Elevated Levels of Urokinase-Type Plasminogen Activator and Plasminogen Activator Inhibitor Type-1 in Malignant Human Brain Tumors

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ABSTRACT

The plasminogen-plasmin system has been found to modulate neoplastic spread and angiogenesis in tumors outside the central nervous system (CNS), but there have been no quantitative studies on the invasive and vascular tumors of the CNS. Quantitative zymography and enzyme-linked immunosorbent assay were used to determine the amounts of urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator, and plasminogen activator inhibitors type 1 and type 2 (PAI-1 and PAI-2) in benign and malignant primary brain tumors (n = 28) as well as nonneoplastic brain (n = 5). u-PA and PAI-1 antigens were undetectable in normal brain but significantly elevated in glioblastoma multiforme (u-PA, 2.86 ± 3.01 ng/ml; PAI-1, 8.19 ± 5.57 ng/ml; P < 0.001). There was no difference, however, in tissue-type plasminogen activator antigen levels among control, benign, or malignant tissues except for a 4- to 7-fold increase in acoustic neuroma. PAI-2 was detected at low levels in 2 of the 33 specimens. These findings indicate that malignancy in primary CNS neoplasms is associated with elevated levels of u-PA and PAI-1, supporting the role of the plasminogen-plasmin system in the pathogenesis of CNS malignancy and as a potential biomarker and therapeutic target.

INTRODUCTION

The malignant phenotype of CNS neoplasms is characterized by local invasion (1, 2) and angiogenesis (3, 4); however, the mechanisms regulating these processes in brain tumors is poorly understood. Proteolytic degradation of the extracellular matrix is an essential first step in the invasive processes of neovascularization and tumor cell invasion (5, 6). The plasminogen activators, t-PA and u-PA, catalyze the conversion of plasminogen to plasmin, a serine protease with broad specificity that degrades noncollagenous components of the extracellular matrix and converts the zymogen procollagenase to active collagenase (6).

Elevated levels of u-PA and PAI-1 in tumor tissue are powerful and independent predictors of poor prognosis in breast cancer (7–10) and have been correlated with histological degree of malignancy in breast, colon, prostate, skin, and other tumors outside of the CNS (11). Nonspecific PA activity in brain tumor extracts has been correlated with the amount of peritumoral brain edema (12); however, the role of u-PA in human brain tumors has been investigated only in vitro (13, 14). In the present study we have examined the amount and activity of u-PA, t-PA, and PA inhibitors by zymography and ELISA in extracts of nonneoplastic human brain and in brain tumor tissues, and now report that u-PA and PAI-1 are selectively increased in glioblastoma.

MATERIALS AND METHODS

Tissue Preparation. Brain tumor samples were collected at the time of surgery, embedded in O.C.T. Compound (Miles Inc. Elkhart, IN), snap frozen in isopentane and stored at -70°C. Frozen sections of each sample were cut, stained with hematoxylin and eosin, and then reviewed so that only well preserved, nonneurotic, representative tissue was included in the study. Samples were then thawed; rinsed in cold phosphate-buffered saline, pH 7.4; and weighed. Tissue fragments were homogenized in an ice bath with a motor-driven Teflon pestle for 3 min in 1 ml of extraction buffer/50 mg of tissue wet weight (0.075 M potassium acetate, 0.3 M NaCl, 0.1 M L-arginine, 10 mM EDTA, 0.25% Triton X-100, pH 4.2) (15). The homogenates were centrifuged at 12,000 × g for 10 min at 4°C and the supernatants were aliquoted and stored at -70°C. Protein concentrations were determined by the method of Bradford (16) using an IgG protein standard (Bio-Rad, Richmond, CA).

