The Plasminogen-Plasminogen Activator (PA) System in Neuroblastoma: Role of PA Inhibitor-1 in Metastasis

Yasuo Sugiura, Liqun Ma, Bo Sun, Hiroyuki Shimada, Walter E. Laug, Robert C. Seeger, and Yves A. DeClerck

Division of Hematology-Oncology, Department of Pediatrics [Y. S. M., B. S., W. E. L., R. C. S., Y. A. D.], Department of Pathology [H. S.], and Department of Biochemistry and Molecular Biology [Y. A. D.], Children’s Hospital Los Angeles, and University of Southern California, Los Angeles, California 90027

ABSTRACT

Proteases of the plasminogen-plasminogen activator (PA) system play an important role in cancer metastasis. We have examined the expression of these proteases and their cell surface receptors and inhibitors in neuroblastoma, a tumor that originates in cells of the neural crest and is the second most common solid tumor in children. Urokinase PA and, in particular, tissue-type PA were expressed in cell lines and in tumor tissues; however, their levels of expression did not correlate with clinical stage. There was little evidence suggesting that neuroblastoma cells concentrate PA activity at their cell surface because urokinase-type PA receptor mRNA was detected in two cell lines and in 5 of 20 tumor samples by reverse transcription-PCR only. PA inhibitor (PAI)-2 was absent in all cell lines and tumor tissue samples examined. However, PAI-1, which was not expressed by the cell lines, was expressed by stromal cells and, specifically, endothelial cells in tumor tissue. By extending the analysis of PAI-1 expression in 64 primary tumor specimens, we found that high PAI-1 expression paradoxically correlated with metastatic stage and tumor recurrence. In vitro experiments indicated that the expression of PAI-1 by human microvascular endothelial cells was stimulated in the presence of SK-N-BE(2) human neuroblastoma cells and neuroblastoma culture medium. Recombinant PAI-1 also promoted SK-N-BE(2) cell detachment from vitronectin and migration from vitronectin toward fibronectin. From these data, we conclude that the up-regulation of PAI-1 expression in endothelial cells may promote rather than inhibit metastasis in neuroblastoma.

INTRODUCTION

Neuroblastoma is a tumor that originates in cells of the neural crest and is the second most common solid tumor in children. Patients with neuroblastoma can present with a localized disease (stage I and II) that has a favorable prognosis or with a locally invasive (stage III) or metastatic disease (stage IV) that has a much worse prognosis despite intensive multimodal therapy (1). However, in children less than 1 year of age, disseminated neuroblastoma confined to the liver, skin, or bone marrow (but not the bone) can undergo spontaneous regression and is designated stage IVS. Age, clinical stage, tumor histology, and amplification of the MYCN oncogene are important prognostic factors that outline therapeutic strategies and patient outcomes (2–4). Although much is known about the biology of neuroblastoma, very little is known about the mechanisms involved in metastasis in this cancer type.

The degradation of the ECM by proteases is an important rate-limiting step during metastasis that permits tumor cells to locally invade and access blood vessels, exit the vasculature, and establish themselves in a distant organ (5, 6). In addition, the synthesis of these proteases by endothelial cells is essential during angiogenesis (7). Among proteases that play an active role in the degradation of ECM during cancer progression are the serine proteases of the plasminogen-PA-plasmin system (7, 8). The main serine protease involved in ECM degradation is plasmin, which degrades glycoproteins such as laminin and fibronectin and also type IV collagen. Plasmin is generated from plasminogen by two activators: (a) tPA, a protease predominantly present in plasma and involved in thrombolysis; and (b) uPA, a protease expressed by a large variety of cells and present in tissues (9).

In addition to its role in thrombolysis and ECM degradation, plasmin has a regulatory function in the activation of other ECM-degrading proteases and, in particular, the MMPs, a family of Zn2+-dependent endopeptidases. For example, plasmin proteolytically activates the precursor forms of collagenase (MMP-1),stromelysin (MMP-3), and gelatinase B (MMP-9; Refs. 10 and 11). The activity of serine proteases in the extracellular space is controlled at two levels. A group of specific inhibitors, members of the serpin family designated PAI-1, PAI-2, and protease-nexin 1, provide a first level of control on plasmin activity. These inhibitors form 1:1 stoichiometric enzyme-inhibitor complexes with PA and therefore prevent the activation of plasminogen (12).

A second level of control is provided by the existence of a specific receptor for uPA, tPA, and plasminogen-plasmin (annexin II), that confine plasmin-mediated proteolysis to the pericellular space (13–15). PAI-1 also regulates the level of uPA bound to uPAR by promoting the rapid endocytosis of the trimolecular uPA/PAI-1/uPAR complex (16). In addition to acting as a protease receptor, uPAR is an adhesion receptor for vitronectin because it has a specific domain that binds to the somatomedin B-like domain of vitronectin. The binding of uPA to uPAR enhances binding to vitronectin (17). Because PAI-1 binds to the same somatomedin B-like domain of vitronectin, it can therefore compete with the binding of the uPA/uPAR complex (18, 19). This latter property allows PAI-1 not only to disrupt the binding of uPAR to vitronectin but also to compete with the binding of vitronectin to integrins (20, 21).

