[CANCER RESEARCH 61, 5587–5594, July 15, 2001]

Plasminogen Activator Inhibitor-1 Promotes Angiogenesis by Stimulating Endothelial Cell Migration toward Fibronectin[1]

Chiho Isogai, Walter E. Laug, Hiroyuki Shimada, Paul J. Declerck, Monique F. Stins, Donald L. Durden,[2] Anat Erdreich-Epstein, and Yves A. DeClerck[3]

Departments of Pediatrics [C. I., W. E. L., M. F. S., D. L. D., A. E-E., Y. A. D.], Pathology [H. S.], and Biochemistry and Molecular Biology [Y. A. D.], Childrens Hospital Los Angeles and Keck School of Medicine, University of Southern California, Los Angeles, California 90027, and Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, 3000 Leuven, Belgium [P. J. D.]

ABSTRACT

Increased expression of plasminogen activator inhibitor-1 (PAI-1) in cancer patients is associated with unfavorable outcome, and the reason for this paradox has been poorly understood. We have previously reported elevated levels of PAI-1 in primary tumors of advanced neuroblastomas (Y. Sugiura *et al.*, Cancer Res., *59:* 1327–1336, 1999). Here we demonstrate that PAI-1 is coexpressed with the angiogenesis marker $\alpha_v\beta_3$ integrin in blood vessels of primary neuroblastoma tumors, suggesting that PAI-1 plays a role in angiogenesis. Using human brain microvascular endothelial cells (HBMECs), we found that PAI-1 inhibits $\alpha_v\beta_3$ integrin-mediated cell adhesion to vitronectin but promotes $\alpha_5\beta_1$-mediated migration from vitronectin toward fibronectin. Inhibition of vitronectin adhesion by PAI-1 did not induce HBMEC apoptosis. PAI-1 also inhibited endothelial tube formation on Matrigel in the presence of vitronectin but had a stimulatory effect in the presence of fibronectin. This effect of PAI-1 on microvascular endothelial cells is primarily related to the ability of PAI-1 to bind to vitronectin via its NH_2-terminal domain and to interfere with cell adhesion to vitronectin. We propose that PAI-1 acts as a positive switch for angiogenesis by promoting endothelial cell migration away from their vitronectin-containing perivascular space toward fibronectin-rich tumor tissue. These observations provide a novel explanation for the enhancing effect of PAI-1 in cancer progression.

INTRODUCTION

Increased expression of PAI-1[4] in cancer patients is associated with unfavorable outcome (1–3). The reason for this phenomenon is presently unknown, but experiments in PAI-1-deficient mice have suggested that PAI-1 may be necessary for local invasion and angiogenesis (4). PAI-1 is a dual function protein that inhibits the activation of plasminogen by plasminogen activators and interferes with cell binding to vitronectin (5). This dual function involves two specific domains in PAI-1. The vitronectin binding domain of PAI-1 is situated in its NH_2-terminal region and includes glutamine at position 123, whereas the antiproteolytic properties of PAI-1 are localized at its COOH-terminal region and include arginine at position 346. Binding of PAI-1 to vitronectin competes with the binding of two cell surface-associated proteins, the uPAR and the $\alpha_v\beta_3$ integrin (6). By interfering with cell adhesion to vitronectin, PAI-1 represses integrin-mediated cell migration on vitronectin (7–9), suggesting an inhibitory rather than a stimulatory role in angiogenesis. This seemingly contradictory role in tumor progression is presently not fully understood.

Elevated levels of PAI-1 have been reported by us in primary tumors of progressive neuroblastoma, and immunohistochemical examination showed that PAI-1 was strictly localized to the vascular endothelial cells, suggesting a possible involvement in angiogenesis (10). Here we have tested this hypothesis and provide evidence for a mechanism supporting a positive function for PAI-1 in angiogenesis and a key role of fibronectin in this process.

MATERIALS AND METHODS

Materials. Recombinant, stable variant of human PAI-1 was purchased from American Diagnostica (Greenwich, Connecticut). Recombinant PAI-1 Q123K and PAI-1 R346A mutants were made in a stable form by P. J. Declerck as described previously (11). These mutants have an impaired vitronectin-binding site (Q123K) or impaired active site (R346A). The active α_v antagonist EMD 121974 cyclo (Arg-Gly-Asp-D-Phe-[N-Met]-Val) was provided by A. Jończyk and S. L. Goodman (Merck KGaA, Darmstadt, Germany).

Tumor Specimens and Immunohistochemistry. Primary tumor specimens of human neuroblastoma were obtained from institutions of the Children's Cancer Group (Arcadia, CA) between 1985 and 1992 as described previously (12). Immunohistochemistry was performed on 6-μm-thick serial cryostat sections prepared from frozen tumor tissues preserved in OCT or on paraffin-embedded tissue sections. Frozen sections were immediately fixed in acetone for 5 min and air dried. Tissue for paraffin embedding was fixed in 4% paraformaldehyde, dehydrated through a graded series of ethanol washes, cleared with xylene, and embedded. Sections were then dewaxed in xylene and rehydrated through an ethanol series of decreased concentrations for 2 min each and rinsed with PBS. The tissue sections were exposed for 10 min to 2% goat serum in Tris-buffered saline (10 mM Tris-HCl, pH 7.6, 150 mM NaCl), washed, and incubated with the primary antibodies overnight at 4°C. The following primary antibodies were used: a rabbit antihuman PAI-1 polyclonal antibody (Molecular Innovations, Inc., Royal Oak, MI) at a 1:50 dilution, a mouse antihuman $\alpha_v\beta_3$ monoclonal antibody (LM609; Chemicon Int., Temecula, CA) at a 1:50 dilution, a mouse antihuman CD31 monoclonal antibody (DAKO, Carpinteria, CA), a mouse antihuman vitronectin monoclonal antibody (Biogenesis, Kingston, New Hampshire) at a 1:1000 dilution, and a rabbit antihuman fibronectin polyclonal antibody (Sigma Chemical Co., St. Louis, MI) at a 1:400 dilution. Slides were then washed three times in Tris-buffered saline prior to incubation with the secondary antibody [multi-link swine antigoat, mouse, rabbit immunoglobulins (DAKO) at a 1:50 dilution] for 30 min at room temperature. The sections were further processed for avidin-biotin horseradish peroxidase reaction using the Vectastain ABC kit (PK 400; Vector Laboratories Inc., Burlingame, CA) for 30 min. After washing three times, the reaction was developed in the presence of 3,3′-diaminobenzidine tetrahydrochloride (0.4 mg/ml). The slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted. A mouse IgG1 monoclonal antibody against *Aspergillus niger* glucose oxidase (DAKO) was used as a negative control.

Immunofluorescence Staining. Cryostat sections were prepared as described above and incubated with a mouse monoclonal antibody against $\alpha_v\beta_3$ (LM609) overnight at 4°C. After washing with Tris-buffered saline, the sections were incubated with a rabbit antihuman PAI-1 antibody for 2 h at room temperature. The sections were washed and incubated with a mixture of Rhodamine-labeled antimouse IgG and FITC-labeled antirabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 60 min at room

Received 11/27/00; accepted 5/16/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants CA84103 and CA82989 from the NIH and by a grant from the T. J. Martell Foundation.

[2] Present address: Herman B. Wells Center for Pediatric Research, Cancer Research Institute, Indiana University School of Medicine, Indianapolis, IN 46202.

[3] To whom requests for reprints should be addressed, at Childrens Hospital Los Angeles, 4650 Sunset Boulevard, MS #54, Los Angeles, CA 90027. Phone: (323) 669-2150; Fax: (323) 664-9455; E-mail: declerck@hsc.usc.edu.

