEM. The cells were labeled for various times with ^{35}S-methionine (100 μCi/ml) in methionine-deficient MEM and then CM, cell extract and ECM were prepared as above and analyzed as described. For pulse–chase experiments, the washed monolayers were exposed to ^{35}S-methionine for only 1 h, then washed and chased in serum-free MEM for up to 24 h. In some experiments, BAEs were labeled during the attachment and spreading process. In these experiments, the confluent monolayer was removed with trypsin and the trypsin was neutralized with soybean trypsin inhibitor (500 μg/ml). After washing, the cells were plated at confluence (10^5 cells/cm^2) in methionine-deficient MEM containing ^{35}S-methionine (100 μCi/ml). After 6 h, the CM, cell extract, and ECM were prepared and analyzed. The labeling medium for HUVEC cultures also was supplemented with endothelial cell growth factor (75 μg/ml).

Immunoprecipitation Analysis

Metabolically labeled cellular samples were mixed with an equal volume of immunoprecipitation buffer (PBS containing casein [1%], aprotinin [10 U/ml], benzamidine hydrochloride [10 mM], and Triton X-100 [0.1%]) and antibodies were added (i.e., polyclonal antiserum [3% vol/vol] raised against bovine PAI-1 [van Mourik et al., 1984]; affinity-purified anti-bovine Vn IgG [10 μg/ml]; rabbit antihuman Vn [3% vol/vol]). The samples were rocked overnight at 4°C, and then incubated for 2 h at room temperature with protein A Sepharose. Nonbound proteins were removed from the Sepharose beads by sequential washing with immunoprecipitation buffer, 2 M NaCl containing 0.1% Triton X-100, and finally with distilled water. Bound proteins were released from the beads by boiling in sample buffer (in the case of Vn, the SDS contained 50 mM DTT) and fractionated by SDS-PAGE. After electrophoresis, the gels were soaked in Amplify, dried, and exposed to Kodak XAR-5 x-ray film with intensifying screens. In some experiments, the autoradiogram was used to localize PAI-1 in the gel. These regions were excised, hydrolyzed, and subjected to liquid scintillation counting.

Ligand Binding Assay

A ligand binding assay to detect PAI-1 binding proteins was performed. Briefly, samples were fractioned by SDS-PAGE and the proteins in the gel transferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose sheets were incubated with guanidine-reactivated PAI-1 (0.25 nM) for 16 h (4°C) and washed. Bound PAI-1 was detected by incubating the sheets with rabbit anti-PAI-1 IgG (van Mourik et al., 1984) followed by ^{125}I-labeled donkey anti-rabbit IgG. After washing, the nitrocellulose sheets were exposed to Kodak XAR-5 x-ray film.

Immunoblotting

Samples were fractionated by SDS-PAGE and transferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose sheet was soaked in PBS containing 1% casein (PBS/casein) for 1 h at room temperature to block additional protein binding sites and then incubated at room temperature for 2 h with affinity-purified anti-Vn IgG (1 μg/ml in PBS/casein). The nitrocellulose sheet was washed three times with PBS/casein and then incubated for 2 h at 22°C with ^{125}I-labeled donkey anti-rabbit IgG (200,000 cpm/ml). The nitrocellulose sheet was washed, dried, and autoradiograms were prepared by using Kodak XAR-5 x-ray film.

Miscellaneous

SDS-PAGE was performed on slab gels according to the procedure described by Laemmli (1970). The upper stacking gel contained 4% acryl-

amide, and the lower resolving gel contained 9% acrylamide. In general, protein concentration was determined by the method of Bradford (1976) using rabbit IgG as a standard protein for IgG and BSA for all other proteins. The protein concentration of laminin and type IV collagen was determined by enzyme immunoassay (Form et al., 1986). Molecular mass markers included carbonic anhydrase (M_r 29,000), ovalbumin (M_r 43,000), BSA (M_r 68,000), and phosphorylase b (M_r 97,000).

Results

Identification of PAI-1 Binding Proteins in the ECM

We showed previously that PAI-1 could bind to the ECM of cultured BAEs (Mimuro et al., 1987) and that bovine plasma contained a protein that also bound to PAI-1 and prevented its interaction with ECM (Mimuro and Loskutoff, 1989*a*). Although the plasma protein was shown to be Vn, no attempt was made in those experiments to establish the identity and source of the PAI-1 binding protein(s) in the ECM itself. Thus, experiments were performed to characterize the PAI-1 binding protein(s) present in the ECM (Fig. 1). Purified Vn was used as a standard for these studies. The Vn contained two polypeptides of M_r of 80,000 and 65,000 (Fig. 1 *A*, lane *1*), and both of these were positive when tested in the PAI-1 ligand binding assay (Fig. 1 *B*, lane *1*). The ECM contained two major polypeptides (M_r 63,000 and 57,000) and a minor polypeptide (M_r 80,000), all of which bound to PAI-1 (Fig. 1 *B*, lane *2*). Similar results were obtained if ECM was prepared from cells removed with EDTA instead of Triton X-100, thus minimizing the possibility that cytoskeletal proteins (Fey et al., 1980) or absorbed cellular debris remained in the ECM fraction (not shown). All of these polypeptides also were recognized by antibodies to Vn (Fig. 1 *A*, lane *2*), suggesting that they are a mixture of native (80 kD) and partially degraded Vn. The 45–50 kD protein seen in Fig. 1 *B*, lane *2* is the endogenous PAI-1 present in ECM (Fig. 1 *B*, compare lanes *2* and *4*). Most of the Vn-related material in the ECM has a lower M_r than the purified Vn (Fig. 1 *A*, compare lanes *1* and *2*) and probably represents partially degraded Vn.