There is abundant evidence in the literature indicating that a high level of expression of uPA and uPAR in many cancers is a predictor of poor clinical outcome (22). uPA was the first protease shown to be a prognostic factor in breast cancer (23) and has since been shown to be a prognostic marker in many other malignancies including cancers of the lung (24), bladder (25), stomach (26), colorectum (27), and brain (28). uPAR also seems to be an important prognostic marker in cancers of the colon (29), breast (23), and lung (30). Although tPA is present in many cancers, its role is less clear, and it has only been shown to be associated with neoplastic transformation and the invasive phenotype in human pancreatic cancer (31). Because of the association between high levels of PA (in particular, uPA) expression...
and tumor progression, it was anticipated that high levels of PAIs would predict a more favorable outcome. Although this has been the case with PAI-2 in breast cancer (32), in the case of PAI-1, high rather than low levels of inhibitor have been shown to predict an unfavorable outcome. For example, high levels of PAI-1 are associated with a higher incidence of lymph node involvement in breast cancer (33, 34) and a poor response to tamoxifen therapy (35). These observations indicate a more complex role for PAI-1 in cancer progression that may be explained by the multifunctional nature of PAI-1. It is conceivable that high levels of PAI-1 may promote cancer cell detachment from vitronectin and migration on vitronectin and thus stimulate the formation of distant metastasis.

In this report, we have examined the expression of members of the plasminogen-PA family in a series of human neuroblastoma cell lines and primary tumor specimens. We present data indicating that high levels of PAI-1 mRNA expression correlate with metastatic stage and tumor recurrence, suggesting that expression of PAI-1 may be a contributing factor to metastasis in this form of cancer.

MATERIALS AND METHODS

Cell Culture. Seven established human neuroblastoma cell lines derived from patients with stage IV and stage III disease were used. The LA-N-1, LA-N-2, LA-N-5, and LA-N-6 cell lines were established in our laboratory. The IMR-32 cell line was provided by Dr. J. Tumilowicz (Institute for Medical Research, Camden, NJ). The SK-N-SH and SK-N-BE(2) cell lines were obtained from Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). The HT1080 human fibrosarcoma cell line was obtained from the American Type Culture Collection (Rockville, MD), and the human melanoma cell line M24met was provided by Dr. R. Reisfeld (Research Institute of Scripps Clinic, La Jolla, CA). These two latter cell lines were used as controls in our assays because of their previously well-characterized protease profile (36, 37). Human dermal microvascular endothelial cells immortalized by transfection with the large T-antigen (HMEC-1) were obtained from the National Center for Infectious Disease, Centers for Disease Control (Atlanta, GA; Ref. 38). Cells were maintained in RPMI 1640 or MEM containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin.

Reverse Fibrin Agarose Overlay. The expression of PA and PAI in the culture media of cell lines was assessed by reverse fibrin overlay (39). Concentrated samples of serum-free conditioned medium from neuroblastoma cell lines were electrophoresed on a SDS (0.1%)-polyacrylamide (10%) gel. After electrophoresis, the gel was washed in 2.5% Trition X-100 for 1 h; placed on a 1% low melting point agarose gel containing bovine fibrinogen (4.4 mg/ml), human plasminogen (10 µg/ml), and uPA (0.06 unit/ml); and incubated at 37°C in humid air. In these gels, a clear zone of digested fibrin that appeared within 4–8 h indicated the presence of PA, whereas the presence of an opalescent zone of undigested fibrin that remained after complete digestion of the fibrin (10–16 h) indicated the presence of PAIs. The presence of receptor-bound PA activity was similarly determined on acidic supernatants of cells lysed in 0.5% NP-40 and centrifuged at 2000 g for 10 min to eliminate nuclei (41). Samples of cytoplasmic RNA (20 µg) were electrophoresed on a 1% formaldehyde agarose gel and blotted onto nylon membranes. Blots were sequentially hybridized at 42°C for 18 h in the presence of 32P-labeled cDNA probes. tPA, uPA, and PAI-1 cDNAs were obtained from Dr. D. Collen (University of Leuven, Leuven, Belgium), anexin II cDNA was kindly provided by Dr. K. A. Hajjar (Cornell University Medical College, New York, NY), and PAI-2 and uPAR cDNAs were obtained from Dr. E. K. O. Knuthof (Geneva, Switzerland). GAPDH cDNA was purchased from the American Type Culture Collection. After hybridization, blots were sequentially washed in 3× SSC [1× SSC = 450 mM NaCl, 45 mM sodium citrate (pH 7.0), 25 mM NaH2PO4, and 0.1% SDS], 1× SSC, 0.3× SSC, and 0.1× SSC at 60°C before being autoradiographed at −80°C.