[4] The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; rPAI-1, recombinant PAI-1; HBMEC, human brain microvascular endothelial cell; uPAR, urokinase plasminogen activator receptor; FACS, fluorescence-activated cell sorter; ECM, extracellular matrix; PI-PLC, phosphatidylinositol-specific phospholipase C; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; VEGF, vascular endothelial growth factor.

temperature. The stained slides were photographed under a fluorescence microscope (Vanox-S, AH-2, Olympus).

Cell Culture, Adhesion, and Migration Assays. HBMECs were isolated from surgically resected brain tissue of children with seizure disorders after appropriate informed consent was obtained from the family as described previously (13). Cells used were between passages 8 and 10 and maintained in RPMI 1640 containing 10% fetal bovine serum supplemented with epidermal and endothelial growth factors, insulin, and transferrin (NuSerum IV; Becton Dickinson, Bedford, MA) and containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids and penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were always split and maintained in subconfluent culture for experiments. Forty-eight well, non-tissue culture treated cluster plates were coated with 150 μl/well of native vitronectin (Promega Corp., Madison, WI; 5 μg/ml) or fibronectin (Promega; 10 μg/ml) at 37°C for 1 h. Nonspecific binding was blocked by the addition of 1% heat-treated BSA for 30 min at room temperature. When indicated, wells were incubated with stable recombinant PAI-1 for 30 min prior to the addition of cells. Cells (5×10^4/well) harvested by brief (1-min) treatment with trypsin, followed by neutralization with serum-containing medium, were washed with PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) and resuspended in 200 μl of adhesion buffer (serum-free culture medium containing 0.5% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM $MnCl_2$). The cells were plated in the wells and allowed to attach for 1 h. Cells were then washed gently three times with adhesion buffer to remove nonadherent cells, and the adherent cells were stained with a 0.1% crystal violet solution at room temperature for 2 min. After washing three times, the insoluble dye taken up by adherent cells was dissolved in 200 μl of methanol, and the absorbance of the solution was read at 595 nm in an ELISA plate reader. Cell migration assays were performed in Transwell plates equipped with a 12-μm pore size polycarbonate filter (Costar, Cambridge, MA). The upper side of the filter was coated with vitronectin (150 ng/filter), and the lower side with vitronectin (750 ng/filter) or fibronectin (1.5 μg/filter) diluted in PBS. After incubation for 1 h at 37°C in the presence of each protein, the filters were treated with 1% heat-treated BSA in PBS to prevent nonspecific binding and washed twice with PBS. Cells suspended in serum-free medium were then placed in the upper chamber (5×10^4 cells/chamber) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 20 h, the cells on the upper side of the filter were removed with a cotton swab, and cells on the lower side of the filter were fixed and stained with Diff-Quik Stain Set (Dade Behring, Newark, DE). The cells on the lower side of the filter were counted in nine randomly selected fields using a ×20 objective.

FACS Analysis. Exponentially growing HBMECs were harvested in dissociation buffer (enzyme free/Hanks based; Life Technologies, Inc.), centrifuged, and resuspended on ice at a concentration of 10^6 cells/ml in PBS supplemented with 0.1% BSA. Cells were then incubated with a primary antibody or a nonspecific IgG at a final concentration of 1 μg/100 ml for 30 min at 4°C. The cells were then washed three times with PBS containing 0.1% BSA, resuspended in 50 μl of PBS/0.1% BSA, and incubated with a FITC-conjugated antimouse IgG antibody in the dark at 4°C for 20 min. After washing three times with PBS/0.1% BSA, the cells were fixed in 4% paraformaldehyde and kept at 4°C in the dark before analysis. FACS analysis was done using a Coulter Epics Elite ESP instrument. For data analysis, we used the Coulter Expo2 software provided by the manufacturer. The primary antibodies used were: a mouse antihuman $\alpha_v\beta_3$ integrin monoclonal antibody (LM609); a mouse antihuman $\alpha_v\beta_5$ integrin monoclonal antibody (P1F6; Chemicon Int.); a mouse antihuman $\alpha_5\beta_1$ integrin monoclonal antibody (HA5 and JBS5; Chemicon Int.); a mouse antihuman β_1 (activated conformation) monoclonal antibody (HUTS-4; Chemicon Int.); a mouse antihuman uPAR monoclonal antibody (American Diagnostica); a mouse antihuman von Willebrand factor (A0082; DAKO) monoclonal antibody; a mouse antihuman fibroblast growth factor receptor monoclonal antibody (VBS1; Chemicon Int.); a mouse anti-Flk-1 monoclonal antibody (CH-11; Chemicon Int.); and a mouse antihuman Flt-1 monoclonal antibody (C-17; Santa Cruz Biotechnology, Santa Cruz, CA).

Treatment with PI-PLC. Treatment with PI-PLC (Sigma Chemical Co.) was used to remove cell surface-associated uPAR. For these experiments, cells were harvested in trypsin-EDTA, followed by neutralization with serum-containing medium. The cells were then washed twice in PBS and resuspended at 5×10^6 cells/ml in RPMI containing 0.1% BSA. PI-PLC was added at a final concentration of 10 units/ml, and cells were further incubated for 90 min at 4°C. Cells were then centrifuged and washed twice in PBS/0.1% BSA. An aliquot of the cell suspension was used to verify the expression of uPAR by FACS analysis as above described, whereas another aliquot was used for adhesion and migration assays. Cells similarly incubated without the addition of PI-PLC were used as control.

Apoptosis. HBMECs were allowed to attach to protein-coated wells used for adhesion assay. After incubation, nonadherent cells were harvested, and adherent cells were collected by trypsinization and added to nonadherent cells. Cytospin preparations of cells were made and fixed in 4% paraformaldehyde for 1 h at room temperature. Apoptotic cells were evaluated using the TUNEL detection kit (Boehringer Mannheim, Indianapolis, IN). Cells were then lightly counterstained with hematoxylin, and the number of TUNEL-positive cells was counted by light microscopy in 10 random fields under a ×40 objective.

Western Blot. Cells were lysed in buffer containing 1% NP40, 0.1% SDS, and a freshly added mixture of protease inhibitors (Roche, Mannheim, Germany). After incubation for 30 min and centrifugation, the protein concentration in the supernatant was measured by a Protein Quantification kit (Bio-Rad). Forty μg of each sample were electrophoresed in 0.1% SDS-12.5% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk at 37°C for 1 h and incubated overnight at 4°C with a rabbit polyclonal anti-Bcl-X_L antibody (Santa Cruz Biotechnology) or a rabbit polyclonal anti-Bax antibody (PharMingen, San Diego, CA) using dilutions recommended by the manufacturer. To ensure that equal amounts of proteins were loaded and blotted, the membranes were stripped and reprobed with a mouse antihuman β-tubulin monoclonal antibody (Sigma Chemical Co.).

Endothelial Tube Assay. Matrigel (Becton Dickinson) was added (300 μl) to each well of a 24-well plate and allowed to polymerize. This Matrigel was found to be free of vitronectin and/or fibronectin by Western blot analysis (data not shown). When indicated, vitronectin (5 μg/ml) and/or fibronectin (10 μg/ml) were added to the Matrigel before polymerization. A suspension of 2×10^4 HBMECs in culture medium was then added into each coated well in the presence or absence of rPAI-1 (100 nM) or Q123K PAI-1 mutant (100 nM). Cells were incubated for 24 h at 37°C, viewed using a Nikon TMS microscope (Tokyo, Japan), and photographed using Matrox Inspector software. The number of tubes were counted in 10 fields and averaged.