Origin of PAI-1 Binding Proteins in the ECM

The detection of significant amounts of Vn in the ECM (Fig. 1), together with the observation that serum Vn absorbs to plastic and promotes cell spreading (Holmes, 1967), raised the possibility that the binding of PAI-1 to ECM was mediated, at least in part, by serum-derived Vn. Experiments were performed to access this possibility (Fig. 2). Serum was adsorbed to plastic tissue culture dishes, the dishes were washed, and the "serum-derived matrices" were prepared using the same procedures employed for the preparation of cell-derived ECM (see Materials and Methods). The serum-derived matrices were then analyzed by immunoblotting with antibodies to Vn (Fig. 2 *A*) and by ligand blotting (Fig. 2 *B*). The PAI-1 binding pattern for this serum-derived ma-

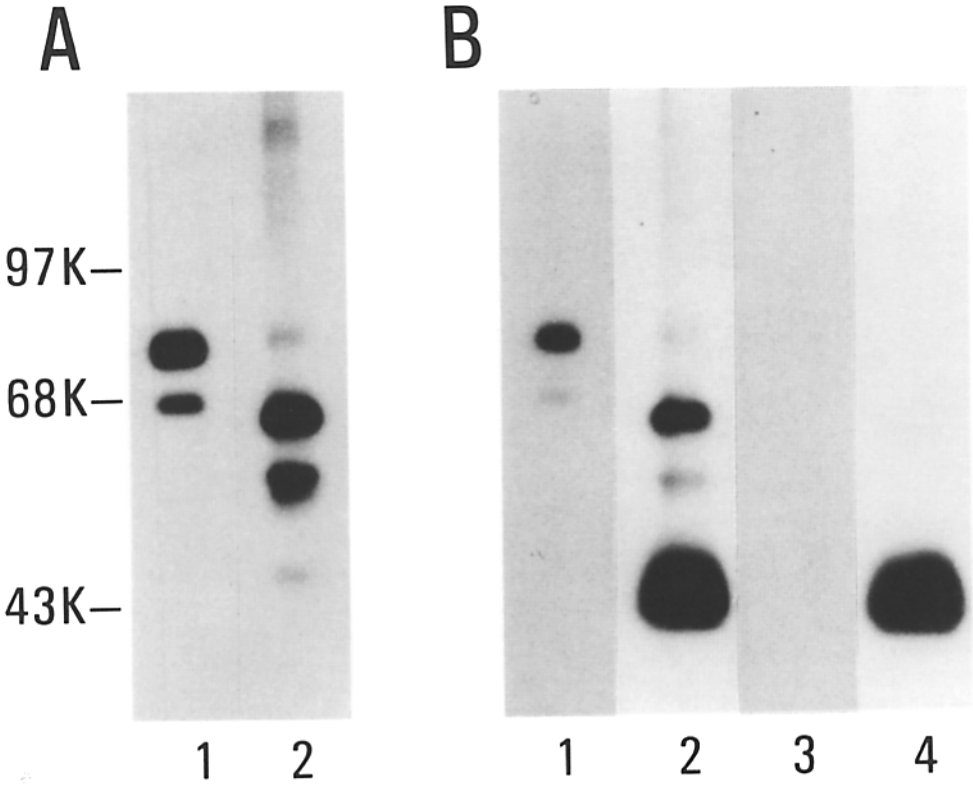

Figure 1. Detection of PAI-1 binding-proteins in the ECM. Purified Vn and ECM prepared as described under Materials and Methods, were chemically reduced (50 mM dithioerythreitol), and then fractionated by SDS-PAGE and transferred to nitrocellulose. (*A*) The membrane was analyzed by immunoblotting by using affinity-purified anti-Vn IgG followed by ^{125}I-labeled donkey anti-rabbit IgG, and subjected to autoradiography. (lane *1*) Purified Vn (500 ng); (lane *2*) ECM (equivalent to 25 cm^2 area of culture dish). (*B*) The nitrocellulose sheets were analyzed by ligand blotting by sequentially incubating them with activated PAI-1 (0.25 nM), followed by rabbit anti-PAI-1 antiserum, and then with ^{125}I-labeled donkey anti-rabbit IgG. The washed nitrocellulose sheets then were subjected to analysis by autoradiography. Controls were treated identically, except that the PAI-1 incubation step was omitted. (Lane *1*) Purified Vn (500 ng); (lane *2*) ECM (equivalent to 25 cm^2); (lane *3*) Vn control; (lane *4*) ECM control.