Neuroblastoma Tissue Samples. Samples of primary tumors from 64 patients with neuroblastoma diagnosed from birth to age 18 years were analyzed. At the time of surgery, these specimens were snap-frozen in isopentane/dry ice and shipped on dry ice to the Children’s Cancer Group Neuroblastoma Biology Laboratory at Children’s Hospital Los Angeles. Upon receipt, a portion of the frozen tissue was removed and placed in OCT compound (Miles, Elkhart, IN), and the remaining material was stored at −80°C until use. OCT-pressed tissues were serially sectioned with a cryostat (10 µm) and homogenized with Trizol (Life Technologies, Inc., Gaithersburg, MD) to extract RNA according to the procedure recommended by the manufacturer. Clinical information about patients from whom these specimens were obtained was available from the Children’s Cancer Group, National Center for Infectious Disease, Centers for Disease Control (Arcadia, CA). Clinical staging for these specimens was done according to the classification of Brodeur et al. (1): stage I, a tumor confined to the organ of origin; stage II, a tumor extending in continuity beyond the organ of origin but not crossing the midline; stage III, a tumor extending beyond the midline; stage IV, a metastatic tumor; and stage IVS, a tumor in a patient less than 1 year of age with metastatic disease confined to the liver, skin, and/or bone marrow but without evidence of bone metastases. The mean follow-up on these patients was 3 years and 10 months, with a range of 17 days to 9 years and 6 months.

RT-PCR Analysis. The RNA extracted from neuroblastoma cells and tumor tissues was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in the presence of random primers. For amplification, the reverse-transcribed cDNA was subjected to 30 or 35 cycles of RT-PCR using the Taq PCR Core Kit (Quagen, Chatsworth, CA) in the presence of 10 pmol of sense and antisense oligonucleotide primers. The primers used were as follows: for uPA, 5'-GGCCAGCAATGAATCTCCGATCAGGTTCGGCGTCT-3' and 5'-ACAGGAGCCTGCCCTCGGAC-3' and 5'-GAGGGGATTTCCAGGTATTAGG-3'; for tPA, 5'-CCAGCAACATCAGTCGACCTGAC-3' and 5'-GCA-TTCTCCAGCACCAATCCTCC-3'; for PAI-1, 5'-CCTTTGGTGAAGGCTGCCTGTC-3' and 5'-CTCCACCTCCTGAAAGTGCCTG-3'; for PAI-2, 5'-ATGGGAGATCTTCTGGTG-3' and 5'-GGGGAAATTTTTTCGACAGAA-3', and for GAPDH, 5'-ACGGATTGTTGCTATTTGG-3' and 5'-TGATTTGGGAGGAGTCTGCCG-3'. Each set of primers corresponded to sequences located on different exons to allow the detection of genomic DNA that may have contaminated the reaction. Each RT-PCR cycle included a denaturation step at 94°C for 1 min; a primer annealing step at 56°C for 30 s at uPA, at 60°C for 1 min (uPAR and GAPDH), at 55°C for 1 min (PAI-1 and PAI-2), or at 62°C for 1 min (GAPDH); and an extension step at 72°C for 1 min. Reactions were performed in a Programmable Thermal Controller model 96 V (MJ Research, Watertown, MA). The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized and photographed under UV light.

Quantitative RT-PCR Analysis. A quantitative analysis of PAI-1 mRNA in tumor tissues was performed using a noncompetitive RT-PCR method in the presence of two pairs of primers for PAI-1 and GAPDH, respectively. Hexa-chlorinated analogue (Applied Biosystems, Foster City, CA) of 6-carboxyfluorescein was used for the direct 5' end fluorescent labeling of the sense primers on a Biosearch 8750 DNA Synthesizer (Milligen/Bioresearch, Novato, CA), using standard phosphoramidite chemistry. The 5' fluorescence-labeled primers were purified by reverse-phase high-performance liquid chromatography using a Dynamax Pure DNA column (Raminin Instrument Co., Woburn, MA). Equal amounts of cDNA were amplified by PCR in two separate reactions each for PAI-1 and GAPDH. Each PCR cycle included a denaturation step at 94°C for 30 s, a primer annealing step at 55°C for 45 s, and an extension step at 72°C for 45 s. To standardize the assay conditions, we performed a time course analysis of the generation of the fluorescence-labeled GAPDH PCR product in the presence of an amount of cDNA corresponding to 1 ng of RNA. This experiment indicated that the fluorescent PCR product remained undetectable (<100 fluorescent units) until cycle 12, when the reaction became detectable and exponential, until it reached a plateau at cycle 31 (>0.5 × 106 fluorescent units). For GAPDH, the analysis of the PCR products was performed at cycle 27. For PAI-1, the analysis of the PCR products was performed at cycle 31. A second sample at cycle 18 (GAPDH) and cycle 27 (PAI-1) was also analyzed to ensure that the analysis was performed during the exponential phase of the reaction. The amount of fluorescence present in each PCR product

