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Human Endothelial Cells Grow Poorly on Vitronectin: Role of PAI-1

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Abstract The cell adhesive protein vitronectin is a common component of interstitial extracellular matrix and circulates in plasma. It competes effectively with other plasma proteins to adsorb to certain biomaterial surfaces, and is likely to represent an important cell adhesion mediator on the luminal surface of vascular grafts. It is also found associated with certain vascular pathologies. We have shown previously that human endothelial cells grow poorly on a vitronectin surface compared with other extracellular matrix molecules. In this paper we show that endothelial cells seeded on vitronectin and fibronectin produced substantially different profiles of extracellular matrix molecules. The most outstanding difference was in the amount of matrix-localised plasminogen activator-inhibitor-1 which was high on vitronectin and negligible on fibronectin. This was correlated with a small but significant inhibition of cell adhesion to vitronectin compared with fibronectin, and very significant interference with dissociation of cell: extracellular matrix contacts, resulting either from direct inhibition of the proteolytic activity of urokinase, or from interference with urokinase-receptor signaling and consequent focal adhesion turnover. Such interference would inhibit cell proliferation by disabling the cells from loosening their matrix contacts in order to proceed through mitosis. This would seriously compromise endothelial recovery in cases of damage to the vascular wall and placement of stents or grafts, where the presence of surface-adsorbed vitronectin is likely to modulate the tissue response. J. Cell. Biochem. 82: 98–109, 2001. © 2001 Wiley-Liss, Inc.

Key words: endothelial; vitronectin; PAI-1; uPA; uPAR; cell adhesion

The cell adhesive protein vitronectin is a common component of interstitial extracellular matrix and circulates in plasma at concentrations around 400 μg/ml [Boyd et al., 1993]. Increased vitronectin deposition has been described at sites of wound healing and pathologies of the vascular system such as atherosclerosis [Nicolescu et al., 1987, 1989]. The influence of vitronectin deposited on the artery wall following damage due to procedures such as angioplasty or stent placement, upon endothelial recovery is unknown. As a molecule with high surface activity, vitronectin is able to outcompete other plasma proteins and adsorb to biomaterial surfaces [Bale et al., 1989; Horbett, 1994]. It is likely to represent an important cell adhesion mediator on the luminal surface of vascular grafts, where it may affect endothelial colonisation of the surface and long term patency rates.

In previous studies we have described poor proliferation of human endothelial cells on coated vitronectin compared with other extracellular matrix (ECM) molecules such as fibronectin, laminin, and collagen types I and IV [Underwood and Bean, 1996]. This poor growth was correlated with relatively low levels of urokinase (uPA), and high levels of its inhibitor plasminogen inhibitor-1 (PAI-1) in the cell layer and ECM of endothelial cells cultured on vitronectin. There has been considerable interest in recent years in the interplay between vitronectin, PAI-1, integrins, and the cellular receptor for uPA (uPAR: for reviews see Hess et al., 1995; Chapman, 1997; Loskutoff et al., 1999). PAI-1 and uPAR each bind to vitronectin in the same region of its N-terminal domain

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[Deng et al., 1996] and occupancy of uPAR with uPA increases its affinity for vitronectin [Wei et al., 1994]. The RGD-containing binding site for αv integrins on vitronectin is adjacent to the N-terminal domain [Suzuki et al., 1985]. When cells attach to vitronectin via integrin receptors, uPA/uPAR is found co-localised with integrins in focal contacts [Ciambrone and McKeown-Longo, 1992; Conforti et al., 1994; Wilcox et al., 1996]. There is some evidence that occupancy of uPAR with uPA results in intracellular signalling, and phosphorylation of proteins regulating focal adhesion turnover [Tang et al., 1998]. Localisation of uPA/uPAR in focal contacts also provides a source of uPA to initiate proteolytic cascades for local dissolution of ECM.

PAI-1 can inhibit the binding of either uPAR or integrins to vitronectin, by steric hindrance [Deng et al., 1996; Stefansson and Lawrence, 1996]. PAI-1 is a labile molecule in solution with a short half life converting the active inhibitor to an inactive form [Hekman and Loskutoff, 1985]. It is the active form of PAI-1 which binds to vitronectin and its activity is stabilised while bound [Loskutoff et al., 1999]. This provides a souce of active PAI-1 in the ECM of cells grown on vitronectin, available for either direct inhibition of uPA activity or steric hindrance of cell adhesion. Furthermore, PAI-1 binding to uPA/uPAR induces internalisation of the whole complex via the low-density-lipoprotein receptor-related protein (LRP) [Chapman, 1997], thus interfering with cytoplasmic signaling via uPA/uPAR.

The cycle of proliferation of tissue cells requires that cells initially attached to the substratum (integrin binding to ECM), spread and flatten to initiate DNA synthesis (focal contact formation), and finally change cell shape and round up to go through mitosis (dissolution and/or disengagement of focal contacts). The daughter cells then migrate out and re-initiate the cycle. Thus excessively high PAI-1: uPA ratios on a vitronectin substratum could interfere with this process at several points; cell adhesion, proteolysis, and focal contact turnover. These alternatives are illustrated in Figure 1. The present report investigates the production and compartmentalisation of PAI-1 and ECM molecules by endothelial cells cultured on vitronectin and fibronectin respectively, and the ability of this endogenous PAI-1 to interfere with cell adhesion and proteolytic release from focal contacts.