Statistical Analysis. The statistical analysis of the results was done using Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

PAI-1 Is Expressed in Angiogenic Endothelial Cells. To determine whether PAI-1 was expressed by every endothelial cell in neuroblastoma tumors or more specifically by endothelial cells that were responding to an angiogenic stimulus, we examined whether PAI-1 was coexpressed in blood vessels with the $\alpha_v\beta_3$ integrin, a specific marker for angiogenesis (14). We observed that PAI-1 and $\alpha_v\beta_3$ colocalized in blood vessels of primary neuroblastoma tumor specimens (Fig. 1, *A–C*). Furthermore, in a total of 18 primary tumors examined, there was a direct correlation between the proportion of blood vessels expressing PAI-1 and those expressing $\alpha_v\beta_3$ (Fig. 1*D*). We also examined these tumors for the presence of vitronectin and fibronectin by immunohistochemistry. Whereas vitronectin was specifically present in endothelial cells and around microvessels (Fig. 1*E*), fibronectin was abundantly present in the tumor tissue and in septae of connective tissues that separated nests of tumor cells (Fig. 1, *F* and *G*).

Integrin-dependent Adhesion of HBMECs to Vitronectin and Fibronectin. To examine the role of PAI-1 in angiogenesis, we initiated a series of *in vitro* experiments using HBMECs established from human biopsy specimens (13). When maintained in subconfluent cultures in the presence of 10% NuSerum IV between passages 8 and 10, these cells expressed several endothelial cell-specific markers including the von Willebrand factor and receptors for fibroblast growth factor and VEGF (Flt-1 and Flk-1). In addition, they also expressed uPAR and integrin receptors for fibronectin ($\alpha_5\beta_1$) and

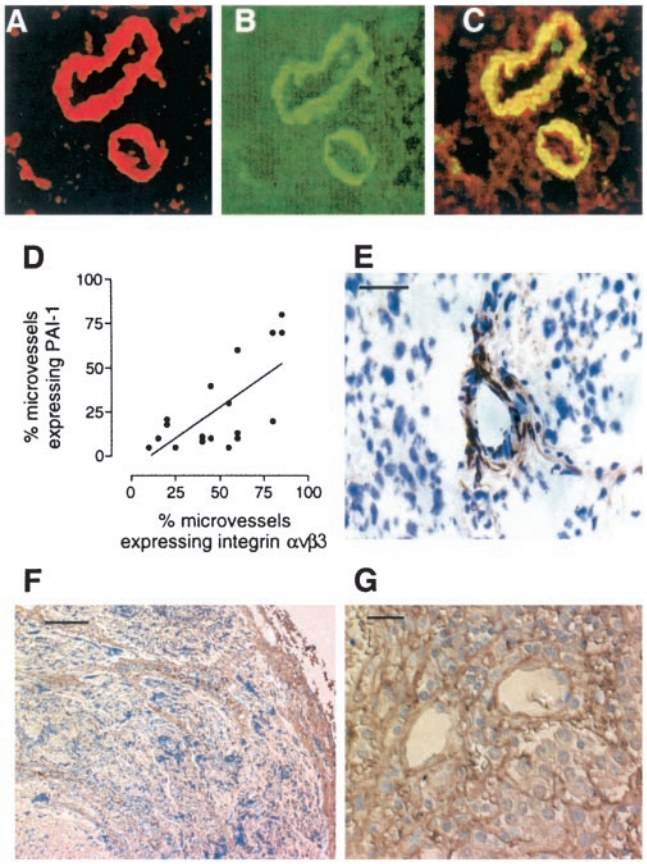

Fig. 1. *A–D*, immunofluorescence and immunohistochemical analysis of PAI-1 and $\alpha_v\beta_3$ expression in human neuroblastoma tumor specimens. *A–C*, fluorescence staining of $\alpha_v\beta_3$ and PAI-1 expression. Cryostat sections of a human neuroblastoma tumor were incubated with a rabbit anti-PAI-1 polyclonal antibody and a mouse anti-$\alpha_v\beta_3$ monoclonal antibody. After washing, the sections were incubated with a mixture of rhodamine-labeled antimouse IgG and FITC labeled antirabbit IgG. The sections were then examined under fluorescence microscopy. *A*, $\alpha_v\beta_3$ expression (rhodamine); *B*, PAI-1 expression (FITC); *C*, colocalization of PAI-1 and $\alpha_v\beta_3$ (*yellow*). *D*, serial cryostat sections from 18 stage III neuroblastoma tumors were examined by immunohistochemistry for the expression of $\alpha_v\beta_3$, PAI-1, and CD31. Positivity of microvessels for $\alpha_v\beta_3$ and PAI-1 was determined as the fraction of blood vessels staining positive for $\alpha_v\beta_3$ or PAI-1 from the total vessels that stained positive for CD31 in consecutive serial sections. The strength of the linear trend was estimated using the Pearson correlation coefficient and indicated an r of 0.675 with a P of <0.01. *E*, expression of vitronectin in neuroblastoma: frozen section stained by immunoperoxidase for vitronectin. *Bar*, 20 μm. *F* and *G*, expression of fibronectin in neuroblastoma. Paraffin-embedded sections were stained by immunoperoxidase for fibronectin. *Bar*, 100 μm in *F* and 20 μm in *G*.

Table 1 *Expression of integrin and cell surface markers in HBMECs*

Subconfluent cultures of HBMECs were harvested in dissociation buffer, and cells in suspension (10^6 cells/ml) were aliquoted and incubated in the presence of primary antibodies against several cell surface proteins as indicated. Analysis of cell surface protein receptor expression was done by FACS analysis in the presence of an FITC-conjugated IgG. The data represent the percentage (±SE) of the cell population with a signal >95% of the control population (background) in triplicate samples.

Receptor	% positive
von Willebrand factor	93.6 ± 0.0
Fibroblast growth factor receptor	33.1 ± 9.8
VEGF-R1 (Flt-1)	26.1 ± 10.7
VEGF-R2 (Flk-1)	26.2 ± 0.0
uPAR	96.1 ± 5.4
$\alpha_5\beta_1$	91.0 ± 10.1
$\alpha_v\beta_3$	88.1 ± 3.8
$\alpha_v\beta_5$	94.3 ± 0.3

vitronectin ($\alpha_v\beta_3$ and $\alpha_v\beta_5$; Table 1). We next verified that these integrins were involved in the adhesion of HBMECs to vitronectin and fibronectin by testing cell adhesion in the absence and presence of integrin-specific blocking monoclonal antibodies. The data (Fig. 2) show that adhesion of HBMECs to vitronectin was specifically inhibited in the presence of an anti-$\alpha_v\beta_3$ blocking antibody (LM609) and to a lesser extent by an anti-$\alpha_v\beta_5$ (P1F6) blocking antibody and not in the presence of an anti-$\alpha_5\beta_1$ (JBS5) blocking antibody. In contrast, adhesion to fibronectin was not affected by anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ antibodies but was inhibited by the anti-$\alpha_5\beta_1$ antibody. The data therefore indicate that under the established culture conditions, these microvascular cells closely mimic angiogenic endothelial cells and adhere to vitronectin and fibronectin via specific integrins.