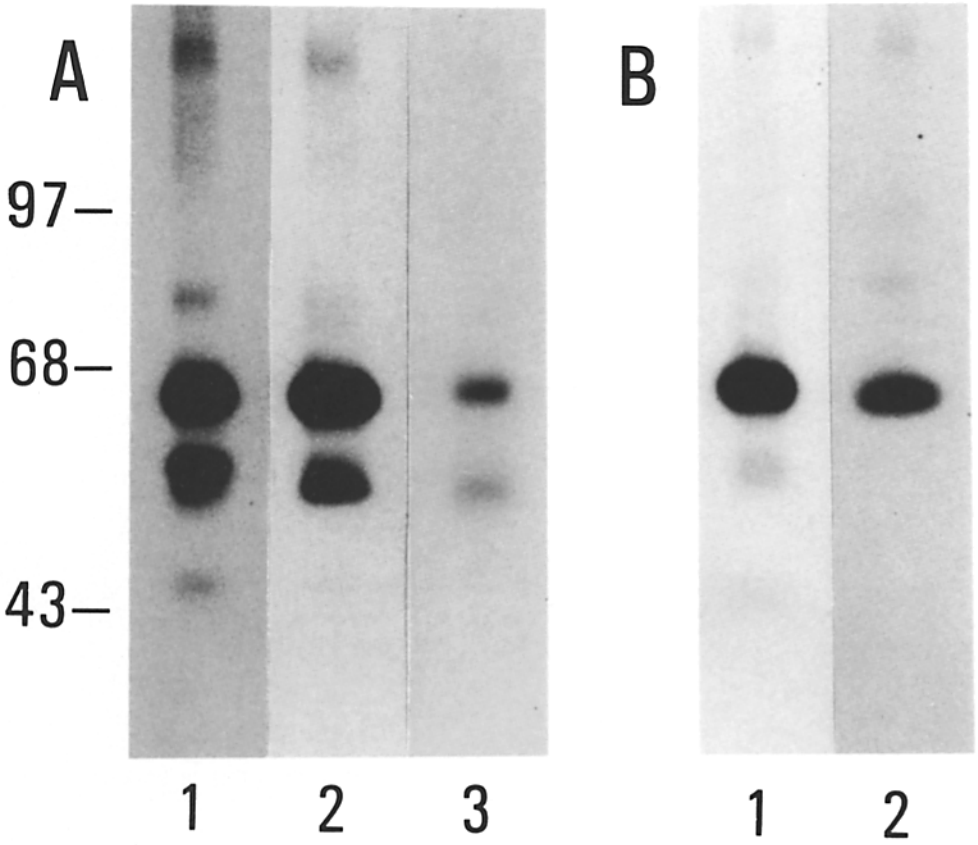

Figure 2. Origin of PAI-1 binding-proteins in the ECM. Bovine serum and the serum-derived matrix fraction were prepared as described under Materials and Methods, fractionated by SDS-PAGE, and transferred to nitrocellulose. The washed nitrocellulose sheets were analyzed by immunoblotting with antibodies to Vn and by PAI-1 ligand binding. (*A*) Analysis by immunoblotting; (lane *1*) ECM (equivalent to 25 cm^2); (lane *2*) serum-derived matrix fraction (equivalent to 25 cm^2); (lane *3*) serum (0.25 μl). (*B*) Analysis by PAI-1 ligand binding; (lane *1*) serum-derived matrix fraction (equivalent to 25 cm^2); (lane *2*) serum (0.5 μl).

trix fraction (Fig. 2 *B*, lane *1*) was very similar to that obtained from the endothelial cell ECM (Fig. 1 *B*, lane *2*). In fact, serum itself contained PAI-1 binding proteins (Fig. 2 *B*, lane *2*) which were similar in molecular size to those present both in the ECM (Fig. 1 *B*, lane *2*) and in the serum-derived matrix fraction (Fig. 2 *B*, lane *1*). Again, both of these peptides were immunologically related to Vn (Fig. 2 *A*, lanes *2* and *3*). The M_r 50,000 polypeptide (i.e., PAI-1) observed in the ECM (Fig. 1 *B*, lane *2*) was missing in the serum-derived matrix fraction (Fig. 2 *B*, lane *1*). Thus, the PAI-1 in the ECM was not serum derived but was produced by the BAEs themselves.