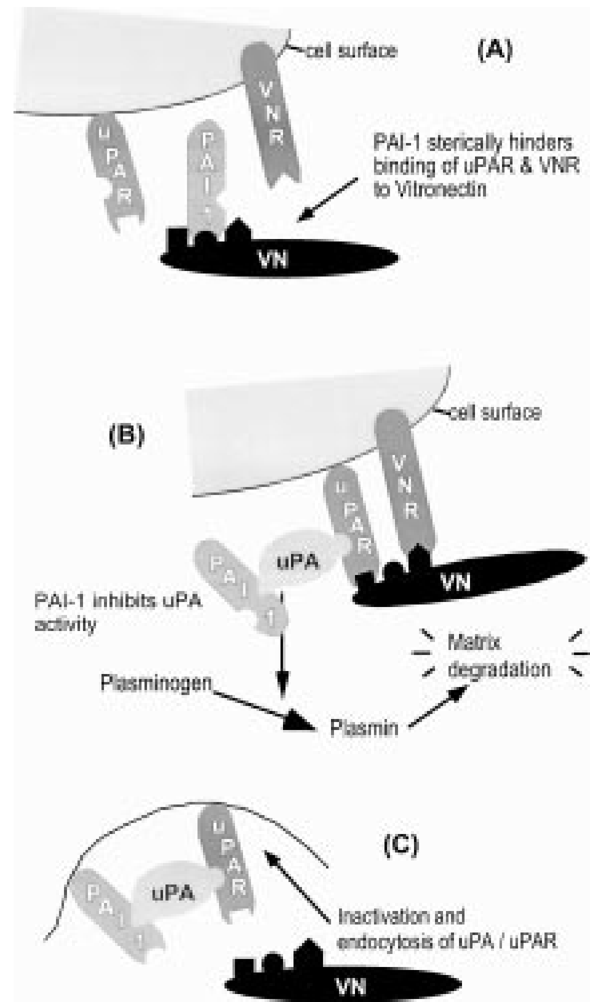

Fig. 1. Diagram of ways in which PAI-1 might interfere with cell surface activity on vitronectin. **A:** Inhibition of cell adhesion to vitronectin. **B:** Prevention of local dissolution of ECM by inhibition of uPA enzyme activity **C:** Prevention of uPAR signalling by internalisation of uPA/uPAR.

MATERIALS AND METHODS

Bovine fibronectin used to coat surfaces for routine cell culture was partially purified from fresh bovine plasma by affinity chromotography on gelatin Sepharose (AMRAD Pharmacia Biotech, Melbourne, Victoria, Australia) as described by Ruoslhati et al., [1982]. Bovine vitronectin was purified from bovine serum using a monoclonal antibody affinity column as previously described [Underwood and Bennett, 1989]. Purified bovine fibronectin (F-4759 from Sigma, Sydney, N.S.W., Australia) and vitronectin (prepared as above), were used to coat test surfaces. Stock solutions of ECM molecules were stored in sterile aliquots at −70°C. Human recombinant PAI-1 active mutant was

from American Diagnostica (Greenwich, CT). Human plasminogen (P5661), plasmin (P4895), tranexamic acid (A6516), and ITS+2 (I2646) were obtained from Sigma. Human recombinant basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were from R&D Systems Inc. (Minneapolis). Fibronectin and laminin peptides GRGDS and YIGSR respectively were from Peninsula Labs (Belmont, CA). All other chemicals were Analar grade. Tissue-culture polystyrene and 24-well bacteriological grade plates were obtained from Nunc (Roskilde, Denmark) and bacteriological grade 96-well Linbro-Titertek microtitration plates were from ICN Biomedicals (Australasia). Flexible polyvinyl U shaped ELISA plates were from Dynex Technologies (Chantilly, VA).

Antibodies

Anti human-specific fibronectin monoclonal antibody (MAb) clone 1, MAb clone III anti laminin a1 chain and rabbit antiserum to decorin were from Gibco BRL Life Technologies (Melbourne, Victoria, Australia). MAb BA4 anti elastin, MAb COL 94 anti type collagen IV and MAb CS56 anti chondroitin sulphate from Sigma (Sydney, N.S.W., Australia). MAb A76 anti perlecan, MAb A65M anti thrombospondin, and MAb A21 anti laminin b1 were prepared in this laboratory from mice immunised with endothelial cell extracellular matrix [Underwood et al., 1990]. The characterisation of the former two has been previously described [Matthias et al., 1996; Whitelock et al., 1996]. The specificity of the anti laminin MAb was determined by immunoprecipitation, blocking of immunoprecipitation of purified laminin by a polyclonal antibody, and comparison of immunostaining of kidney and vascular tissue with that reported for anti b1 laminin antibodies by Sanes et al. [1990]. These MAbs were affinity purified from mouse ascites on protein A Sepharose (AMRAD Pharmacia Biotech, Melbourne, Australia). MAb D7 anti laminin b2 chain [Sanes et al., 1990] was a gift from Dr. J. Sanes, Washington University School of Medicine, St Louis, Missouri, U.S.A. MAb 5D5 anti versican [Hakkinen et al., 1993] was a gift from Dr. F. Rahemtulla, University of Alabama, Burmingham, Alabama, U.S.A. MAbs 5D8/G9, 2G8/B1, and 1E2/E4 anti collagen types I, III and V respectively, [Werkmeister et al., 1989], were gifts from Dr. J. Werkmeister, CSIRO Molecular Science. Anti PAI-1 Mab (No. 3785) was from American Diagnostica. Non-specific mouse immunoglobulins or rabbit serum were used as negative controls. Biotinylated anti mouse or rabbit immunoglobulins and peroxidase-conjugated streptavidin were from Amersham Pharmacia Biotech (Melbourne, Australia). Peroxidase-conjugated rabbit anti mouse immunoglobulins were from Dako (Sydney, Australia).

Treatment for Removal of Endotoxins

All solutions used in tissue culture were prepared using pyrogen free water (Baxters Diagnostics, Arcadia Ridge, Queensland, Australia) and filtered through Zetapore 0.2 μm nylon membranes (Cuno Pacific Pvt. Ltd., Sydney, N.S.W., Australia). All re-usable glassware was treated with E-toxa-clean (Sigma) to remove pyrogens, and following several washes in tap water and distilled water, was rinsed in pyrogen free water before sterilization by autoclaving at 120°C for 1 h followed by dry heat sterilization at 170°C for 3 h.