Plasminogen Activator Determinations. Quantification of antigen was performed using commercially available ELISA kits (t-PA, Diagnostica Stago, Asnieres-Sur-Seine, France; u-PA, PAI-1, PAI-2, Biopool, Umea, Sweden). All determinations were made in duplicate on separate ELISA plates. The u-PA ELISA detects u-PA, pro-u-PA, and u-PA-PAI complexes. Zymography was carried out as a modification of the method of DePetro et al. (17). Equivalent protein amounts of tissue extract were electrophoresed under nonreducing conditions on a 10% sodium-dodecyl-sulfate polyacrylamide gel. Each gel included lanes loaded with two-chain t-PA (M, 68,000; American Diagnostica, Greenwich, CT) and varying amounts of human high-molecular weight u-PA (M, 54,000; American Diagnostica) to quantify u-PA activity in the tissue extracts. The gels were washed twice for 3 min in 0.1 M Tris·2.5% Triton X-100, pH 8.1 and overlaid on a 10 × 10 cm casein-agarose gel made with 10 ml of Bio-Rad protease gel substrate tablets dissolved in distilled water supplemented with 10 mg/ml of nonfat dry milk and 20 μg/ml of affinity-purified human plasminogen (18). The zymograms were incubated overnight at 4°C then at 37°C and photographed hourly as the lytic bands developed. Quantification of zymographic activity was done by computer-assisted image analysis on an imaging densitometer (Bio-Rad Model GS-670) (19). A numerical value, representing both size and density, was calculated as the sum of the gray scale units per square millimeter of each lytic band, adjusted by subtraction of background. A standard curve for determination of u-PA activity in the tissues extracts was generated from the four u-PA activity standards included on each gel. As a control, casein-agarose underlay gels were prepared without plasminogen to visualize any intrinsic caseinolytic activity in the tissue extracts. In addition, polyclonal anti-t-PA or anti-u-PA antibodies (American Diagnostica) were incorporated into underlay gels to verify the identity of the lytic bands.

Calculations and Statistics. Antigen concentrations of u-PA, t-PA, and PAI-1 were expressed as nanograms of antigen per milligram of total protein. Enzyme activities were expressed as milli-international units (mlU) of u-PA per milligram of total protein. Comparisons between tumor types were made by two-tailed analysis of variance, except in the case of normal brain, which had undetectable u-PA and PAI-1 (and therefore no intragroup variance). Differences were considered significant at P values < 0.05. To determine significance of difference from normal brain, levels of u-PA and PAI-1 in each tumor type were compared to zero by Student’s t test. Correlations between u-PA, t-PA, PAI-1, and u-PA activity were determined by a Pearson correlation matrix with confidence levels determined by Bonferroni probabilities. The nonparametric Spearman rank test produced similar correlations with equivalent levels of significance.

RESULTS

The levels of u-PA, t-PA, and PAI-1 antigens in extracts of 28 brain tumors and five normal brain specimens are summarized in Fig. 1. The
an extract of nonneoplastic brain contained 4.8 ng/mg, barely exceeding the threshold of detection (3.0 ng/mg) of the assay.

The u-PA levels were significantly correlated with the levels of PAI-1 among the 20 astrocytic tumors (r = 0.655; P < 0.01) (Fig. 2). There were no significant correlations, however, between the levels of t-PA and either PAI-1, or u-PA.

A representative zymogram is shown in Fig. 3. There was no caseinolytic activity in any of the tissues when plasminogen was omitted from the underlay gel, indicating that the tissue extracts lacked intrinsic plasmin activity. The u-PA and t-PA lytic bands on the zymogram were identified by comigration with u-PA and t-PA standards, and by their disappearance after inclusion of polyclonal anti-human-u-PA or anti-human-t-PA antibodies in the casein-agar underlay (Fig. 4). A significant elevation of u-PA activity was detected in glioblastoma compared to normal brain (P < 0.001) (Fig. 5). The zymographic u-PA activity was correlated with the u-PA antigen levels determined by ELISA (r = 0.652; P < 0.005) (Fig. 6).

DISCUSSION

The finding of significantly increased u-PA and PAI-1 in glioblastoma provides a new biomarker for malignant tumors of the CNS. In several human carcinomas, high levels of u-PA in tumor tissue are average u-PA content of the glioblastoma tissue was increased more than 10-fold in comparison to less malignant forms of glioma (anaplastic astrocytoma, and oligodendroglioma and anaplastic astrocytoma) and P < 0.001 compared to normal brain. b, minimum sensitivity of assay for u-PA: <0.04 ng/mg of total protein. c, P < 0.001 compared to all other groups. d, P < 0.005 compared to glioma. e, P < 0.003 compared to meningioma, and P < 0.001 compared to normal brain. e, minimum sensitivity of assay for PAI-1 <0.20. f, P < 0.01 compared to normal brain. Values are the mean ± SD of each group.
no antibody  
anti - u-PA  
anti - t-PA

Fig. 4. Verification of the lytic bands in thecasein-agarose underlay. Three identical sodium-dodecyl-sulfate polyacrylamide gel electrophoresis gels were prepared. Four lanes of each gel were loaded with purified high-molecular weight u-PA extracts of glioblastoma and acoustic neuroma, and purified t-PA. Polyclonal anti-u-PA or anti-t-PA blocking antibodies were incorporated in two of the underlay gels, obliterating the respective u-PA and t-PA lytic bands.