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Fig. 1. Reverse fibrin agarose overlay analysis of PAs and PAIs in neuroblastoma cell lines. A, top panel, concentrated samples of conditioned medium corresponding to $5.25 \times 10^6$ cells were electrophoresed on a 0.1% SDS-10% acrylamide gel before being overlaid on a fibrin agarose gel as described in “Materials and Methods.” Positions of migration of molecular weight markers are indicated (in thousands) on the left. uPA, tPA, and PA:PAI complexes present in HT1080 and M24met cell are shown on the right. A, bottom panel, samples of acidic eluate corresponding to $1 \times 10^6$ cells were electrophoresed as indicated in “Materials and Methods.” The figures are reproductions of photo negatives in which dark zones represent zones of fibrinolysis. The number of MYCN copies for each cell line according to data published by Reynolds et al. (61) is indicated at the bottom. B, samples (10 μl) of concentrated conditioned medium from LA-N-6 and SK-N-BE(2) cells ($6.5 \times 10^5$ cells) were preincubated with 4 μg of a nonspecific mouse IgG1 (control) or of a function-blocking anti-tPA monoclonal antibody (anti-tPA) before being placed in a well in a fibrin-agarose gel and incubated at 37°C for 4 h. The fibrin gel was then incubated for 24 h at room temperature and observed for the development of a circular zone of fibrinolysis. The number of lytic zones was determined by laser spectrometry using a 373A DNA sequencer (Applied Biosystems) after separation of the products on a 6% acrylamide gel. The relative amount of the PAI-1 PCR product was then calculated as the ratio of PAI-1:GAPDH (fluorescent units).

**Immunohistochemistry.** Cryostat sections (6-μm thick) were prepared from frozen tumor tissues embedded in OCT and fixed in acetone for 5 min at room temperature. The tissue sections were exposed for 5 min to 2% goat from frozen tumor tissues embedded in OCT and fixed in acetone for 5 min at room temperature. The tissue sections were exposed for 5 min to 2% goat serum in Tris-buffered saline [10 mM Tris-HCl (pH 7.6) and 130 mM NaCl] at a concentration of 40 μg/ml and a rabbit polyclonal antibody against PAI-1 (number 380; American Diagnostica, Inc., Greenwich, CT) at a concentration of 40 μg/ml and a rabbit polyclonal antibody against factor VIII-related antigen (A0082; DAKO) at a 1:400 dilution for 2 h at room temperature. Slides were then washed in Tris-buffered saline before incubation with the second biotinylated swine antimouse IgG1 antibody (DAKO) at a 1:50 dilution for 30 min. After washing in Tris-buffered saline, the reaction was developed in the presence of 3,3' diaminobenzidine tetrahydrochloride. 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 negative control.

**PAI Determination.** PAI-1 was measured by ELISA (number 822; American Diagnostica, Inc.) using multiwell plates precoated with an anti-human PAI-1 mouse monoclonal IgG1 and the conditions suggested by the manufacturer. Monoclonal antibodies against human VEGF receptor or bFGF receptor were obtained from Chemicon International, Inc. (Temecula, CA).

**Cell Adhesion Assays.** The 48-well non-tissue culture-treated cluster plates (Costar, Cambridge, MA) were coated with 100 μl/well vitronectin (10 μg/ml) or fibronectin (10 μg/ml) at 37°C for 1 h. Nonspecific binding was then blocked by the addition of 1% heated BSA. Cells were resuspended in adhesion buffer (serum-free culture medium containing 1% BSA, 1 mM CaCl₂, 1 mM MgCl₂, and 0.5 mM MnCl₂) at a final concentration of 5 × 10⁵ cells/ml, and 100 μl of cell suspension were seeded into each well. The cells were allowed to attach for 90–120 min, after which they were gently washed three times by hand pipetting to remove nonadherent cells. The adherent cells were then stained with a thiazol blue solution at 37°C for 2 h. After two washings, the insoluble dye taken by adherent cells was dissolved in 200 μl of DMSO, and the absorption was read at 550 nm in an ELISA plate reader.

**Cell Migration Assays.** Cell migration assays were performed in Transwell plates with a 12-μm pore size polycarbonate membrane (Costar). The upper side of the membrane was coated with vitronectin (150 ng), and the lower side was coated with fibronectin (2 μg/membrane) or vitronectin (2 μg/membrane) diluted in serum-free RPMI 1640. After being incubated for 1 h at 37°C, the filters were treated with 1% heated BSA in PBS to prevent nonspecific binding. Cells suspended in serum-free medium were placed in the upper chamber (5 × 10⁴ cells/chamber), whereas the lower chamber was filled with serum-free conditioned medium from SK-N-BE(2) cells. After a 20-h incubation at 37°C, cells on the upper side of the membrane were removed with a cotton swab, and cells on the lower side of the membrane were fixed and stained with Diff-Quik stain (Baxter, McGaw Park, IL). The cells on the entire lower surface of the membrane were counted using a 20× objective.