Cell Culture

Primary cultures of human umbilical arterial endothelial cells (HUAECS) were prepared from fresh umbilical cords delivered by Caesarian section at Royal North Shore Hospital, Sydney, as described [Weis et al., 1991] using 0.1% collagenase (Sigma C6885). Cells were routinely grown in tissue culture flasks precoated for 2 h at 37°C with bovine FN at 10 μg/ml (5 ml/75 cm^2 flask). The culture medium was Medium 199 with Earle's Salts (Gibco, Life Technologies Inc.) containing 100 U/ml penicillin, 100 μg/ml streptomycin sulphate, 100 μg/ml heparin (Sigma H3149), 2% bovine brain extract, prepared according to Maciag et al. [1979], and 20% low endotoxin foetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Victoria, Australia). Cells were passaged at a 1:3 split ratio after disaggregation with 0.125% trypsin, 0.02% EDTA (T/EDTA), and used between passages 6 and 10. In most experiments cells were cultured in medium containing FCS depleted of vitronectin and fibronectin. Depletion was sequential using MAb and gelatin Sepharose affinity columns as described above for the purification of these molecules. The double-depleted serum (DDS) was tested for residual vitronectin and fibronectin using dot blots and ELISAs. Remaining activity was less than 1% of the starting level,

and was considered negligible for the present work.

Cell Proliferation

Wells of 96-well tissue culture plates were coated with 50 μl volumes of vitronectin or fibronectin at 10 μg/ml in phosphate buffered saline (PBS), for 2 h at 37°C. The wells were then blocked with 1% bovine serum albumin (BSA) in PBS (blocking buffer) for 1 h at 37°C and washed with serum-free medium 199. HUAEC were suspended in culture medium containing 10% DDS (experimental medium) and seeded at 3×10^3 cells /well. Cultures were maintained for 6–7 days with medium changes at Day 3 or 4. Cell density was measured on Day 0, 1, 4, and 6/7 by fixing the cells with 4% formaldehyde in PBS and staining with crystal violet (C.I. 42555) as previously described [Kueng et al., 1989; Underwood et al., 1992]. Absorbances of solubilised dye were read on a Bio Rad 3550 plate reader at T 595 nm, R 405 nm. Replicate wells were used to prepare cell-free extracellular matrix (ECM) for the estimation of ECM-bound PAI-1 (see below). Conditioned medium collected on Day 3 and 6 was used to estimate the concentration of fluid phase active PAI-1 (see below).

Preparation of ECM for Assay of Components

96-well tissue culture plates were coated with vitronectin or fibronectin as described for the proliferation assay above, and seeded with HUAEC at 10^4/well (approaching confluence) in experimental medium. Cultures were maintained for 6 days with a medium change at Day 3. On Day 1, 3, and 6 triplicate wells were treated with hypotonic ammonium hydroxide to remove cells and expose underlying ECM, as described by Gospodarowicz and Lui [1981]. Wells were washed with PBS and stored under minimal PBS at 4°C overnight or −70°C for longer periods. The presence of various ECM molecules was detected by ELISA as described by Underwood et al. [1998] using concentrations of primary antibodies selected to detect changing concentrations of ECM deposition with time (i.e., not reagent limited). Secondary antibodies (all at 1/1000 dilution) were peroxidase conjugated rabbit anti mouse for antibodies to PAI-1, human fibronectin and type IV collagen; biotinylated anti rabbit for the antibody to decorin, and biotinylated anti mouse for the rest. Peroxidase conjugated streptavidin was used following biotin conjugates, at 1/500 dilution. Reagents were diluted in blocking buffer and intervening washes were in PBS. Primary, secondary and tertiary reagents were incubated at room temperature for 2 h, 1.5 h, and 0.75 h respectively on a rotary shaker. The substrate was diammonium-2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, Sigma) at 1.1 mg/ml in 0.05M citrate pH 4.5, stopped by the addition of $\frac{1}{4}$ volume of 6% oxalic acid. Absorbance was read at T 405 nm and R 490 nm.

Preparation of ECM for Cell Adhesion and Cell Spreading Assays

96-well tissue culture plates were coated with vitronectin or fibronectin as described for proliferation assays. HUAEC were added at 10^4 / well and cultured for 2 days in experimental medium. Replicate coated wells were incubated with experimental medium or PBS in the absence of cells for the same period. All wells were treated with ammonium hydroxide for preparation of ECM as above, washed with PBS and used immediately for cell adhesion and cell spreading assays or assay of ECM-bound PAI-1.

Assays of PAI-1

a) Binding of PAI-1 active mutant to coated vitronectin. The PAI-1 active mutant is stable and does not convert to the latent form. It shows the same inhibitory activity for uPA and ability to bind vitronectin as native active PAI-1. 96-well tissue culture plates were coated with vitronectin or fibronectin and blocked as for proliferation assays described above. Serial dilutions of PAI-1 mutant from 2 μg/ml, in 1% BSA in PBS were added and incubated for 2 h at room temperature on a rotary shaker. Bound PAI-1 was detected using anti PAI-1 antibody at 1/500 dilution and ELISA conditions as described above for ECM components. b) PAI-1 secreted by HUAEC and bound to the ECM was assayed directly on ECM-wells by ELISA as described above. c) PAI-1 secreted by HUAEC into the overlying medium. Vitronectin was coated at 5 μg/ml into the wells of flexible polyvinyl ELISA plates, overnight at 4°C to give a saturated protein monolayer. Following blocking with 1% BSA in PBS, HUAEC-conditioned medium was incubated in the wells for 2 h at room temperure. Bound PAI-1 was detected by ELISA as described above.

Note that in (b) and (c) only active PAI-1 is detected since vitronectin in the ECM or coated on wells only binds the active form [Loskutoff et al., 1999].

Cell Adhesion

96-well polystyrene plates were coated with fibronectin or vitronectin at 2.5 and 1.5 μg/ml, respectively, in PBS overnight at 4°C. These concentrations were predetermined for maximal sensitivity of HUAEC cell adhesion. Wells were blocked with 1% BSA in PBS for 1 h at 37°C. HUAECs were seeded at 3×10^4 /well in experimental medium containing the peptides GRGDS or YIGSR at 200 μg/ml, or concentrations of PAI-1 active mutant between 0 and 2 μg/ml. After 2 h incubation at 37°C, adherent cells were fixed and stained with crystal violet. Adhesion of HUAECs was estimated colorimetrically as described for cell proliferation above, having validated this method for HUAECs by comparison with directly counted trypsinised cells. Cell adhesion on HUAEC ECM was done on prepared ECM plates (see preparation of ECM above).