PAI-1 Inhibits Integrin-mediated Adhesion of HBMECs to Vitronectin but not to Fibronectin. Considering the known ability of PAI-1 to interfere with cell binding to vitronectin (5), we tested the effect of PAI-1 on the adhesion of HBMECs to vitronectin and fibronectin. For these experiments, human rPAI-1 was incubated at final concentrations of 40 and 100 nM in vitronectin- or fibronectin-precoated tissue culture wells for 30 min prior to the addition of HBMECs (Fig. 3*A*). The data show a 76 and 77% inhibition of adhesion to vitronectin in the presence of 40 and 100 nM of rPAI-1, respectively, and no effect on adhesion to fibronectin. When the amount of vitronectin in the well was lowered from 750 μg/well to 400 ng/well, lower concentrations of rPAI-1 (up to 1 nM) inhibited HBMEC adhesion to vitronectin (Fig. 3*B*). This is consistent with the requirement of rPAI-1 to occupy cell binding sites on vitronectin to inhibit adhesion. Cell binding to vitronectin is mediated by at least two types of cell surface receptors, integrins of the α_v family and uPAR (6). Because both receptors were present on the surface of HBMECs (Table 1), we asked which one of these two receptors was primarily affected by PAI-1 (Fig. 3*C*). We first tested whether removal of uPAR on the cell surface by treatment with PI-PLC would affect vitronectin binding in the absence or presence of rPAI-1. Treatment of HBMECs with PI-PLC reduced the level of uPAR expression from 82 to 17% (Fig. 3*C*, *inset*). This treatment had no effect on basal adhesion of HBMECs to vitronectin and did not alter the inhibitory effect of PAI-1 on cell binding to vitronectin. Next, we

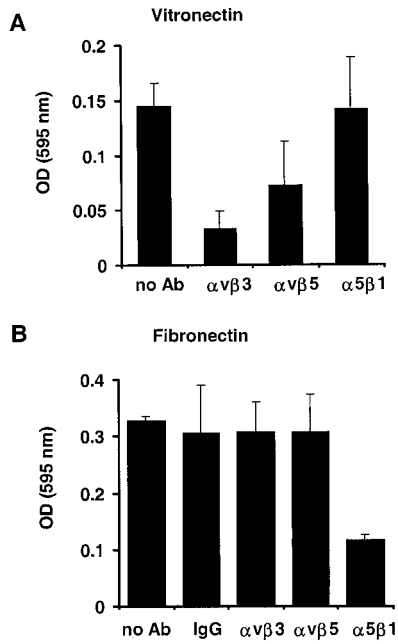

Fig. 2. Integrin-mediated adhesion of HBMECs to vitronectin and fibronectin. HBMECs (10^6 cells/ml) suspended in adhesion buffer were incubated in the presence of 0.1% BSA (*no Ab*, no antibody), mouse IgG, or integrin-specific blocking antibodies (10 μg/ml) for 30 min prior to plating at 5×10^4 cells/well on vitronectin-coated (*A*) or fibronectin-coated (*B*) wells. After 1 h incubation, nonadherent cells were washed, and the amount of adherent cells was determined by staining with crystal violet as indicated in "Materials and Methods." The data represent the mean absorbance of triplicate wells; *bars*, SE.

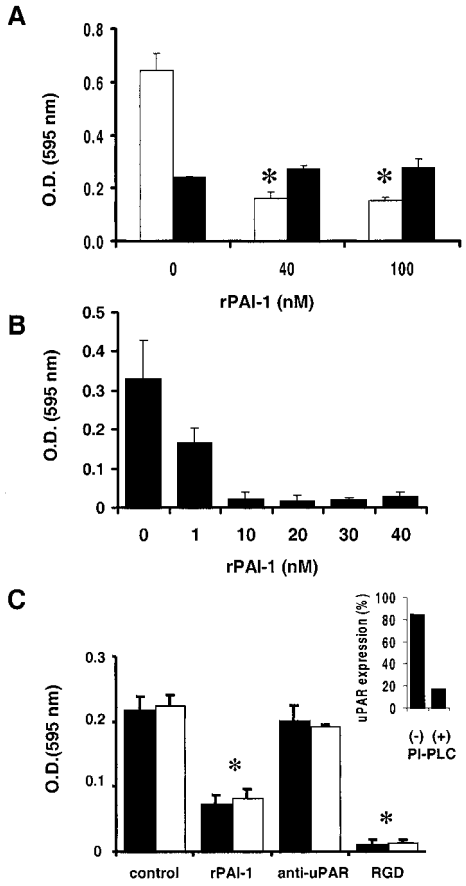

Fig. 3. Effect of rPAI-1 on HBMEC adhesion. *A*, vitronectin-coated (0.75 μg; □) and fibronectin-coated (1.5 μg; ■) wells were incubated in the presence or absence of rPAI-1 for 30 min at 37°C. HBMECs (5×10^4 cells/well) suspended in adhesion buffer were then added to the wells and incubated for 1 h at 37°C. The amount of adherent cells was then determined as described in "Materials and Methods." The data represent the mean absorbance of triplicate wells; *bars*, SE. The data represent one experiment from a total of three similar experiments. *, $P < 0.01$ in comparison with control (0 nM). *B*, rPAI-1 was preincubated at indicated concentrations in vitronectin-precoated tissue culture wells (400 ng of vitronectin/well) for 30 min at 37°C. HBMECs (5×10^4 cells/well) were then added and incubated for 1 h at 37°C. The amount of adherent cells was determined. The data represent the mean absorbance of triplicate wells; *bars*, SE. *C*, HBMECs were incubated in the absence (■) or presence (□) of PI-PLC prior to being plated on vitronectin-coated wells in the absence (control) or presence of rPAI-1 (100 nM, added to the wells prior to the addition of endothelial cells). *anti-uPAR*, cells were incubated in the presence of a function-blocking anti-uPAR monoclonal antibody for 30 min prior to being plated on vitronectin. *RGD*, cells were added to vitronectin-coated wells in the presence of the α_v antagonist EMD 121974 cyclic peptide (25 μg/ml). The amount of adherent cells was determined after 1 h by absorbance measurement. The data represent the mean absorbance of triplicate wells; *bars*, SE. *, $P < 0.05$ in comparison with control. *Inset*, cells were analyzed for uPAR expression by FACS analysis before and after PI-PLC treatment.

observed that addition of a function-blocking monoclonal antibody against uPAR did not inhibit binding to vitronectin of cells treated or untreated with PI-PLC. Then, we found that an α_v antagonist cyclic penta-peptide (EMD 121974) had a strong inhibitory effect on HBMECs binding to vitronectin, independent of PI-PLC treatment. Altogether, the data indicate that the binding of HBMECs to vitronectin is primarily mediated by integrins and that PAI-1 specifically interferes with this binding.

PAI-1 Does Not Induce Endothelial Cell Apoptosis. Inhibition of endothelial cell adhesion to vitronectin induces apoptosis as a result of loss of contact with the ECM (15). This phenomenon, known as anoikis, has been the basis for the development of inhibitors of angiogenesis, the mechanism of action of which consists of interfering with $\alpha_v\beta_3$ integrin-mediated endothelial cell binding to vitronectin (16, 17). Considering the inhibitory effect of PAI-1 on $\alpha_v\beta_3$-mediated HBMEC binding to vitronectin, a negative rather than a positive role for PAI-1 on angiogenesis would be predicted. To resolve this paradox, we designed a series of experiments to compare the effects of PAI-1 and the α_v antagonist EMD 121974 on HBMEC adhesion and apoptosis. We first asked whether these two inhibitors of vitronectin adhesion would have a similar inhibitory activity when added prior to or after cell contact with vitronectin, because differences in inhibition of Hep-2 human epidermoid carcinoma-derived cell adhesion to vitronectin by PAI-1 and RGD peptides have been reported previously (18). The data (Fig. 4*A*) indicated that PAI-1 and EMD 121974 effectively inhibited the attachment of HBMECs to vitronectin when they were added to vitronectin-coated wells prior to plating the endothelial cells. However, when PAI-1 was added after the endothelial cells were plated on vitronectin, no inhibition was seen, and the cells did not detach from the substrate. In contrast, addition of the EMD 121974 cyclic peptide to HBMECs attached to vitronectin resulted in a rapid loss of adhesion, even when the pentapeptide was added 3 h after cells had adhered to vitronectin. In a second set of