These rather unexpected results indicate that much of the Vn in the ECM is serum derived, and raise the possibility that BAEs do not produce the Vn to which PAI-1 binds. Experiments were performed to measure Vn biosynthesis by these cells. BAEs were cultured in the presence of ^{35}S-methionine and the resulting CM was subjected to immunoprecipitation by using antibodies against Vn (Fig. 3). Purified, ^{125}I-labeled Vn was used as a positive control in these experiments (Fig. 3, lane *1*). No radiolabeled Vn was detected in immunoprecipitations obtained from CM (800,000 TCA precipitable cpm; not shown), cell lysates (3,000,000 cpm; not shown), or ECM (120,000 cpm; Fig. 3, lane *2*). Exogenously added radiolabeled Vn could be quantitatively immunoprecipitated from the ECM, CM, and cell lysates (not shown), indicating that these cellular fractions did not interfere with the immunoprecipitation reaction. The experiments with ^{125}I-labeled Vn indicate that the lower detection limit for this approach was 0.2 ng Vn. In contrast to these results, radiolabeled Vn was readily immunoprecipitated from the CM of the human hepatoma cell line, HEP 3B (Fig. 3, lane *3*). In fact, analysis of as little as 1,000 cpm (TCA precipitable) of the CM from HEP 3B was sufficient to obtain a positive signal (data not shown). These results suggest that the binding of PAI-1 to the ECM of cultured BAEs is dependent on the presence of Vn in the culture serum, and not on Vn biosynthesis by the cells themselves.

Experiments were performed to assess the generality of this conclusion. Two human cell lines, HUVECs (Levin and Santell, 1987) and HT 1080 cells (Laiho et al., 1987), also were reported to deposit PAI-1 into their ECM. The ability of these cells to produce Vn was investigated as above. Confluent cultures were metabolically labeled with ^{35}S-methionine for 6 h and the CM, cell lysates, and ECM were prepared and immunoprecipitated using antibodies to Vn. Again, no radiolabeled Vn was detected in the CM of these cells (Fig. 3, lanes *4* and *5*) or in cell lysates and ECM prepared from them (data not shown). The rate of Vn biosynthesis by HUVEC and HT 1080 cells was at least 100-fold less than that of HEP 3B cells. Thus, the presence of PAI-1 in the ECM of HUVECs (Levin and Santell, 1987) and HT-1080 cells (Laiho et al., 1987) also is mediated by serum-derived Vn.

Effect of Various Matrix Proteins on the Pericellular Localization of PAI-1

The effect of different matrix proteins on the pericellular distribution of PAI-1 was investigated. Cells were plated on culture dishes coated with either Vn or Fn and then metabolically labeled during the attachment and spreading process (Fig. 4). A major protein of M_r 50,000 was detectable in

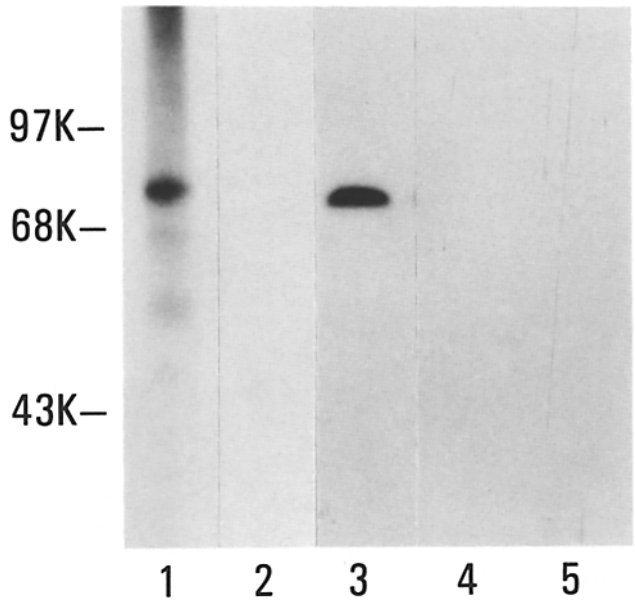

Figure 3. Biosynthesis of Vn by BAEs, Hep 3Bs, HUVECs, and HT 1080 cells. Confluent cells were metabolically labeled with ^{35}S-methionine and the resulting CM and ECM were prepared as described under Materials and Methods. The radiolabeled Vn was collected by immunoprecipitation using protein A Sepharose, analyzed by SDS-PAGE and autoradiography, and compared to purified ^{125}I-labeled bovine Vn. (lane *1*) Purified, ^{125}I-labeled Vn (10,000 cpm); (lane *2*) ^{35}S-labeled ECM (equivalent to 1 cm^2; 120,000 TCA precipitable cpm); (lane *3*) CM from Hep 3B cells (results from the immunoprecipitation of 5,000 cpm of TCA-precipitable protein); (lane *4*) CM from HUVECs (100,000 cpm); (lane *5*) CM from HT 1080 cells (100,000 cpm).

both the CM and ECM of cells which adhered to Vn (Fig. 4, lanes *1* and *3*), and this protein was shown to be PAI-1 (Fig. 4, lane *5*). PAI-1 was also present in the CM of cells plated on Fn (Fig. 4, lane *2*), but was absent from ECM (Fig. 4, lane *4*). The rate of cell attachment and spreading was reduced when BAEs were plated on laminin or type IV collagen coated-dishes compared to Vn, Fn, matrigel, or serum-coated tissue culture dishes (not shown). However, no significant differences in the overall rate of protein synthesis by the cells could be demonstrated on the different substrates over this time interval (not shown).