**Statistical Analysis.** Statistical analysis of the measurements of PAI-1 levels in tumor samples was performed after normalization of the data by natural log transformation. Both parametric (t test) and nonparametric (Kruskal-Wallis and Mann-Whitney U test) tests were used for statistical comparison of recurrent and survival analysis. The ANOVA test was used for...
Expression of PAs and PAIs in Neuroblastoma Cell Lines. The expression of PAs and PAIs in seven human neuroblastoma cell lines was first examined by reverse fibrin agarose overlay on aliquots of serum-free culture medium. The data (Fig. 1A) revealed the secretion of two fibrinolytic proteins of $M_r$ 53,000 and $M_r$ 66,000 in four of seven cell lines [LA-N-6, LA-N-1, LA-N-2, and SK-N-BE(2)]. The size of the $M_r$ 53,000 band was consistent with uPA, and size of the $M_r$ 66,000 band was consistent with tPA, as shown in comparison with the uPA and tPA present in the culture medium of HT1080 and M24met cells. To determine whether there was any cell surface-associated PA in these cell lines, a similar analysis was performed on acidic eluates, and this analysis indicated an absence of cell surface-associated PA in all of the neuroblastoma cell lines tested. In this limited study, there was no obvious correlation between the expression of uPA or tPA and the degree of amplification of the MYCN proto-oncogene. Further evidence that tPA is the major fibrinolytic enzyme expressed by neuroblastoma was obtained by testing the fibrinolytic activity of samples of conditioned medium from two cell lines [LA-N-6 and SK-N-BE(2)] in the presence of a function-blocking anti-tPA antibody. The data (Fig. 1B) showed a significant inhibition of fibrinolysis in the presence of an anti-tPA antibody. Thus, neuroblastoma cells secrete predominantly tPA and little uPA in the absence of inhibitors and do not concentrate PA activity at their cell surface. Additional information was obtained by performing a Northern blot analysis (Fig. 2). The data revealed the presence of tPA mRNA in four cell lines that expressed the $M_r$ 70,000 fibrinolytic protein. In addition, the LA-N-5, SK-N-SH, and IMR-32 cell lines, which were negative by fibrin overlay, did express some tPA mRNA. Several cell lines were positive for annexin II mRNA, the tPA/plasminogen-plasmin receptor. No mRNA for uPAR, PAI-1, and PAI-2 was detected, an observation consistent with the fibrin overlay. No uPA mRNA was observed, despite the presence of a detectable amount of protein in the culture medium. Because uPA is considerably more active than tPA in the fibrin overlay assay (42), it is conceivable that the Northern blot was not sensitive enough to detect low copy numbers of uPA mRNA. This possibility was confirmed by RT-PCR analysis (Fig. 3), which detected the presence of small amounts of uPA mRNA in the LA-N-6, IMR-32, LA-N-2, and SK-N-BE(2) cell lines. This analysis also revealed the presence of small amounts of uPAR mRNA in the LA-N-6 and SK-N-BE(2) cell lines.

Expression of PAs and PAIs in Tumor Tissues. To obtain a better insight into the potential function of PA and PAI in neuroblastoma metastasis, we examined their expression in a series of 20 primary tumor specimens obtained at diagnosis from patients with various stages of disease. This initial analysis was performed by RT-PCR as indicated in “Materials and Methods” (Fig. 4). tPA and uPA mRNAs were detected in 85% and 90% of the specimens, respectively, independent of their clinical stage. uPAR was weakly detected in 5 of 20 (25%) samples, and PAI-2 was undetected. PAI-1 mRNA was detected in 11 of 20 (55%) specimens, particularly those from patients with stage IV disease, which were all positive. To further examine the possibility that there are higher levels of PAI-1 expression in the metastatic stage, a quantitative RT-PCR method based on fluorescence-labeled oligonucleotides was developed to examine the expression of PAI-1 in a total of 64 tumor specimens. Although the PAI-1:GAPDH ratio was above 2 in only four samples, all four samples were derived from patients who had stage IV disease (Fig. 5A) and experienced a recurrence of their disease (Fig. 5B). The mean PAI-1:GAPDH ratio in samples from patients with stage IV disease was 1.13, which was statistically significantly higher than that in patients with stage I and II disease (0.33; $P < 0.001$). However, the difference with stage III (mean ratio, 0.34) and IVS (mean ratio, 0.32) disease was not statistically significant ($P = 0.074$ and $P = 0.525$, respectively) because of the lower number of specimens in these groups. The mean PAI-1:GAPDH ratio in samples from patients who experienced recurrent disease was 1.28; which was statistically significantly higher than the mean ratio of 0.33 found in patients who did not experience recurrent disease ($P = 0.031$).
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**Fig. 4.** RT-PCR analysis of PA, uPAR, and PAI expression in neuroblastoma tissues. RT-PCR was performed on cDNA obtained from RNA extracted from 20 samples of neuroblastoma tissues. We used 30 cycles for tPA, PAI-1, and GAPDH and 35 cycles for uPA, uPAR, and PAI-2. cDNA from HT1080 cells and plasmids (10 ng) containing the corresponding cDNAs was used as a positive control. The PCR products were examined by agarose gel electrophoresis and ethidium bromide staining and scored as detected or undetected. The size of the DNA fragments indicated by arrows is 215 bp for tPA, 130 bp for uPA, 1046 bp for uPAR, 409 bp for PAI-1, 1280 bp for PAI-2, and 230 bp for GAPDH. In the uPA reaction, PCR products of higher size represent contamination with genomic DNA, and the arrow is positioned according to the size of the PCR product generated with the plasmid and with HT1080 cells.