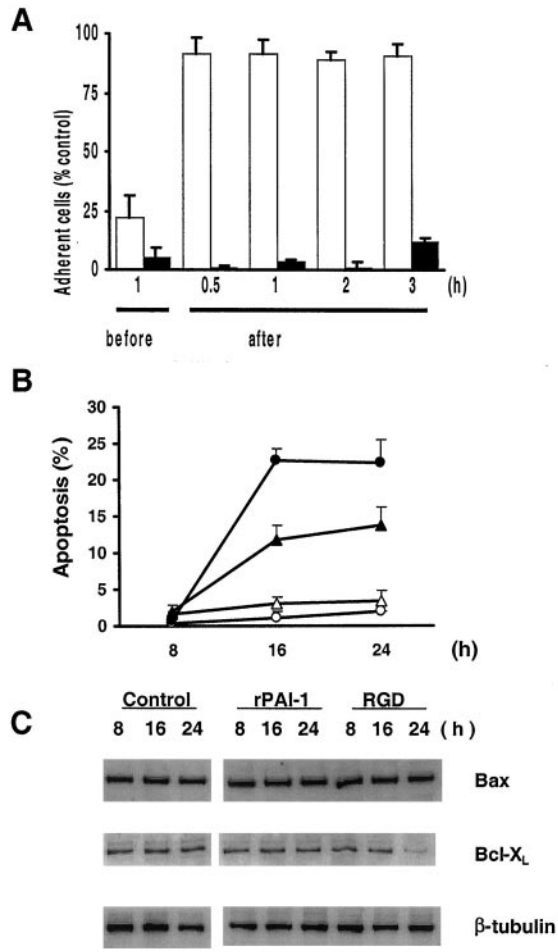

Fig. 4. Effect of rPAI-1 and EMD 121794 on HBMEC attachment and detachment from vitronectin and apoptosis. *A*, rPAI-1 (□; 100 nM) or EMD 121974 (■; 25 μg/ml) was added to vitronectin-precoated wells before (1 h) or after (30 min, 1 h, 2 h, and 3 h) the addition of HBMECs. Cells were then incubated for 1 additional h before the amount of adherent cells was determined by absorbance measurement. The data represent the number of adherent cells as the mean percentage of the control (absence of PAI-1 or EMD 121974) from triplicate wells; *bars*, SE. *B*, rPAI-1 (100 nM) or EMD 121974 (25 μg/ml) was added to vitronectin-precoated wells 1 h before or 1 h after the addition of HBMECs. At the indicated times after cell plating, adherent and nonadherent cells were collected and analyzed for the presence of apoptotic nuclei by TUNEL assay. The data represent the mean percentage of positive cells from triplicate wells and represent one experiment from a total of two similar experiments; *bars*, SE. △, rPAI-1 added 1 h before cells; ▲, EMD 121974 added 1 h before cells; ○, rPAI-1 added 1 h after cells; ●, EMD 121974 added 1 h after cells. *C*, cell lysates (40 μg) from HBMECs were obtained at the indicated times after plating and analyzed by Western blot for the expression of Bax, Bcl-X_L, and β-tubulin. rPAI-1 and EMD 121974 (*RGD*) were added 1 h before plating the cells.

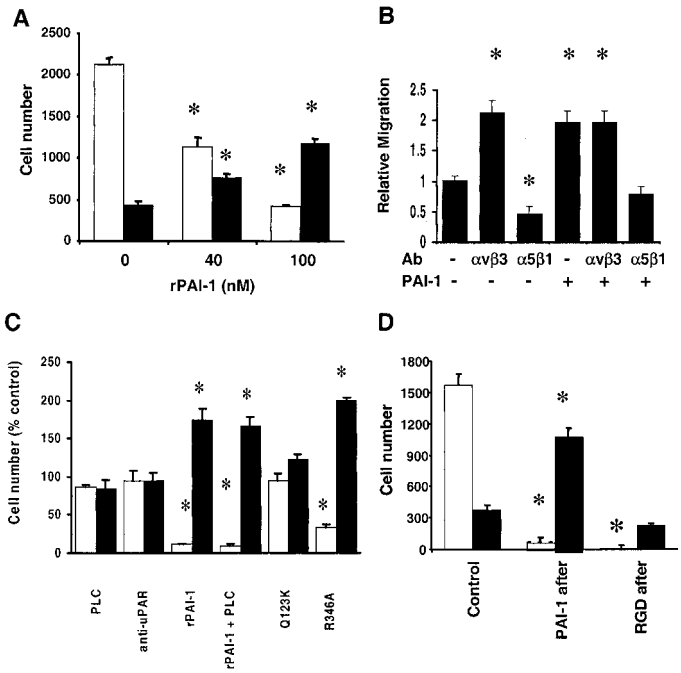

Fig. 5. Effect of rPAI-1 on HBMEC migration. *A*, HBMECs (5×10^4 cells/chamber) were added to Transwells in which the upper side of the filter was coated with vitronectin and the lower side with vitronectin (□) or fibronectin (■). rPAI-1 was added to the upper chamber at the indicated concentrations 30 min prior to the addition of cells. The data represent the mean number of cells that migrated to the lower side of the filter in triplicate samples for one experiment among three separate experiments showing similar data. *, $P < 0.02$ in comparison with control (0 nM PAI-1). *B*, HBMECs were preincubated with murine IgG1 (no Ab), an anti-$\alpha_v\beta_3$ monoclonal antibody (LM609) or an anti-$\alpha_5\beta_1$ monoclonal antibody (JBS5) prior to being plated in Transwells in which the filter was coated with vitronectin (upper side) and fibronectin (lower side). When indicated, rPAI-1 (100 nM) was added to the upper chamber for 30 min prior to cell plating. The data represent the mean number of cells that migrated to the lower side of the filter in triplicate samples; *bars*, SE. *, $P < 0.05$ in comparison with control (no PAI-1 and no Ab). *C*, HBMECs were plated in Transwells in which the upper side was coated with vitronectin and the lower with vitronectin (□) or fibronectin (■). *PLC*, cells were incubated with PI-PLC prior to being plated in the Transwells. *Anti-uPAR*, cells were preincubated with an anti-uPAR blocking monoclonal antibody prior to being plated. *rPAI-1*, rPAI-1 (100 nM) was added to the upper chamber 30 min prior to plating the cells. *Q123K* and *R346A*, stable rPAI-1 mutants (100 nM) were added to the upper chamber 30 min prior to plating the cells. The data represent the mean percentage of migrating cells from the control (no treatment) in triplicate samples of one experiment among two separate experiments showing similar data; *bars*, SE. *, $P < 0.01$. *D*, HBMECs were plated in Transwells in which the upper side of the filter was coated with vitronectin and the lower side with vitronectin (□) or fibronectin (■). rPAI-1 (100 nM) or EMD 121974 (RGD; 25 μg/ml) was added 30 min after plating the cells. The data represent the mean number of cells that migrated to the lower side in triplicate samples; *bars*, SE. *, $P < 0.01$ when compared with controls.

experiments, we compared the effect of PAI-1 and EMD 121974 on endothelial cell apoptosis (Fig. 4, *B* and *C*). Whether added before or after cell plating, PAI-1 had no effect on endothelial cell apoptosis up to 24 h after cells were plated. In contrast, the percentage of cells going into apoptosis significantly increased 16 and 24 h after plating the cells in the presence of the EMD 121974 cyclic peptide. This increase in apoptosis was observed independently, whether the α_v antagonist was added before or after the cells were in contact with vitronectin. Consistently, rPAI-1 had no effect on the expression of the antiapoptotic protein Bcl-X_L. In contrast, in the presence of the EMD 121974 cyclic peptide, a decrease in the expression of Bcl-X_L was observed after 24 h. Neither EMD 121974 nor PAI-1 had any effect on the expression of Bax. Bcl-2 was not detectable by Western blot in HBMECs and was not induced in response to PAI-1 or EMD 121974 (not shown). Thus, the data point to a significant difference between the two inhibitors of vitronectin binding, with regard to their ability to detach cells from vitronectin and to induce apoptosis.