A substrate for u-PA-generated plasmin is not immediately apparent in brain tumors because, aside from basement membrane around blood vessels and at the glial limits externa, the extracellular matrix of normal brain lacks the glycoproteins and interstitial collagens typically found in the stroma of other tissue types (33). The interstitial space of glioblastoma, however, has been found to contain vitronectin, a potential substrate for plasmin (34). Increased vascular permeability allows extravasation of serum proteins into the tumor bed and is an early finding in the pathogenesis of malignant brain tumors (4). Thus, serum-derived plasminogen would be readily available as a substrate for u-PA in glioblastoma.

Prominent angiogenesis with vascular endothelial proliferation is a hallmark of glioblastoma multiforme (35). Despite ample evidence for u-PA production in cultured malignant glioma, the primary source of u-PA in vivo may be the vascular endothelium. Endothelial cell-mediated proteolytic degradation of basement membrane is a necessary step in angiogenesis (36). Glioblastomas have been found to contain

correlated with malignancy by histological criteria (20–23). The current study demonstrates that a similar pattern is true for primary human CNS tumors.

Astrocytic tumors including astrocytoma, oligodendroglioma, and glioblastoma multiforme differ from non-CNS neoplasms by their lack of fibroblastic stromal cells. Because there was no u-PA in normal brain, the question arises as to what are the cellular sources of u-PA in malignant glioma. In colon cancer, in situ hybridization demonstrates that fibroblastic stromal cells express u-PA that is subsequently bound to u-PA receptors on the surface of tumor cells (24). By contrast, in squamous cell carcinoma of the skin, u-PA is produced and bound by the tumor cell (22). In CNS tumors, the potential sources of u-PA include either the tumor or endothelial cell subpopulations.

The local invasion of neoplastic cells into the brain adjacent to tumor is the principal reason for failure of all local therapies in the treatment of malignant primary CNS tumors. In vitro, u-PA activity has been found to be essential for tumor cell invasion (25–27). Normal and neoplastic glial cells in vitro produce u-PA and PA inhibitors (12, 13, 28–32). The present finding of increased u-PA in the highly invasive glioblastoma multiforme suggests a role for u-PA-mediated proteolysis in tumor cell invasion in the brain.

Fig. 5. u-PA activity levels in tissue extracts as determined by quantitative zymography. a, P < 0.05 as compared to glioma and P < 0.001 compared to normal brain. b, minimum sensitivity of assay 5 mlU/mg. Values are the mean ± SD of each group.

Fig. 6. u-PA antigen as determined by ELISA versus u-PA activity determined by quantitative zymography in tissue extracts of benign and malignant brain tumors and nonneoplastic brain (n = 25). Pearson r = 0.652; Bonferroni P < 0.005.
several angiogenic factors including basic fibroblast growth factor (37) and vascular endothelial growth factor (38, 39) that not only are mitogens, but also potent inducers of u-PA production in endothelial cells (40, 41).

In addition to a marked increase in u-PA, there was also a prominent elevation of PAI-1 in glioblastoma compared to less aggressive gliomas. For cancers of the breast, recent reports show that levels of PAI-1 are elevated in parallel to u-PA and serve as a powerful predictor of prognosis (7, 10). The up-regulation of both u-PA and its inhibitor, PAI-1, appears paradoxical. Angiogenesis, however, requires a tightly controlled balance between production of u-PA and PAI-1 (36). PAL1 elevation of PAI-1 in glioblastoma compared to less aggressive gliotic neuroma (42). The similar t-PA content in normal brain and gliotic tissue may also play a role in protection of tumor stroma from autodegradation by tumor cell u-PA (7).

The data also confirm the previous report of elevated t-PA in acoustic neuroma (42). The similar t-PA content in normal brain and gliotic multiforme. This provides a rationale for the use of inhibitors of u-PA for the inhibition of angiogenesis and neoplastic infiltration in CNS tumors. Measurement of u-PA in tumor tissue or in serum, recently reported to be clinically valuable in detection of colorectal cancer (44), may be useful to determine prognosis and evaluate efficacy of treatment in patients with malignant gliomas.

REFERENCES


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