Additional experiments were performed to more completely characterize the influence of ECM proteins on the pericellular deposition of PAI-1, and to assess the specificity of PAI-1 binding to Vn (Fig. 5). Similar amounts of PAI-1 were detected in the CM (Fig. 5 *A*) and cell lysates (data not shown) when cells were plated on laminin (lane *1*), type IV collagen (lane *2*), serum (lane *3*), and matrigel (lane *4*). As expected from the results in Fig. 2, PAI-1 was deposited into the culture substratum when cells were plated on serum-coated culture dishes (Fig. 5 *B*, lane *3*). However, no PAI-1 could be detected in the ECM when the cells were cultured on laminin (Fig. 5 *B*, lane *1*), type IV collagen (Fig. 5 *B*, lane *2*), or matrigel (Fig. 5 *B*, lane *4*). Moreover, direct binding studies using ^{35}S-labeled PAI-1 failed to demonstrate PAI-1 binding to Fn, type IV collagen, laminin, or matrigel (not shown). Matrigel is a matrix preparation containing laminin, type IV collagen, entactin, and proteoglycan (Kleinman et al., 1986).

Influence of Vn and Fn on the Kinetics of PAI-1 Accumulation in CM and ECM

Pulse–chase experiments were performed to investigate the influence of matrix composition on the kinetics of PAI-1 deposition into ECM and appearance in CM (Fig. 6). BAEs were plated on Vn- or Fn-coated dishes under serum-free conditions with ^{35}S-methionine for 1 h (pulse). The monolayers were washed again and then incubated for various times in serum-free MEM in the absence of label (chase), and both CM and ECM were prepared. The overall rate of protein biosynthesis by cells plated on Vn or Fn was approximately equal as judged by the amount of TCA-precipitable radioactivity in each culture (data not shown). Immunoprecipitation experiments indicated that cells plated on Vn rapidly deposited PAI-1 into the ECM but released it into the CM only after a lag period of 1–2 h (Fig. 6 *A*). In these experiments, the binding of radiolabeled PAI-1 to Vn was transient: ~50% of the bound PAI-1 disappeared from the ECM within 5 h and radiolabeled PAI-1 could not be detected in the ECM after 24 h. The appearance of radiolabeled PAI-1 in CM occurred at approximately the same time as the amount of PAI-1 in the ECM began to decrease, and the rate of PAI-1 accumulation in the CM was similar to the rate of PAI-1 loss from the ECM. These results suggest that the accumulation of PAI-1 in CM resulted from the release of PAI-1 from ECM. Similar results were obtained when serum was absorbed to the culture substratum in place of Vn (data not shown). In contrast to these results, when the cells were plated on Fn, no lag period was observed. In this instance, the PAI-1 appeared to be synthesized and then rapidly and directly released into the culture medium (Fig. 6 *B*). No PAI-1 was detectable in the ECM over the course of the experiment, consistent with the results shown in Fig. 4.

Discussion

Although large amounts of biologically active PAI-1 have been detected in the ECM derived from a variety of cultured cells (Laiho et al., 1986; Rheinwald et al., 1987; Knudsen et al., 1987; Mimuro et al., 1987; Levin and Santell, 1987; Pollanen et al., 1987), the identity of the PAI-1 binding protein(s) in ECM remains unclear. PAI-1 circulates in plasma complexed to the adhesive glycoprotein Vn (Wiman et al., 1988; Declerck et al., 1988), and purified Vn competes with ECM for binding to PAI-1 (Mimuro and Loskutoff, 1989*a*). These observations have led to the suggestion that PAI-1 in ECM is bound to Vn (Mimuro and Loskutoff, 1989*a*). In this report, we confirm this hypothesis by showing that Vn is indeed the major PAI-1 binding protein of ECM (Fig. 1), and also extend it by showing that the binding of PAI-1 to Vn is fairly specific. The inhibitor was not detected in ECM when cells were plated on tissue culture dishes coated with other basement membrane proteins (i.e., laminin, Fn, type IV collagen and matrigel; Figs. 4–6), and direct binding studies failed to demonstrate the binding of PAI-1 to any of these matrix proteins. Thus, while these proteins were able to mediate the adhesion and spreading of BAEs, they were unable to replace Vn in the ECM as PAI-1 binding proteins.