Measurement of Areas of Individual Spread Cells

Wells of 24-well polystyrene plates were coated with 1 ml volumes of fibronectin or vitronectin as described for adhesion. 1×10^4 HUAECs were added per well in experimental medium with or without PAI-1 at 4 μg/ml and incubated at 37°C for 2 h. The cells were then fixed and stained with crystal violet as described for cell adhesion. The cells were photographed in the light microscope at 20 fold magnification. Images from the negatives were transfered to a Quantimet 570 image analysis system (Cambridge Instruments) and the areas of at least 50 randomly selected cells per treatment were determined. Cell areas were measured on prepared ECM (see above) in the same way.

Effect of Plasminogen on Cell Adhesion

24-well tissue culture plates were coated with 1 ml/well of vitronectin or fibronectin at 10 μg/ml in PBS for 2 h at 37°C and blocked with 1% BSA in PBS. HUAEC were treated as described by Wilcox et al. [1996]. Briefly, they were dispersed with trypsin/EDTA, washed twice with serum-free 199, resuspended in this medium supplemented with 1 mM tranexamic acid for 2 min to remove surface plasmin, centrifuged and resuspended in 199 containing 1% ITS+2, 20 ng/ml bFGF and 10 ng/ml EGF plus antibiotics. They were seeded at 5×10^4/well in 1 ml medium and incubated for 24 h at 37°C. The medium was replaced with fresh medium containing either plasmin or plasminogen at 0, 10, or 100 μg/ml and incubated further for 24 h. The cultures were then washed with PBS and fixed and stained with crystal violet. Three non contiguous areas per well were photographed at 20 fold magnification and the negatives were imaged in the image analyser. Percent of the culture area covered with cells was estimated for a total of nine areas per treatment.

Statistical Analysis

Each experiment was done a minimum of three times. Statistical significance was estimated using the Analysis of Variance (ANOVAR) and Student Newman Keul's (SNK) test or Student's t test where appropriate.

RESULTS

As we have previously shown [Underwood and Bean, 1996] the proliferation rate of HUAEC was significantly reduced on a vitronectin substratum compared with fibronectin ($P < 0.01$, Student's t test). The difference in growth rates became more pronounced with culture time, growth on vitronectin at 7 days culture being only 50 – 60% of that observed on fibronectin. The corresponding ECM profiles from cells seeded close to confluence and cultured for 6 days are shown in Figure 2. There were considerable differences in the nature of the ECM laid down on these two surfaces and these differences also became more marked between 1 and 6 days culture. Significantly less fibronectin and thrombospondin were secreted onto vitronectin, but more elastin. ($P < 0.05$, Student's t tests). Components of laminin 1 and s-laminin were all elevated on vitronectin. Chondroitin sulphate was elevated on vitronectin but the heparan sulphate proteoglycan, perlecan was reduced. Significantly more collagen types I and III were secreted onto vitronectin but less collagen type IV. The most outstanding difference in the ECM profile was in PAI-1 which was present at much higher levels on vitronectin than fibronectin. The amounts of PAI-1 bound to the ECM, or active PAI-1 present in the overlying medium, from