GAPDH ratio was also higher in tumors obtained from patients who did not survive (1.23) than in tumors obtained from patients who survived (0.37), but this difference was not statistically significant ($P = 0.059$; Fig. 5C). The levels of PAI-1 did not correlate with the degree of MYCN amplification, age, or the histopathological grade (data not shown). Thus, the data indicate that high PAI-1 levels are associated with metastatic and recurrent disease; however, the absence of a high PAI-1 level does not predict the absence of metastasis or disease recurrence because low PAI-1 levels were observed in many samples obtained from patients with stage IV disease or with recurrent disease.

**PAI-1 Is Expressed by Host-derived Endothelial and Stromal Cells.** The absence of PAI-1 expression in tumor cell lines led us to suspect that PAI-1 was expressed in primary tumors by host-derived stromal cells rather than by malignant cells. This possibility was examined by immunohistochemical analysis of PAI-1 expression in a series of fresh tumor specimens (Fig. 6). In stage IV tumors, there was an abundant expression of PAI-1 in vascular endothelial cells, in perivascular cells, and in stromal cells surrounding the nests of tumor cells, but there was no expression in tumor cells (Fig. 6, B, G, and H). In contrast, specimens from stage I or stage II disease showed no reactivity with an anti-PAI-1 antibody (Fig. 6A). However, the degree of PAI-1 expression did not seem to reflect vascular density in tumor tissue because abundant blood vessels that stained for factor VIII-related antigen were found in both nonmetastatic (stage I and II) and metastatic (stage IV) tumors (Fig. 6, E and F).

**Neuroblastoma Cells Stimulate PAI-1 Expression by Endothelial Cells.** Thus, the data suggest that in stage IV neuroblastoma, the malignant cells stimulate the expression of PAI-1 by adjacent endothelial and stromal cells. To explore this possibility, we performed a series of coculture experiments in which SK-N-BE(2) cells were cultured in the presence of human dermal microvascular endothelial cells (HMEC-1 cells; Fig. 7). In these experiments, we compared culture conditions that allowed cell contact with conditions in which cells were separated by a 12 μm nucleopore filter (transwell assay) and measured the amount of PAI-1 secreted in the medium after 24 and 48 h (Fig. 7A). As anticipated, neuroblastoma cells did not express PAI-1, but the HMEC-1 cells produced 3.1 and 10.4 ng/ml PAI-1 after 24 and 48 h, respectively. The amount of PAI-1 produced in the medium was more than doubled when HMEC-1 cells were cocultured with neuroblastoma cells, regardless of whether or not the two cell types were in contact. Thus, it appeared that neuroblastoma cells may stimulate the production of PAI-1 by endothelial cells via the expression of a soluble factor. This possibility was further demonstrated by testing the effect of serum-free conditioned medium from SK-N-BE(2) cells on PAI-1 production by endothelial cells. The data (Fig. 7B) revealed a 3.1-fold increase in PAI-1 expression when endothelial cells were incubated in the presence of SK-N-BE(2) conditioned medium for 48 h. This stimulatory effect was inhibited by 46% and 31% in the presence of an anti-bFGF receptor or an anti-VEGF receptor antibody, respectively.

PAI-1 Inhibits Neuroblastoma Cell Adhesion to Vitronectin and Promotes Cell Migration toward Fibronectin. Because of the known property of PAI-1 to interfere with vitronectin binding, we investigated the possibility that PAI-1 could affect the binding of SK-N-BE(2) cells to vitronectin (Fig. 8). We observed a 32% and 78% inhibition of cell binding to vitronectin after 90 min in the presence of 40 and 100 nM rPAI-1, respectively (Fig. 8A). This inhibition was specific to vitronectin because rPAI-1 had no inhibitory effect on the binding of SK-N-BE(2) cells to fibronectin (Fig. 8B).

Next we performed a series of experiments to determine whether the inhibition of adhesion to vitronectin would affect cell migration on ECM proteins (haptotaxis). For these experiments, we used transwell chambers with a 12-μm pore size membrane in which the upper side was coated with vitronectin and the lower side was uncoated or coated with vitronectin or fibronectin. In the absence of ECM protein on the lower side of the membrane, cells did not migrate (data not shown), and either vitronectin or fibronectin was required for the cells to migrate. A significant difference was observed between vitronectin and fibronectin in the presence of PAI-1. Whereas rPAI-1 inhibited cell haptotaxis on vitronectin (Fig. 8C), it stimulated migration toward fibronectin (Fig. 8D). Thus, the data show that PAI-1 inhibits neuroblastoma cell adhesion to and migration on vitronectin but stimulates neuroblastoma cell migration from vitronectin toward fibronectin.

**DISCUSSION**

To understand the potential role of the plasminogen-PA system in neuroblastoma metastasis, we have examined the expression of PAs and their receptors and inhibitors in cell lines and primary tumor...
The statistical analysis was performed as indicated in “Materials and Methods.” The number of specimens analyzed in each group is indicated in parentheses. We provide data suggesting that the expression of uPA and tPA in neuroblastoma cells does not indicate a degree of tumor aggressivity but rather reflects their origin in the nervous system.