Vn was also found to influence the relative rate of accumulation of PAI-1 in ECM and CM. For example, when BAEs were plated on Vn, the majority of secreted PAI-1 was rapidly

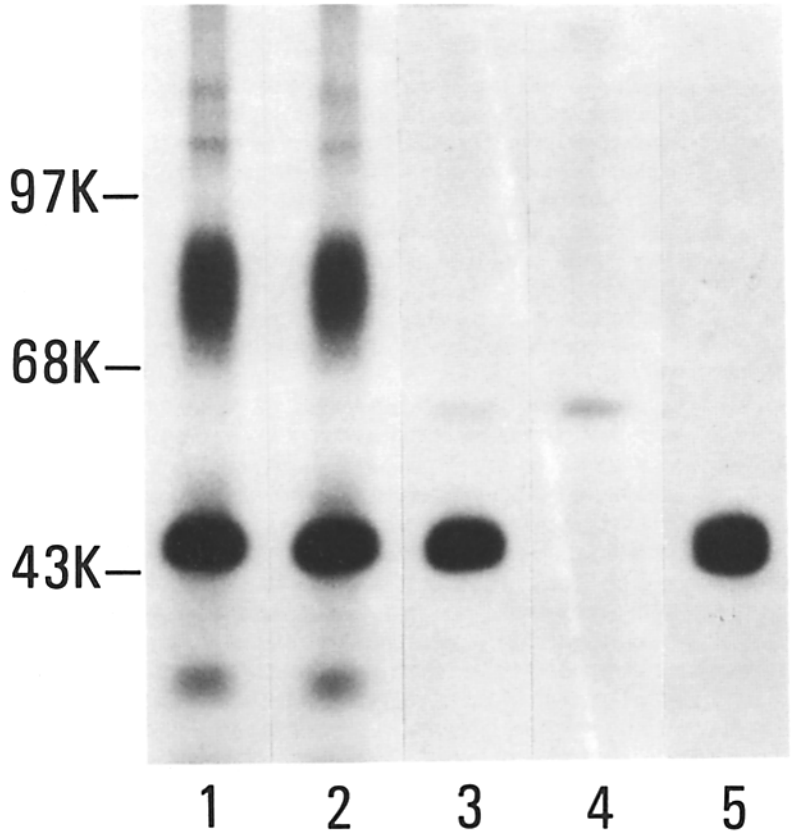

Figure 4. Effect of Vn- or Fn-enriched matrices on the pericellular deposition of PAI-1. BAEs were removed from the culture dish by trypsin, washed, and then plated on Vn- or Fn-coated dishes in serum-free medium containing ^{35}S-methionine. After 6 h the CM was collected, the cells were lysed with Triton X-100, and the ECM was prepared (see Materials and Methods). The samples were fractionated by SDS-PAGE, and the gels were dried and analyzed by fluorography. CM (lane *1*) and ECM (lane *3*) from cells plated on Vn-coated dishes; CM (lane *2*) and ECM (lane *4*) and from cells plated on Fn-coated dishes. Lane *5* shows PAI-1 immunoprecipitated from the CM of cells plated on Vn-coated dishes (see Materials and Methods).

deposited into the ECM but appeared in the CM only after a significant lag period (Fig. 6). This behavior is similar to that observed previously for PAI-1 secretion by mesothelial cells (Rheinwald et al., 1987) and HT 1080-cells (Laiho et al., 1987), and has raised the possibility that PAI-1 may be released in a polar manner. However, this apparent polar deposition of PAI-1 was only observed when the cells were plated on Vn (Fig. 6 *A*). When the cells were cultured on Fn-coated culture dishes, PAI-1 was rapidly and directly released into the medium, and none was detected in the ECM (Fig. 6 *B*). Thus, the pericellular distribution of PAI-1 actually reflects the composition of the ECM itself.

The observation that three cell types previously reported to deposit PAI-1 into their ECM (i.e., BAEs, HUVECs, and HT-1080 cells) do not synthesize detectable amounts of Vn (Fig. 3), was rather unexpected in view of this requirement that Vn be present for PAI-1 deposition into ECM (Fig. 6). It now seems clear that the majority of PAI-1 in the ECM of