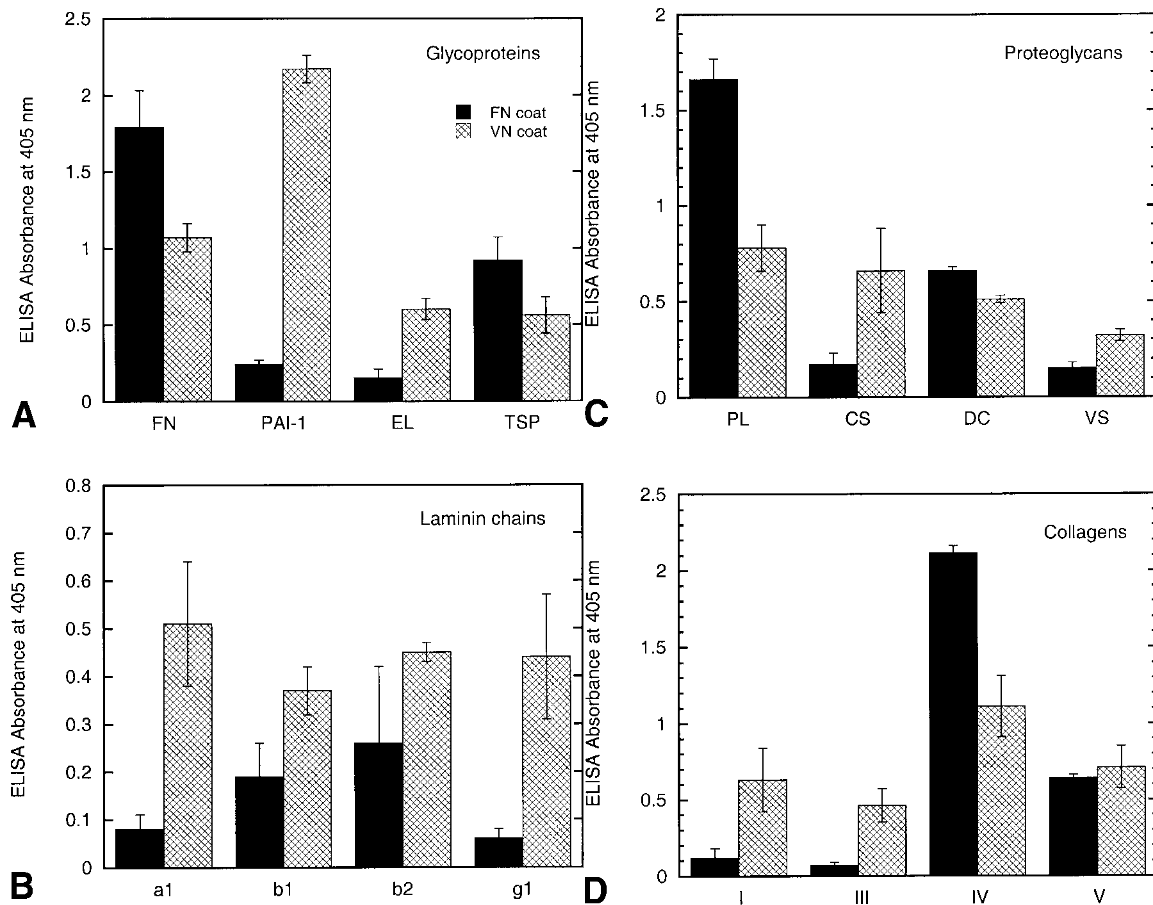

Fig. 2. Profile of ECM components from HUAEC grown on vitronectin or fibronectin coated surfaces for 6 days. Wells of 96-well tissue culture plates were coated with vitronectin or fibronectin for 2 h at 37°C as described in Materials and Methods. HUAEC were seeded at 10^4/well (near confluence) and cultured at 37°C. ECM was prepared from replicate samples at 1, 3, and 6 days incubation. Individual ECM components were detected by ELISA with specific antibodies. Bars are mean ABTS absorbances and SEM of 6-day determinations from three separate experiments. Key to abbreviations: FN fibronectin, EL elastin, TSP thrombospondin, a1 laminin alpha 1 chain, b1 laminin beta 1 chain, b2 laminin beta 2 chain g1 laminin gamma 1 chain, PL perlecan, CS chondrotin sulphate, DC decorin, VS versican, collagen types I, III, IV & V.

cells grown under the same experimental conditions as for estimating proliferation are shown in Figure 3. On the vitronectin surface most of the PAI-1 was bound in the ECM, with very low levels present in the medium. On fibronectin the reverse was true with most of the PAI-1 present in the medium and very little bound in the ECM.

Since the PAI-1 sequestered in the ECM was presumably binding to vitronectin, we estimated the saturation level of PAI-1 bound to coated vitronectin, using a constitutively active mutant of PAI-1. The binding curves of this mutant to vitronectin and fibronectin (as the non-specific control) are shown in Figure 4. Specific binding of PAI-1 to vitronectin saturated at a loading concentration of 0.2–0.3 μg/ml PAI-1. The level of PAI-1 measured in the ECM during the growth of HUAEC on vitronectin (Fig. 3) suggested that the coated vitronectin readily became saturated with PAI-1 during the period of the growth assay, and that the surface concentration of PAI-1 was unlikely to exceed that obtained from an overlying concentration of 0.2–0.3 μg/ml. We then investigated the effects of a range of concentrations of added mutant PAI-1 on HUAEC adhesion to coated vitronectin and fibronectin. The purpose of this was to determine whether levels of PAI-1 secreted by HUAEC and bound locally by coated vitronectin, were sufficient to significantly interfere with cell adhesion, and to compare the results with other published studies using exogenous PAI-1. The results are shown in Figure 5. It is clear that added PAI-1

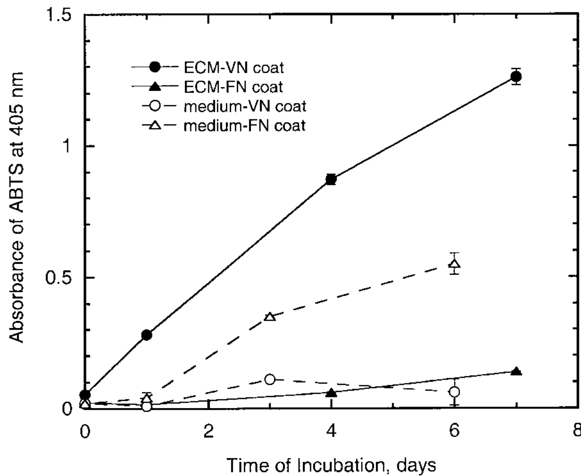

Fig. 3. Estimation of active PAI-1 secreted into the ECM or overlying medium from HUAEC proliferating on vitronectin or fibronectin. Wells of 96-well tissue culture plates were coated with vitronectin or fibronectin for 2 h at 37°C as described in Materials and Methods. HUAEC were seeded at 3×10^3 cells/well and cultured at 37°C. At the indicated time intervals ECM was prepared or conditioned medium sampled. PAI-1 in the ECM was estimated by ELISA as for Figure 2. Active PAI-1 in the culture medium was estimated by capture on coated vitronectin and ELISA. Points are the mean ABTS absorbances and SEM of triplicate determinations in one representative experiment.C

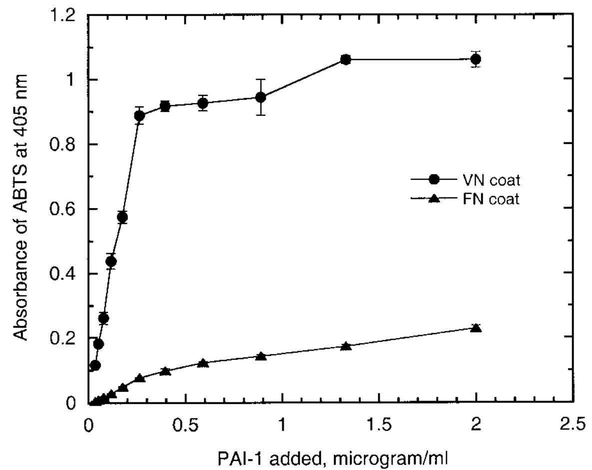

Fig. 4. Binding of active mutant PAI-1 to coated vitronectin and fibronectin. Tisue culture plates were coated with vitronectin or fibronectin as for Figure 2. Serial dilutions of active mutant PAI-1 were added and bound PAI-1 detected by ELISA. Points are the mean ABTS absorbances and SEM for four replicates per treatment in one representative experiment.

did not affect the adhesion of HUAEC to fibronectin ($P > 0.05$, ANOVAR and SNK test). At a concentration of 2 μg/ml of added PAI-1 there was substantial (~30%) inhibition of cell adhesion to vitronectin, while at 0.2 μg/ml the inhibitory effect was still significant but smaller. This implies that at saturating concentrations (~0.3 μg/ml) on vitronectin, ECM-bound PAI-1 would be likely to exert a minor inhibitory effect on HUAEC adhesion to vitronectin. The positions of Y and R in Figure 5 are the percent cell adhesion to vitronectin in the presence of the laminin peptide YIGSR (negative control) or vitronectin/fibronectin peptide GRGDS, resccpectively. The complete inhibition of cell adhesion to vitronectin by GRGDS suggests that adhesion was integrin mediated. Added PAI-1 was only capable of inhibiting cell adhesion to vitronectin when added together with the cells and was ineffective if added to the coated vitronectin first, with removal of unbound PAI-1 before addition of cells. This implies a competitive inhibition mechanism with affinity of binding in favour of the integrin. Mutant PAI-1 added at 2 μg/ml reduced HUAEC adhesion to vitronectin but did not affect the subsequent spreading of adhered cells, which was similar on fibronectin and vitronectin +/− PAI-1 (data not shown).