PAI-1 Inhibits Endothelial Cell Migration on Vitronectin and Stimulates Migration toward Fibronectin. Cell migration is in part dependent on the nature of the ECM proteins and involves cell attachment and detachment. We therefore examined whether PAI-1 could affect the migration of HBMECs on several ECM proteins (haptotaxis). These experiments were performed in Transwell assays, where the filters were precoated with vitronectin on the upper side and with vitronectin or another ECM protein on the lower side. We first observed that PAI-1 inhibited migration toward vitronectin in a dose-dependent manner, whereas it had a stimulatory effect on migration toward fibronectin (Fig. 5*A*). The stimulatory effect of PAI-1 on migration toward fibronectin was specific because it was not observed when the lower side of the filter was coated with other ECM proteins including laminin and types I and IV collagens (data not shown). Basal migration from vitronectin toward fibronectin in the absence of rPAI-1 was also stimulated in the presence of an anti-$\alpha_v\beta_3$ blocking antibody (LM609), confirming that inhibition of integrin-mediated vitronectin binding is essential to promote migration toward fibronectin (Fig. 5*B*). Basal migration toward fibronectin was, however, inhibited in the presence of an anti-$\alpha_5\beta_1$ blocking antibody (JBS5), indicating the involvement of the fibronectin binding integrin $\alpha_5\beta_1$ in fibronectin haptotaxis. The addition of an anti-$\alpha_v\beta_3$ antibody to rPAI-1 had no additional stimulatory effect on HBMEC migration from vitronectin toward fibronectin, whereas the addition of an anti-$\alpha_5\beta_1$ antibody abolished the stimulatory effect of rPAI-1 on migration. To confirm that the primary role of PAI-1 in promoting HBMEC migration from vitronectin toward fibronectin involves its vitronectin binding inhibitory function and does not involve changes in $\alpha_5\beta_1$ expression or activation, we examined the effect of PAI-1 on the expression of $\alpha_5\beta_1$ and on the active confirmation of β_1 in HBMECs by FACS analysis. The data (Table 2) indicated no difference in expression of $\alpha_5\beta_1$ or active β_1 by PAI-1.

We then asked whether inhibition of migration on vitronectin and stimulation of migration toward fibronectin by PAI-1 required uPAR, and whether it involved the antiproteolytic activity of PAI-1 (Fig. 5*C*). Removal of uPAR by treatment of HBMECs with PI-PLC or blocking uPAR with an antibody did not affect basal endothelial cell migration toward vitronectin or fibronectin, indicating therefore an absence of involvement of uPAR in HBMEC migration. Furthermore, treatment of HBMECs with PI-PLC did not modify the inhibitory activity of PAI-1 on migration on vitronectin nor its stimulatory activity on migration toward fibronectin. To confirm that the effect of PAI-1 on HBMEC migration involves its vitronectin binding inhibitory function and to test whether the antiproteolytic function of PAI-1 is required, we tested the effect of two rPAI-1 mutants on HBMEC migration from vitronectin to fibronectin. These experiments indicated that a mutant PAI-1 (Q123K) that lacks vitronectin binding activity had lost its effect on HBMEC migration. Conversely a mutant PAI-1 (R346A) that lacks antiproteolytic activity but retains vitronectin binding capacity had an activity similar to the wild-type protein. Altogether the

Table 2 *Effect of rPAI-1 on $\alpha_5\beta_1$ and active β_1 expression in HBMECs*

HBMEC cells were plated on BSA, vitronectin, or fibronectin-coated wells in the presence or absence of rPAI-1 (100 nM) for 1 h. Cells were then harvested in dissociation buffer and analyzed for $\alpha_5\beta_1$ expression and active β_1 expression by FACS analysis. The data represent the percentage of the cell population with a signal above background.

Conditions	% of cells expressing	
	$\alpha_5\beta_1$	Active β_1
BSA	83.9	55.3
Vitronectin	85.0	63.9
Vitronectin + PAI-1	85.2	64.7
Fibronectin	85.0	62.1
Fibronectin + PAI-1	86.3	63.9

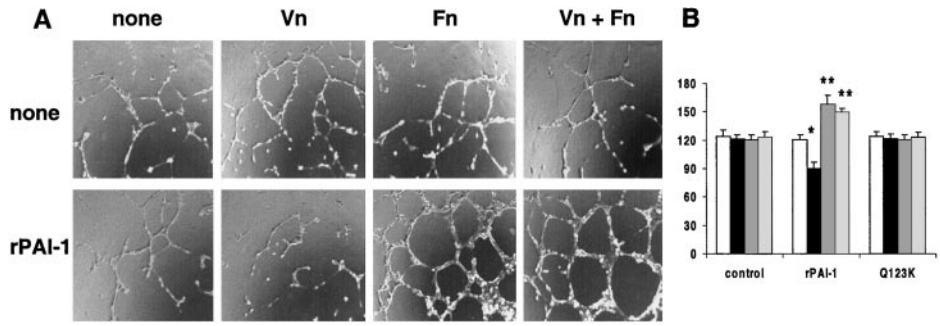

Fig. 6. Effect of PAI-1 on endothelial cell tube formation. HBMECs (2×10^4/well) were plated on Matrigel-coated dishes to which vitronectin (5 μg/ml), fibronectin (10 μg/ml), or vitronectin and fibronectin were added as indicated. Stable rPAI-1 and stable rPAI-1 Q123K (100 nM) mutant were added with the cells as indicated. After 24 h, the dishes were microphotographed, and the number of tubes formed was counted. *A*, photomicrographs of cells after 24 h in culture in the absence (*none*) or presence of rPAI-1. *B*, average number of tubes per microscopic field from triplicate dishes; *bars*, SE. The data represent one experiment among two separate experiments with similar data. □, Matrigel; ■, Matrigel supplemented with vitronectin; ■, Matrigel supplemented with fibronectin; ■, Matrigel supplemented with vitronectin and fibronectin. *, $P < 0.02$; **, $P < 0.01$.

data indicate that the stimulatory effect of PAI-1 on HBMEC migration is primarily related to its inhibitory function on integrin-mediated vitronectin binding and does not require its antiproteolytic activity.

The inability of PAI-1 to detach endothelial cells already bound to vitronectin (Fig. 4*A*) suggests that it cannot compete with existing $\alpha_v\beta_3$-vitronectin binding and that it can only bind to unoccupied sites on vitronectin. We therefore asked whether PAI-1 would have any effect on endothelial cell migration when added after the cells were attached to vitronectin (Fig. 5*D*). Under these conditions, PAI-1 retained its ability to inhibit migration toward vitronectin and to stimulate migration toward fibronectin. In contrast, the delayed addition of the EMD 121974 cyclic-peptide inhibited migration under both conditions. Because endothelial cells need to form new contacts with the ECM at their leading edge to advance, these experiments suggest that occupation of cell-binding sites on vitronectin by PAI-1 prevents further engagement with vitronectin and favors engagement with fibronectin.