Fig. 5. Correlation between PAI-1 expression in tumor tissues and clinical stage, recurrent disease, and survival. The amount of RNA for PAI-1 in each tumor sample was determined using a relative quantitative RT-PCR method in the presence of fluorescence-labeled 5’ end primers as described in “Materials and Methods.” The relative amount of PAI-1 in each sample was calculated as the ratio of the fluorescence of the PAI-1 RT-PCR product to the fluorescence of the GAPDH product. The data are a plot of the calculated PAI-1:GAPDH ratio according to clinical stage (A), tumor recurrence (B), or survival (C). The statistical analysis was performed as indicated in “Materials and Methods.” Numbers in parentheses indicate the number of specimens analyzed in each group.

We demonstrate that tPA and uPA are present in both cell lines and tumor tissues. However, there is little evidence that neuroblastoma cells concentrate PA at their cell surface because we found no cell-associated PA activity in acidic eluates of cell lines and little evidence of uPAR expression in cell lines and tumor tissues. PAI-2 was also absent in cell lines and tumor specimens; however, in contrast, PAI-1 was expressed in tumor tissues, and its expression paradoxically correlated with metastatic stage. We also observed that in tumors, PAI-1 is expressed not by tumor cells but by stromal cells and, in particular, endothelial cells. We provide data suggesting that PAI-1 is up-regulated in endothelial cells by the soluble factor(s) secreted by neuroblastoma cells and that it promotes cell detachment from vitronectin and cell migration toward fibronectin.

The association of the plasminogen-PA system with cancer metastasis has been well established (22). However, in most cancers, uPA and uPAR have been mainly associated with tumor progression (23–30). There is much less evidence that tPA also plays a role in metastasis, with the exception of a recent report that has shown that high tPA levels are associated with neoplastic transformation and invasion in pancreatic cancer (31). In neuroblastoma, we found evidence for the expression of predominantly tPA and some uPA; however, there is no suggestion that their level of expression correlates with other indicators of progression such as amplification of MYCN, histology, or clinical stage. The data therefore raise the question of whether these proteases play an active role in metastasis. As an alternative, the expression of uPA and tPA may reflect the origin of these tumors, which are derived from primitive neuroepithelial cells of the neural crest. For example, both uPA and tPA are expressed by murine neuroepithelial cells of day 8.5 embryos (43), and tPA is produced in rats by cells of the floor plate of the neural tube in day 10.5 embryos and thereafter (44). tPA is also synthesized by the neurons of most brain regions of the adult mouse (45). It has been postulated that the expression of these proteases facilitates axon extension and Schwann cell migration during development and regeneration of the peripheral nervous system (46). In addition, tPA may also play a role in neuronal cell death because neuronal cells of the hippocampus in tPA-deficient mice are resistant to kainate-induced neurodegeneration (47). Finally, tPA expression in neuroblastoma is induced during morphological differentiation by retinoic acid, phospholipid esters, or nerve growth factors (48). Altogether, our data and these observations suggest that the expression of uPA and tPA in neuroblastoma cells does not indicate a degree of tumor aggressivity but rather reflects their origin in the nervous system.

In many cancers, high levels of PAI-1 have been shown to paradoxically correlate with an unfavorable outcome (33–35, 52, 53). We have made a similar observation in neuroblastoma tumors because our data indicate higher levels of PAI-1 in specimens derived from stage IV disease or from patients who experienced recurrent disease. Although the number of specimens with stage IVS was too small (seven) to be conclusive, it is interesting to note that none of these specimens had a PAI-1:GAPDH ratio of >2, as observed in some stage IV disease. In contrast with stage IV disease, which carries a poor clinical outcome, stage IVS disease has a better outcome and is often associated with spontaneous regression of metastatic disease. The absence of high levels of PAI-1 in stage IVS disease may therefore reflect an important biological difference between these two metastatic stages. Interestingly, as observed by others (54, 55), PAI-1 was specifically expressed not by the tumor cells but by stromal cells and, in particular, by endothelial cells in tumor tissues. In this regard, we provide evidence supporting the involvement of soluble paracrine factors such as bFGF and VEGF because partial inhibition of PAI-1 up-regulation was obtained in the presence of antibodies against the receptors for these growth factors. bFGF and VEGF have been shown to promote...
Fig. 6. Immunohistochemical analysis of PAI-1 expression in neuroblastoma tumors. Sections from frozen tumor tissue preserved in OCT compound were incubated in the presence of an anti PAI-1 monoclonal antibody (A, B, G, and H), an anti-factor VIII-related antigen monoclonal antibody (E and F), and a nonspecific mouse IgG1 (C and D). A, C, and E, stage I disease. B, D, F, G, and H, stage IV disease. Bars, 105 (A, B, C, D, E, and F) and 42 μm (G and H). Arrows indicate the presence of PAI-1-positive endothelial cells (EC), perivascular cells (PVC), smooth muscle cells (SMC), stromal cells (S), and PAI-1 negative tumor cells (T).
tumor angiogenesis and PAI-1 expression in endothelial cells (56), and preliminary investigations in our laboratory have shown that neuroblastoma tumor tissues express these two growth factors.