Having established that saturation binding of PAI-1 to vitronectin was likely to cause minimal

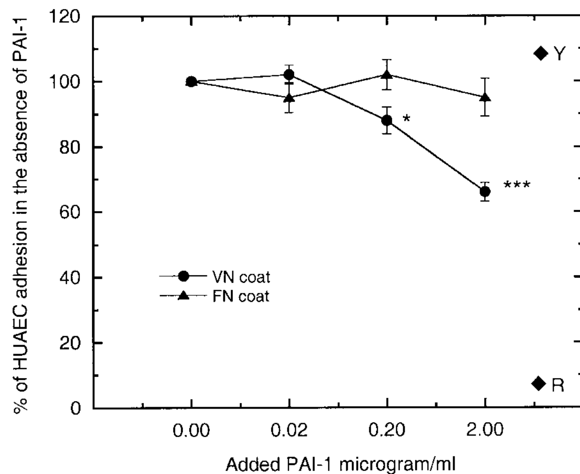

Fig. 5. Effect of added PAI-1 on HUAEC adhesion to coated vitronectin or fibronectin. Wells of 96-well polystyrene plates were coated with vitronectin or fibronectin overnight at 4°C as described in Materials and Methods. HUAEC (at 3×10^4/well) were added in the presence of active mutant PAI-1 at the concentrations indicated and cell adhesion estimated after 2 h by crystal violet staining of adhered cells. Points are the mean percent cell adhesion (100% = level of adhesion in absence of inhibitor) and SEM of determinations from three separate experiments. The letters Y and R denote the percent adhesion in the presence of 200 μg/ml YIGSR and GRGDS peptides respectively. * and *** indicate significant inhibition of adhesion to vitronectin by PAI-1 ($P < 0.05$ and < 0.001 respectively, Analysis of Variance and Student Newman Keul's test).

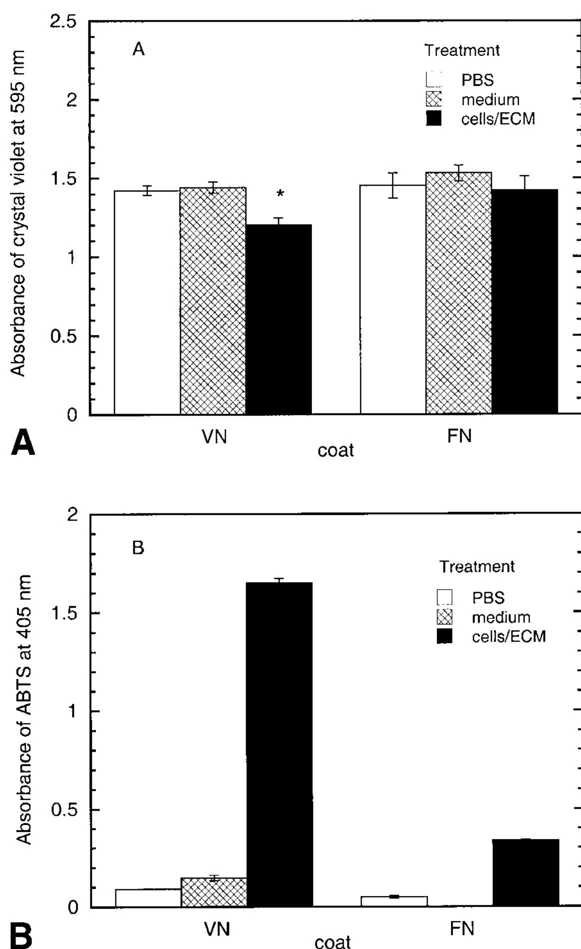

Fig. 6. Effect of underlying coating on HUAEC adhesion to ECM, and level of PAI-1 in ECM. Wells of 96-well tissue culture plates were coated with vitronectin or fibronectin as for Figure 2. Some wells were seeded with HUAEC at 10^4/well (near confluence) and incubated for 2 days. Replicate wells were treated with experimental medium lacking cells, or PBS for 2 days. ECM was prepared for all wells by hypotonic ammonium hydroxide treatment. **A:** The wells were used to estimate cell adhesion of fresh HUAEC as for Figure 5 or **B:** assayed for bound PAI-1 by ELISA as for Figure 2. Bars are the mean crystal violet (A) or ABTS (B) absorbances and SEM for four replicate treatments in one representative experiment. Filled bars are HUAEC ECM, hatched bars are medium only wells and clear bars are PBS treated wells. * Significant difference from PBS control ($P < 0.05$, Analysis of Variance and Student Newman Keul's test).

inhibition of adhesion of HUAEC, we investigated the adhesive capacity of ECM secreted onto vitronectin or fibronectin by HUAEC seeded at near-confluence. Surfaces were coated with vitronectin or fibronectin and subsequently incubated with PBS, experimental medium, or HUAEC in experimental medium for 2 days to allow ECM deposition by the cells. After incubation the surfaces were treated to remove the cells, leaving the ECM intact. The adhesion of fresh HUAEC to these surfaces was compared. The results are shown in Figure 6a. There was no significant difference between HUAEC adhesion to vitronectin or fibronectin coated surfaces treated with PBS or culture medium, or fibronectin coated surface with HUAEC ECM deposited on top ($P > 0.05$, ANOVAR & SNK test). There was a small but significant ($P < 0.05$, ANOVAR & SNK test) reduction in HUAEC adehsion to ECM deposited on vitronectin, similar to that observed with addition of exogenous PAI-1 at 0.2 μg/ml (Fig. 5). Saturation levels of PAI-1 were present in the ECM on the vitronectin surface (Fig. 6b). These results, like those with added PAI-1 (above) suggest that PAI-1 bound to the ECM exerted a minimal inhibitory effect on HUAEC adhesion.