Effect of PAI-1 on Endothelial Cell Tube Formation. To demonstrate that the effect of PAI-1 on HBMEC migration is responsible for its stimulatory role on angiogenesis, we examined the effect of PAI-1 on the formation of tubes by HBMECs plated on Matrigel supplemented with vitronectin and/or fibronectin. The data (Fig. 6) reveal a remarkable difference in the effect of PAI-1 on tube formation between vitronectin and/or fibronectin. Whereas neither vitronectin nor fibronectin altered tube formation in the absence of PAI-1, the addition of PAI-1 inhibited tube formation in vitronectin-supplemented Matrigel but stimulated it in fibronectin-supplemented Matrigel. Consistent with our hypothesis, the inhibitory effect of PAI-1 on tube formation in vitronectin-supplemented Matrigel was converted to a stimulatory effect when fibronectin was simultaneously added to vitronectin in the Matrigel. This effect required the vitronectin-binding capacity of PAI-1 as demonstrated by an absence of effect with the stable Q123K PAI-1 mutant. This stimulatory effect on tube formation in fibronectin-supplemented Matrigel did not involve growth stimulation because PAI-1 had no effect on [^{3}H]thymidine incorporation in HBMECs grown on plastic dishes or on fibronectin-coated dishes (data not shown). Thus altogether, these experiments support a positive role for PAI-1 in angiogenesis.

DISCUSSION

Many studies have shown that high expression of PAI-1 in various cancers correlates rather with unfavorable than favorable prognosis (1, 2, 19–21). The reason for this apparent paradox is still poorly understood. It has been initially suggested that increased PAI-1 expression in tumors could reflect a general up-regulation of the plasminogen activator system in proliferating cancer cells and a stromal reaction aimed at limiting excessive degradation of the ECM in more aggressive and invasive cancer forms (22). More recently, Bajou *et al.* (4) have demonstrated in PAI-1-deficient mice the key role of stromal-derived PAI-1 in tumor invasion and angiogenesis. Using murine skin carcinoma cells implanted with a silicone transplantation chamber in mice, these investigators have elegantly demonstrated a total absence of invasion in PAI-1-deficient mice compared with wild-type mice. The lack of invasion was a consequence of the absence of vascularization of the implants by host derived-endothelial cells. Reestablishment of PAI-1 expression in host cells by adenovirus-mediated gene transfer restored tumor vascularization and invasion, whereas PAI-1 produced by the cancer cells was not sufficient to overcome the host deficiency. These data together with our finding that in neuroblastoma tumors PAI-1 is highly expressed in angiogenic endothelial cells suggest that PAI-1 has a stimulatory function in angiogenesis.

Here we have examined whether the proangiogenic action of PAI-1 involves control of specific proteolysis or a modulation of cell migration, and we show that PAI-1 acts as a stimulator of angiogenesis by promoting the migration of endothelial cells from vitronectin toward fibronectin without triggering apoptosis. Our findings resolve apparently conflicting data indicating that PAI-1 can inhibit as well as promote cell migration (8, 9, 23) by demonstrating that the effect of PAI-1 on endothelial cell migration is dependent on the composition of the extracellular matrix. Although PAI-1 inhibits migration on vitronectin, it stimulates migration from vitronectin toward fibronectin. This effect is primarily related to the inhibitory function of PAI-1 on vitronectin binding and does not involve changes in $\alpha_5\beta_1$ expression or activation. Further supporting the importance of fibronectin is our observation that the addition of fibronectin reverts the inhibitory effect of PAI-1 on endothelial cell tube formation in vitronectin-supplemented Matrigel to a stimulatory effect. In view of the abundance of fibronectin within tumor tissues and the more selective presence of vitronectin in close proximity to endothelial cells, as illustrated in neuroblastoma, this modulatory effect of PAI-1 is likely critical. It allows endothelial cells to escape the vitronectin-rich environment of their perivascular space and to penetrate the unvascularized and fibronectin rich tumor stroma. Accordingly, it has been shown previously that fibronectin promotes the elongation of microvessels during angiogenesis (24). Fibronectin-deficient mice have also been reported to have a poor development of endothelial structures (25).

Our findings also demonstrate that the modulatory effect of PAI-1 on endothelial cell migration involves an effect on cell attachment to vitronectin and not a control over plasminogen-mediated proteolysis.

Mutation of the glutamine residue at position 123 to lysine in PAI-1 suppressed its capacity to bind to vitronectin and resulted in a loss of stimulatory effect on migration toward fibronectin and tube formation in fibronectin-supplemented Matrigel. In contrast, a mutation that suppressed the antiproteolytic activity of the inhibitor (R346A) maintained the stimulatory activity of PAI-1 on endothelial cell migration. Interference with $\alpha_v\beta_3$-mediated adhesion to vitronectin is therefore required to promote endothelial cell migration toward fibronectin, whereas control of plasminogen activator-mediated proteolysis is not. However, *in vivo*, modulation of proteolysis by PAI-1 may still play an additional regulatory function, and therefore our data do not necessarily rule out a possible contribution of the antiproteolytic function of PAI-1 in angiogenesis.

Our finding that PAI-1 affects the interaction between $\alpha_v\beta_3$ and vitronectin in endothelial cells without triggering apoptosis is also significant because inhibition of this interaction by anti-$\alpha_v\beta_3$ antibodies or RGD-based peptides has been shown to induce endothelial cell apoptosis and to inhibit angiogenesis (15–17). This absence of effect of PAI-1 on apoptosis may be attributable to an absence of anoikis because our data demonstrate that an important difference between PAI-1 and the α_v antagonist cyclic peptide EMD 121974 is that PAI-1 does detach endothelial cells from vitronectin. However, we show that the addition of PAI-1 to vitronectin before endothelial cells are in contact with vitronectin inhibits adhesion without inducing apoptosis. Thus, lack of adhesion alone does not explain the absence of apoptosis in the presence of PAI-1. Interference with $\alpha_v\beta_3$-mediated contact with vitronectin is achieved in the case of PAI-1 by binding to the ligand (vitronectin) and in the case of EMD 121974 by binding to the receptor ($\alpha_v\beta_3$). Thus, the fact that PAI-1 does not interfere with the receptor may be responsible for the lack of a proapoptotic signal, as suggested by the absence of changes in the level of Bcl-X_L and Bax in endothelial cells exposed to PAI-1. An additional direct protective effect of PAI-1 on induced apoptosis as demonstrated recently in tumor cells and endothelial cells cannot be eliminated at present (26). In contrast, upon exposure to EMD 121974, HBMECs undergo apoptosis, and Bcl-X_L levels decrease. The decrease, however, was only detected after 24 h and not at 16 h, when a significant increase in apoptosis was already noticed, suggesting that other mechanisms may be involved. We have, for example, shown previously that treatment of bovine brain endothelial cells with an RGD function-blocking cyclic peptide was associated with a 50% increase in endogenous ceramide, a lipid second messenger that can mediate apoptosis (12). Whether a similar pathway is involved in the effect of EMD 121974 on cell apoptosis is being investigated.[5]

As this report was in preparation, Stefansson *et al.* (27) reported recently that PAI-1 inhibits VEGF-induced angiogenesis in the chicken chorioallantoid membrane assay. This effect was attributed to inhibition of endothelial cell vitronectin binding. The data do not contradict our observation which also indicates that PAI-1 inhibits endothelial cell adhesion and migration on vitronectin. However, these investigators have not explored the presence and distribution of fibronectin in their model.