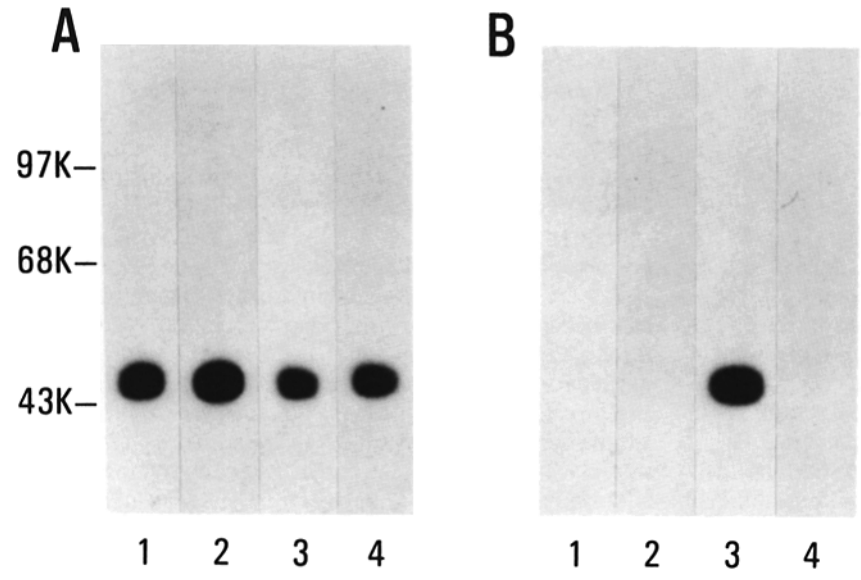

Figure 5. Effect of laminin, type IV collagen, matrigel, and serum on the deposition of PAI-1 in the ECM. Tissue culture dishes were coated with laminin (lane *1*), type IV collagen (lane *2*), serum (lane *3*), and matrigel (lane *4*) as described in Materials and Methods. BAEs were plated on these matrixes as in Fig. 4 and labeled with ^{35}S-methionine under serum-free conditions. After 6 h, the CM (*A*) and ECM (*B*) were prepared. PAI-1 was isolated from each sample by immunoprecipitation and analyzed by SDS-PAGE and autoradiography.

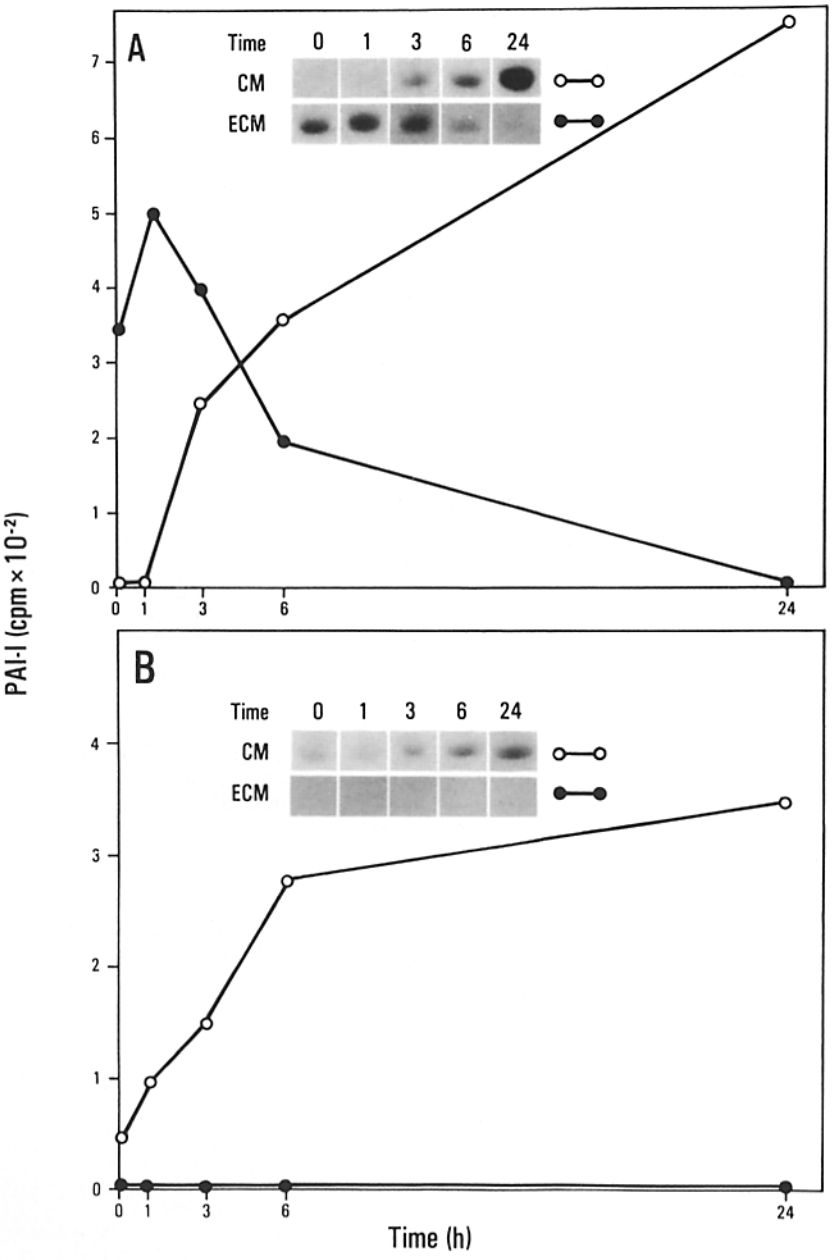

Figure 6. Influence of Vn and Fn on the release of PAI-1 from cells and deposition into ECM. BAEs were plated on Vn (*A*) or Fn (*B*) matrixes in serum-free media for 24 h, washed, and then incubated with ^{35}S-methionine for 1 h. The cultures were washed with serum-free medium and then incubated in serum-free medium for the indicated times and CM (○) and ECM (●) were prepared. The PAI-1 present in each sample was collected by immunoprecipitation and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. The radiolabeled PAI-1 was excised from the gel and the amount of radioactivity present in each sample was determined by liquid scintillation counting. The amount of PAI-1-specific cpm present in the CM at the end of the 1-h labeling period also was determined by immunoprecipitation. The cpm present in these samples were added to the PAI-1-specific cpm at each time point and the combined cpm are shown in the figure. The insets show the actual autoradiograms. The data shown are representative of three independent experiments.