In order to investigate the degree of interference of endogenous PAI-1 with plasminogen activation and pericellular proteolysis, we investigated the sensitivity of HUAEC seeded on vitronectin and fibronectin to added plasminogen in serum-free conditions. The results are shown in Figure 7. There was no significant

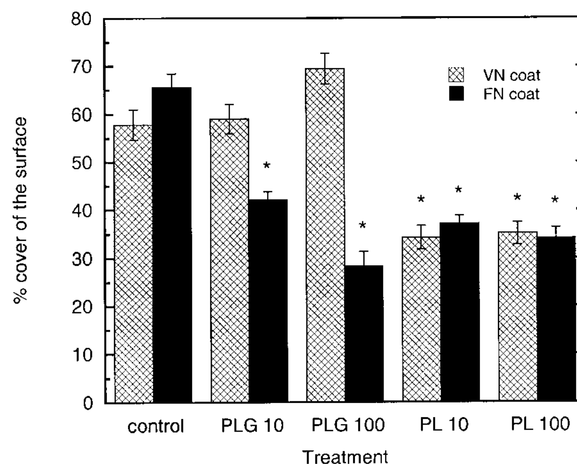

Fig. 7. Effect of plasminogen on HUAEC persistence on fibronectin or vitronectin. Wells of 24-well tissue culture plates were coated with vitronectin or fibronectin as described in Materials and Methods. HUAEC were seeded in serum-free medium as described in Methods and incubated overnight. Medium was replaced with fresh medium containing additions of plasminogen (PLG) or plasmin (PL) at 10 or 100 μg/ml and incubation continued for a further 24 h. The extent of cell coverage of the culture area was determined by image analysis of crystal violet stained cells. Bars are the mean % cell covers and SEM of 9 replicate areas per treatment for one representative experiment. * Significantly different from control ($P < 0.05$, Analysis of Variance and Student Newman Keul's test).

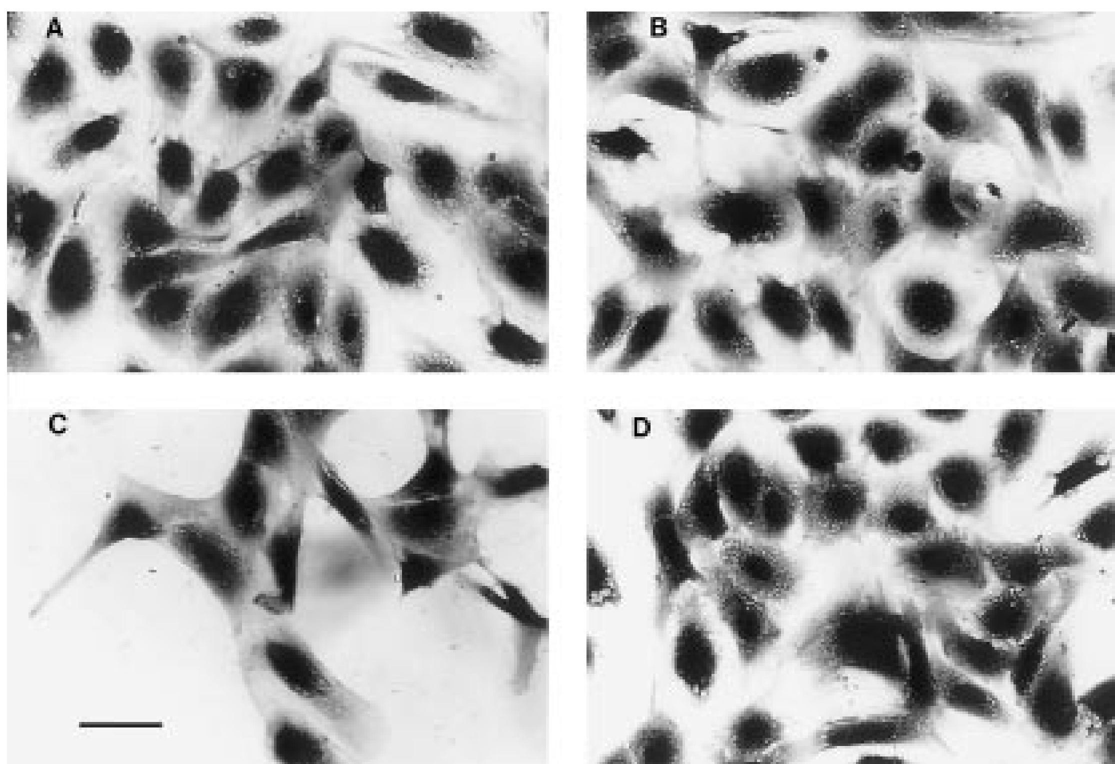

Fig. 8. Light micrographs of effect of plasminogen on HUAEC persistence on fibronectin or vitronectin. Tissue culture surfaces were coated with vitronectin or fibronectin as for Figure 7. HUAEC were seeded and treated with plasminogen at 10 μg/ml as for Figure 7. Light micrographs of stained cells. Bar is 50 microns. **A**, **C** fibronectin coat; **B**, **D** vitronectin coat; C, D treated with plasminogen.

difference in the extent of cell cover between vitronectin or fibronectin substrata in control media ($P > 0.05$, ANOVAR SNK test). In the presence of plasminogen, cells on fibronectin retracted their surface contacts and some became detached (also shown in Figure 8), reducing the extent of cell cover significantly ($P < 0.05$, ANOVAR & SNK test). In contrast, there was no change in the shape or surface contact with cells on vitronectin (Figs. 7 and 8). When active plasmin was added to the cultures a similar degree of cell retraction and detachment was seen on both surfaces. This result suggests that the PAI-1 bound to the vitronectin surface inhibited the activation of plasminogen by cell-surface uPA, whereas the fibronectin surface afforded no such protection. In contrast, active plasmin had a similar effect on both surfaces.