In summary, our data provide a mechanism to explain the essential role of stromal-derived PAI-1 in angiogenesis and tumor invasion reported by Bajou *et al.* (4) and to support the paradoxical clinical finding made by many investigators, including our group, that there is a strong relationship between the levels of PAI-1 in cancer and a poor clinical outcome. PAI-1 is increasingly expressed during tissue remodeling and wound healing, where it maintains the integrity of the fibrin matrix required for cellular activities such as migration (28). Our observation suggests an additional role for PAI-1 as a stimulator of neovascularization in wounded tissues. Our studies also suggest that interfering with the capacity of PAI-1 to bind to vitronectin may suppress the stimulatory effect of PAI-1 on angiogenesis *in vivo* (11, 29), which might be of future therapeutic value.

[5] A. Suzuki, C. Isogai, I. Gonzalez-Gomez, F. H. Gilles, Y. A. DeClerck, and W. E. Laug. Apoptosis induced by a cyclic α_v antagonist pentapeptide, comparison between dermal and brain microvascular endothelial cells, manuscript in preparation.

ACKNOWLEDGMENTS

We thank Drs. A. C. Jończyk and S. L. Goodman for the gift of the cyclic peptide EMD 121974, J. Rosenberg for typing the manuscript, and Dr. Z. Werb (University of California-San Francisco, San Francisco, CA) for critical review of the manuscript.

REFERENCES

1. Foekens, J. A., Peters, H. A., Look, M. P., Portengen, H., Schmitt, M., Kramer, M. D., Brunner, N., Janicke, F., Meijer-van Gelder, M. E., Henzen-Logmans, S. C., van Putten, W. L., and Klijn, J. G. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res., *60:* 636–643, 2000.
2. Chambers, S. K., Ivins, C. M., and Carcangiu, M. L. Plasminogen activator inhibitor-1 is an independent poor prognostic factor for survival in advanced stage epithelial ovarian cancer patients. Int. J. Cancer, *79:* 449–454, 1998.
3. Reuning, U., Magdolen, V., Wilhelm, O., Fischer, K., Lutz, V., Graeff, H., and Schmitt, M. Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis. Int. J. Oncol., *13:* 893–906, 1998.
4. Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat. Med., *4:* 923–928, 1998.
5. Chapman, H. A. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr. Opin. Cell Biol., *9:* 714–724, 1997.
6. Seiffert, D., Ciambrone, G., Wagner, N. V., Binder, B. R., and Loskutoff, D. J. The somatomedin B domain of vitronectin. Structural requirements for the binding and stabilization of active type 1 plasminogen activator inhibitor. J. Biol. Chem., *269:* 2659–2666, 1994.
7. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J. Cell Biol., *134:* 1563–1571, 1996.
8. Stefansson, S., and Lawrence, D. A. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin Nature (Lond.), *383:* 441–443, 1996.
9. Kjoller, L., Kanse, S. M., Kirkegaard, T., Rodenburg, K. W., Ronne, E., Goodman, S. L., Preissner, K. T., Ossowski, L., and Andreasen, P. A. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp. Cell Res., *232:* 420–429, 1997.
10. Sugiura, Y., Ma, L. Q., Sun, B., Shimada, H., Laug, W. E., Seeger, R. C., and DeClerck, Y. A. The plasminogen-plasminogen activator (PA) system in neuroblastoma: role of PA inhibitor-1 in metastasis. Cancer Res., *59:* 1327–1336, 1999.
11. Nar, H., Bauer, M., Stassen, J. M., Lang, D., Gils, A., and Declerck, P. J. Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and implications for serpin inactivation. J. Mol. Biol., *297:* 683–695, 2000.
12. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K. S., Stins, M. F., Seeger, R. C., and Durden, D. L. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. Cancer Res., *60:* 712–721, 2000.
13. Stins, M. F., Gilles, F., and Kim, K. S. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J. Neuroimmunol., *76:* 81–90, 1997.
14. Brooks, P. C., Clark, R. A., and Cheresh, D. A. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. Science (Wash. DC), *264:* 569–571, 1994.
15. Frisch, S. M., and Ruoslahti, E. Integrins and anoikis. Curr. Opin. Cell Biol., *9:* 701–706, 1997.
16. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell, *79:* 1157–1164, 1994.
17. Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F. H., and Cheresh, D. A. Antiintegrin $\alpha_v\beta_3$ blocks human breast cancer growth and angiogenesis in human skin. J. Clin. Investig., *96:* 1815–1822, 1995.
18. Germer, M., Kanse, S. M., Kirkegaard, T., Kjoller, L., Felding-Habermann, B., Goodman, S., and Preissner, K. T. Kinetic analysis of integrin-dependent cell adhesion on vitronectin. The inhibitory potential of plasminogen activator inhibitor-1 and RGD peptides. Eur. J. Biochem., *253:* 669–674, 1998.
19. Kuhn, W., Schmalfeldt, B., Reuning, U., Pache, L., Berger, U., Ulm, K., Harbeck, N., Spathe, K., Dettmar, P., Hofler, H., Janicke, F., Schmitt, M., and Graeff, H. Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. Br. J. Cancer, *79:* 1746–1751, 1999.

20. Look, M. P. Pooled analysis of uPA and PAI-1 for prognosis in primary breast cancer patients. EORTC Receptor and Biomarker Study Group. Int. J. Biol. Markers, *15:* 70–72, 2000.
21. Harbeck, N., Thomssen, C., Berger, U., Ulm, K., Kates, R. E., Hofler, H., Janicke, F., Graeff, H., and Schmitt, M. Invasion marker PAI-1 remains a strong prognostic factor after long-term follow-up both for primary breast cancer and following first relapse. Breast Cancer Res. Treat., *54:* 147–157, 1999.
22. Bianchi, E., Cohen, R. L., Dai, A., Thor, A. T., Shuman, M. A., and Smith, H. S. Immunohistochemical localization of the plasminogen activator inhibitor-1 in breast cancer. Int. J. Cancer, *60:* 597–603, 1995.
23. Waltz, D. A., Natkin, L. R., Fujita, R. M., Wei, Y., and Chapman, H. A. Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. J. Clin. Investig., *100:* 58–67, 1997.
24. Nicosia, R. F., Bonanno, E., and Smith, M. Fibronectin promotes the elongation of microvessels during angiogenesis *in vitro*. J. Cell. Physiol., *154:* 654–661, 1993.
25. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development (Camb.), *119:* 1079–1091, 1993.
26. Kwaan, H. C., Wang, J., Svoboda, K., and Declerck, P. J. Plasminogen activator inhibitor 1 may promote tumour growth through inhibition of apoptosis. Br. J. Cancer, *82:* 1702–1708, 2000.
27. Stefansson, S., Petitclerc, E., Wong, M. K., McMahon, G. A., Brooks, P. C., and Lawrence, D. A. Inhibition of angiogenesis *in vivo* by plasminogen activator inhibitor-1. J. Biol. Chem., *276:* 8135–8141, 2001.
28. Providence, K. M., Kutz, S. M., Staiano-Coico, L., and Higgins, P. J. *PAI-1* gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. J. Cell. Physiol., *182:* 269–280, 2000.
29. Sharp, A. M., Stein, P. E., Pannu, N. S., Carrell, R. W., Berkenpas, M. B., Ginsburg, D., Lawrence, D. A., and Read, R. J. The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. Structure, *7:* 111–118, 1999.

Plasminogen Activator Inhibitor-1 Promotes Angiogenesis by Stimulating Endothelial Cell Migration toward Fibronectin

Chiho Isogai, Walter E. Laug, Hiroyuki Shimada, et al.

Cancer Res 2001;61:5587-5594.