these cells is bound to Vn adsorbed to the plastic tissue culture dish from the growth serum (Figs. 1 and 2). The high concentration of Vn in the serum used for cell culture, together with the tendency of Vn to adsorb to plastic surfaces (Holmes, 1967; Hayman et al., 1985), raises the possibility that serum-derived Vn may be responsible, in large part, for the presence of PAI-1 in matrices prepared from other cells as well. This hypothesis is supported by immunofluorescence studies which indicate that matrix-associated PAI-1 exists as a "rather homogenous carpet" under cells (Pollanen et al., 1987; Schleef et al., 1990). This is the predicted behavior of proteins adsorbed to the plastic substratum, and not characteristic of matrix fibrils. We are currently investigating the possibility that Hep 3B cells which produce both PAI-1 and Vn (Fig. 3) may be able to deposit PAI-1 into the ECM in the absence of serum.

It should be mentioned that there may be some PAI-1 in the ECM that is not associated with Vn. For example, preliminary examination of cross sections of BAEs by immunogold EM using antibodies to PAI-1 (Podor and Loskutoff, unpublished observation) indicates that PAI-1 is distributed in two distinct pools in the ECM. Although the majority is clearly associated with Vn on the culture substratum, a second class of PAI-1 was detected in clusters between fibronectin fibrils. This second minor class of PAI-1 may be associated with another protein, one actually synthesized by the cells and deposited into the ECM. This possibility is currently under investigation.

The generality of the observation that PAI-1 is present in the ECM of cultured cells has raised the possibility that PAI-1 may also be present in the ECM in vivo, and has led to the suggestion that cells may deposit PAI-1 into their matrix as a specific mechanism to protect protease-sensitive structures from cell- and plasma-derived proteinases (Dano et al.,

1985; Laiho et al., 1986; Knudsen et al., 1987). The demonstration that much of this PAI-1 is adsorbed to serum-derived Vn would seem to decrease the physiological significance of this observation in vivo. In spite of this, Vn has been detected in the ECMs of various tissues (Hayman et al., 1983), including the vessel wall (Guettier et al., 1989; Niculescu et al., 1989). The unique distribution of Vn in the vessel wall suggests that it is actually produced by vascular cells. The observation that bovine and human endothelial cells do not produce Vn (Fig. 3) implies that the Vn in the vessel wall is synthesized by other vascular cells (e.g., smooth muscle cells). Whether PAI-1 is also present in the vessel wall remains to be investigated. The actual site of synthesis of Vn in vivo in unclear, although plasma Vn levels are reduced in patients with liver disease (Kemkes-Matthes et al., 1987) suggesting a liver origin. The observation that two hepatoma cell lines (Hep 3B; Fig. 3) and Hep G2 (Barnes and Reing, 1985) produce significant amounts of Vn, supports this idea.

In summary, these results indicate that the pericellular distribution of PAI-1 in cultured cells actually reflects the composition of the ECM itself, and that this composition is, in turn, influenced by serum. According to this hypothesis, if PAI-1 binding proteins (i.e., Vn) are present in the ECM but not in the culture medium, then newly synthesized PAI-1 will bind to them and rapidly accumulate there. This would be the situation for cells cultured initially in complete medium and then placed in serum-free medium for various times. Many of the matrix preparations previously shown to contain substantial amounts of PAI-1 (Laiho et al., 1986; Levin and Santell, 1987; Mimuro et al., 1987; Rheinwald et al., 1987) were in fact prepared from cells cultured in this way. Since the interaction between Vn and PAI-1 is reversible (Mimuro et al., 1987; Declerk et al., 1988; Mimuro and Loskutoff, 1989*a*; Salonen et al., 1989; Wun et al., 1989), and since latent PAI-1 has a relatively low affinity for Vn (Declerk et al., 1988; Mimuro and Loskutoff, 1989*b*), latent PAI-1 should accumulate in the medium as active PAI-1 is spontaneously released from the ECM, and this seems to be the case (Fig. 6). It is well known that latent PAI-1 accumulates in the serum-free conditioned medium of a variety of cells (Hekman and Loskutoff, 1985; Loskutoff et al., 1989). Another prediction from this model is that when cells are cultured continuously in the presence of serum, active PAI-1 will accumulate directly into the medium. In this case, serum Vn should compete with the Vn in ECM for binding to active PAI-1 and thus minimize its deposition into ECM. The active PAI-1 in the medium should then be stabilized (Mimuro et al., 1987; Declerck et al., 1988) by the high concentrations of Vn present in the serum. Preliminary observations indicate that active PAI-1 accumulates in the medium of cells cultured in the presence of serum (Lindahl and Wiman, 1989).

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