DISCUSSION

In this study we have demonstrated that HUAEC cultured on vitronectin and fibronectin substrata produce significantly different profiles of ECM molecules. In previous studies we have shown that different profiles of ECM molecules produced by different cell types significantly affect the behaviour of HUAEC [Underwood et al., 1998]. The most striking difference in ECM output from HUAEC on vitronectin compared with fibronectin was in the relative amounts of uPA (high on fibronectin) and PAI-1 (high on vitronectin) [Underwood and Bean, 1996 and present study]. These molecules play important roles in both cell adhesion and local extracellular proteolysis [reviewed by Hess et al., 1995; Chapman, 1997]. We have attempted to determine the effects of physiologically relevant concentrations of PAI-1 on endothelial cell function. Cells can adhere to vitronectin either via cell surface integrins or uPAR. Our experiments suggested that HUAEC were adhering via integrins, as has been reported for other endothelial cells [Kanse et al., 1996], epithelial cells [Kjoller et al., 1997; Germer et al., 1998], and smooth muscle cells [Stefansson and Lawrence, 1996]. The concentration of PAI-1 required to inhibit integrin-mediated cell adhesion to vitronectin

by 30–50% has been reported as 100–200 nM (5–10 μg/ml) as compared to our finding of 30% inhibition of HUAEC adhesion at 2 μg/ml (40 nM). These concentrations of PAI-1 are 10–50 fold higher than the concentration of PAI-1 required to saturate coated vitronectin, determined in our experiments. We found that this lower concentration of PAI-1 (4–6 nM), still significantly inhibited HUAEC adhesion to vitronectin, but at the level of 10–15% inhibition, this was unlikely to make a major biological impact. Furthermore we found that it was necessary for PAI-1 to be present in solution at the time of addition of cells for inhibition to occur, and pre-incubated PAI-1 was insufficient. These results point to competition between PAI-1 and integrins for binding to vitronectin with affinity driving the interaction in favour of the integrins. Once bound to its ligand, the integrin-mediated adhesion is strengthened by the formation of focal contacts, making subsequent dissociation by PAI-1 less likely. This is in agreement with the findings of Germer et al. [1998] with epithelial cells, who reported that cell adhesion could not be reversed by later addition of PAI-1. This is in contrast to the efficiency of inhibition by PAI-1 of the uPAR-mediated adhesion of myeloid cells to vitronectin, where the affinity of PAI-1 for vitronectin is 30 fold higher than the affinity of uPAR for vitronectin, no focal contacts are formed and cell adhesion can be reversed as well as directly inhibited [Deng et al., 1996]. Our results show that HUAEC in culture secrete active PAI-1 when grown on a substratum of either vitronectin or fibronectin. Whereas on fibronectin the vast majority of active PAI-1 remains in the culture medium, on vitronectin it is all bound to the substratum. These findings are supported by those of others [Preissner et al., 1990; Seiffert et al., 1990; Ciambrone and McKeown-Longo, 1992], who demonstrated specific and selective binding of PAI-1 to vitronectin compared with other ECM proteins. Furthermore, HUAEC readily saturate the vitronectin surface with endogenous PAI-1. As we have shown, however, with both exogenous and endogenous PAI-1, these saturating concentrations (4–6 nM) are too low to exhibit substantial inhibition of HUAEC adhesion to vitronectin. It is therefore unlikely that interference by PAI-1 with cell adhesion is the major underlying mechanism for poor HUAEC growth on this substrate.

It is more likely that poor proliferation is due to PAI-1 interference with uPA activity. There are several findings supporting this proposition. Our results indicate that endogenous HUAEC PAI-1 secreted onto vitronectin inhibited the activation of added plasminogen to plasmin and protected the cell layer against local dissolution of ECM. PAI-1 secreted into the medium overlying cells cultured on fibronectin was unable to prevent cell-associated uPA from activating plasminogen to plasmin, with resultant dissolution of ECM and release of cells from the surface. This observation is in agreement with similar reports for other cell types [Ciambrone and McKeown-Longo, 1990; Wilcox et al., 1996]. While this result does not directly demonstrate that the enzyme activity of uPA is required for HUAEC proliferation per se, it does indicate that endogenous PAI-1 bound to vitronectin under these cells is capable of binding to uPA and inactivating it. There are a number of reports which suggest that the enzyme activity of uPA is required for proliferation of a number of human cell types such as epidermal and melanoma cells [Kirchheimer et al., 1989a, 1989b] and vascular smooth muscle cells [Stepanova et al., 1999]. It is still unclear whether this activity is required for dissolution of focal contacts, mobilisation of growth factors from ECM storage sites, or activation of other key proteins.

Dissolution, dissociation, or turnover of focal contacts is a critical pre-requisite of cell division. Conditions which increase the density of focal contacts decrease proliferation rates of endothelial cells. One example is high surface density of adhesion proteins such as fibronectin leading to formation of excessive focal contacts [Madri et al., 1988; Podesta et al., 1997]. We are beginning to understand some of the conditions controlling focal adhesion turnover. There are a number of reports of co-localisation of uPA-occupied uPAR and integrins in focal adhesions on vitronectin [Pollanen et al., 1987; Ciambrone and McKeown-Longo, 1992; Conforti et al., 1994]. A recent report by Tang et al. [1998] demonstrated that uPA occupancy of uPAR in endothelial cells resulted in activation (phosphorylation) of focal adhesion kinase (FAK). Evidence for the involvement of FAK in focal adhesion turnover comes from cells isolated from FAK-deficient mouse embryos, which display reduced mobility and significantly increased numbers of focal contacts [Ilic et al., 1995].

When PAI-1 binds to receptor-bound uPA, the whole complex is internalised by the low density lipoprotein related protein, thus interfering with uPA/uPAR signalling [Chapman, 1997]. This could provide an alternative mechanism whereby interaction of PAI-1 with cell-associated uPA could interfere with focal adhesion turnover and hence cell division, separately from its effect on uPA enzyme activity. In either event the outcome for the cells is likely to depend on the local balance between uPA and PAI-1. On a vitronectin substratum this is heavily skewed in favour of PAI-1 [Ciambrone and McKeown-Longo, 1992; Underwood and Bean, 1996].

In summary HUAEC seeded on vitronectin and fibronectin produced substantially different profiles of ECM molecules. The most outstanding difference was in the amount of ECM-localised PAI-1 which was high on vitronectin and negligible on fibronectin. This was correlated with very significant interference with dissociation of cell: ECM contacts, resulting either from direct inhibition of the proteolytic activity of uPA, or from interference with uPAR signaling and consequent focal adhesion turnover. This would seriously compromise endothelial recovery in cases of damage to the vascular wall and placement of stents or grafts, where the presence of surface-adsorbed vitronectin is likely to modulate the tissue response.

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