The observation of high levels of PAI-1 expression in metastatic neuroblastoma raises the possibility that, in these tumors, PAI-1 may have a function unrelated to its antiprotease activity. In this regard, we provide data indicating that PAI-1 specifically inhibits the adhesion of neuroblastoma cells to vitronectin. This function is related to the ability of PAI-1 to bind to the somatomedin B-like domain of vitronectin and to interfere with cell binding as proposed by others (19 –21). Two types of receptors for vitronectin could be competed by PAI-1: (a) uPAR, which has a specific binding domain that recognizes the somatomedin B-like domain of vitronectin (22); and (b) integrins of the \( \alpha_v \) family (\( \alpha_v\beta_1 \), \( \alpha_v\beta_3 \), and \( \alpha_v\beta_5 \)), which bind to the RGD domain of vitronectin that is masked by binding of PAI-1 to the somatomedin B-like domain (20). Interestingly, the effect of PAI-1 on cell migration (haptotaxis) dramatically depended on the type of ECM protein that serves as attractant. With vitronectin, PAI-1 inhibited not only adhesion but also migration, whereas with fibronectin, PAI-1 did not affect adhesion but stimulated migration from vitronectin toward fibronectin. A similar inhibitory effect of PAI-1 on smooth muscle cell migration on vitronectin has been reported previously (57).

Fig. 7. Stimulation of PAI-1 expression in endothelial cells by neuroblastoma cells. A, SK-N-BE(2) cells (1.6 \( \times \) 10^4 cells/dish) were cultured in the bottom (contact) or upper (no contact) chamber of a transwell dish in which HMEC-1 cells (5 \( \times \) 10^4) had been preplated in the bottom chamber for 24 h in the presence of serum-free medium. The amount of PAI-1 secreted in the culture medium after 24 and 48 h of coculture was determined by ELISA. The data represent the mean \( \pm \) SD of triplicate dishes. B, HMEC-1 cells (5 \( \times \) 10^4 cells/dish) were cultured in serum-free medium (Control) or in the presence of serum-free conditioned medium from SK-N-BE(2) cells. When indicated, the conditioned medium was supplemented with a monoclonal antibody against bFGFR receptor (+ anti bFGFR), VEGFR receptor (+ anti VEGFR) or a murine nonspecific IgG1 (+ IgG1) at 2.5 ng/ml (1:400 dilution). After 48 h, the amount of PAI-1 present in the culture medium was determined by ELISA. The data represent the mean amount of PAI-1 secreted (\( \pm \) SD) from duplicate aliquots obtained from duplicate dishes.

Fig. 8. Effect of PAI-1 on SK-N-BE(2) cell adhesion and migration. A and B, active, rPAI-1 (American Diagnostic, Inc.) was added to (A) vitronectin- or (B) fibronectin-coated dishes at the indicated concentrations before adding SK-N-BE(2) cells. After 90 min (vitronectin) or 120 min (fibronectin), the plates were carefully washed, and the amount of remaining adherent cells was determined by triazol blue staining as indicated in “Materials and Methods.” The data represent the mean OD (\( \pm \) SD) of two independent experiments performed in triplicate. C and D, the upper side of the nuclospore membrane of a transwell dish (12-\( \mu \)m pore size) was coated by vitronectin (150 ng/filter), and the bottom side was coated with vitronectin (2 \( \mu \)g; C) or fibronectin (2 \( \mu \)g; D). Active rPAI-1 was added at the indicated concentrations to the upper chamber of the transwell dish (vitronectin-coated side) before the addition of 5 \( \times \) 10^5 SK-N-BE(2) cells/well in serum-free medium. After a 20-h incubation at 37°C, the cells present on the lower side of the filter were stained and counted. The data show the number of cells/filter and represent the mean (\( \pm \) SD) of two filters. The experiment is representative of three experiments showing similar results.
in neuroblastoma, up-regulation of PAI-1 in endothelial cells may be a mechanism used by the tumor cells to detach from vitronectin, migrate toward fibronectin-rich matrices, and metastasize.

We have recently reported that neuroblastoma tumors express two MMPs, MMP-2 and MMP-9 (58). Whereas MMP-2 was present almost exclusively in a proform, MMP-9 was detected in both the active and inactive forms and was selectively expressed by stromal cells. Thus, our data raise the question of the collaboration between MMPs and the plasminogen-PA system. The activation of proMMP-2 is not mediated by plasmin but rather by a membrane type-MMP (MT1-MMP) that is not expressed by neuroblastoma. In contrast, plasmin is implicated in the activation of proMMP-9 (59, 60), and it is therefore conceivable that uPA- and tPA-generated plasmin plays the role of activator for proMMP-9 in neuroblastoma.

In summary, our analysis of the plasminogen-PA system in neuroblastoma has indicated that these tumors express uPA and tPA independently of their clinical stage or of the expression of other prognostic markers. In contrast, PAI-1 is associated with metastatic stage and tumor recurrence. It is expressed in stromal cells, likely as a result of the production of paracrine factor(s) by the malignant cells, and may promote metastasis by allowing cells to detach from vitronectin and migrate.

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