Expression and Localization of Urokinase-Type Plasminogen Activator in Human Astrocytomas in Vivo


Abstract

Plasminogen activators regulate a variety of processes involved in tissue morphogenesis, as well as cell differentiation, migration, and invasion. We examined the relative amounts of mRNA and protein and localization of urokinase-type plasminogen activator (uPA) in human astrocytomas in vivo. Using fibrin zymography and densitometric quantitation, we found that uPA activity was significantly higher in malignant astrocytomas, especially in glioblastomas, than it was in normal brain tissues or low-grade gliomas. The amounts of uPA mRNA, as determined by Northern blot analysis, were higher in anaplastic astrocytomas and glioblastomas than in normal brain tissues and low-grade gliomas, consistent with the amounts of uPA activity. To investigate the cellular source of uPA in various tissues, we performed immunocytochemical localization of uPA protein and in situ hybridization of uPA mRNA with astrocytomas and normal brain tissues. Immunocytochemical staining for uPA showed strong immunoreactivity in the tumor cells and vasculature of glioblastomas and anaplastic astrocytomas but undetectable or very low immunoreactivity for uPA in low-grade gliomas and normal brain tissues. uPA mRNA was located in astrocytomas and endothelial cells and was heterogeneously distributed within glioblastoma, with preferential localization near vascular proliferation and at the leading edge of the tumor. uPA expression was dramatically higher in highly malignant astrocytomas, especially glioblastomas, and was correlated with malignant progression of astrocytomas.

Introduction

uPA is synthesized by a variety of tissues, including cells of the central nervous system. uPA is thought to play a pivotal role in the tissue-remodeling process in several physiological and pathological conditions, including wound healing, inflammatory cellular migration, neovascularization, neurite outgrowth, and cancer cell invasion and metastasis, by activating a proteinase cascade that requires localized degradation of the extracellular matrix (1–6). uPA is secreted as an enzymatically inactive single-chain proenzyme or is bound to uPA receptor; it is more readily converted into the active two-chain serine protease by plasmin, kallikrein, and cathepsin B, or nerve growth factor (7–9). Receptor-bound two-chain serine protease activates plasminogen by its cleavage to plasmin, the broad spectrum trypsin-like protease that degrades extracellular matrices such as fibrin, fibronectin, proteoglycans, and laminin (3, 9). Plasmin also indirectly activates latent forms of collagenases, which hydrolyze plasmin-resistant collagenous elements of the extracellular matrix (10).

For many malignant tumors, including lung, breast, and colon carcinomas, there is a significant correlation between the production of uPA and tumor invasion (11–13). Murine tumor cells can be inhibited in their metastatic properties (14) and human tumor cells can be inhibited in their invasive properties (15) with antibodies specific for uPA, demonstrating that uPA activity is essential for tumor cell invasion and metastasis. In addition, tumor cells induce neovascularization in vivo (16); and during the formation of new vessels, endothelial cells degrade the basement membrane and migrate into the surrounding tissue, a process that requires these cells to produce serine proteinases (17).

The most common and malignant brain tumor, glioblastoma multiforme, is distinguishable from astrocytomas by the presence of necrosis, vascular proliferation, and invasion into the surrounding normal brain tissue (18). Increased uPA activity in more malignant cell phenotypes of an astrocytoma cell line in vitro have been described previously (19–21) but the relationship of these in vitro observations to the invasive properties of glioblastomas in vivo was not determined. We previously showed that uPA activity is dramatically higher in human malignant brain tumors in vivo (22, 23). We sought to determine whether uPA is expressed in the tumor cells of human glioblastoma and whether it is associated with tumor progression, invasion, and angiogenesis. We examined the level of uPA activity and the cellular source of uPA in human astrocytomas and normal brain tissues. Our results indicate significantly higher levels of uPA mRNA and uPA protein in anaplastic astrocytomas and glioblastomas than those that appear in normal brain tissues and low-grade gliomas.

Materials and Methods

Surgical Specimens. For fibrin zymography and Northern blot analysis, fresh human brain tumor tissue and normal brain tissue samples were collected in the operating room from patients undergoing craniotomy. The samples were flash-frozen in liquid nitrogen immediately after surgical removal and kept at −80°C. Tissue samples for immunochemistry of uPA protein and in situ hybridization of uPA mRNA were fixed in 10% formalin and embedded in paraffin. Tissue sections were from surgical specimens obtained from patients operated on in 1992 and 1993 and provided by the Department of Pathology, M. D. Anderson Cancer Center. The histological diagnosis was confirmed for each tissue block by standard light-microscopic evaluation of sections stained with hematoxylin and eosin. The samples included 10 glioblastomas, 7 anaplastic astrocytomas, 7 low-grade gliomas, and 8 normal brain tissue samples. None of the patients had been subjected to chemotherapy or radiation therapy before resection.

Tissue Extracts. The frozen tissues were homogenized in Tris buffer (50 mM Tris-HCl, pH 7.5, containing 75 mM NaCl) and centrifuged at 15,000 × g
Fibrillar Zymography. The enzymatic activity and molecular weight of electrophoretically separated forms of PA were determined in tumor extracts and normal brain extracts by SDS-PAGE as described previously (22). Briefly, the SDS-PAGE gel contains acrylamide to which purified plasminogen and fibrinogen were added as substrates before polymerization. After polymerization, tissue extracts (50 μg) of the samples were electrophoresed and the various types of PA were separated based on differences in their molecular weight. The SDS-PAGE gel was then washed twice with 2.5% Triton X-100 for 30 min each time, and the gel was incubated at 37°C overnight with glycine buffer (pH 7.5). After staining with Coomassie blue and destaining, the final gel had a uniformly blue background except in regions to which PA have migrated and activated the plasminogen to plasmin. The levels of enzymatic activity were quantitated in the SDS-PAGE, M, 55,000 band in various tumor and normal brain tissue extracts. To ensure that the intensity of the bands fell within a linear range, the gels were incubated for different time intervals before quantitative estimation by densitometry.

Immunohistochemistry. Sections (4 μm) of fixed tissues embedded in paraffin were mounted on silane-coated slides. The slides were dewaxed with a solution containing 3 parts of xylene and 5 parts of acetone and blocked with normal goat serum. The sections were then incubated with rabbit anti-human von Willebrand factor (DAKO Corp., Carpinteria, CA) for 1 h at room temperature. Then the sections were exposed to biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc., Burlingame, CA) and alkaline phosphatase-conjugated streptavidin (DAKO Corp.), followed by a naphthol AS-BI phosphate and Newfuchsin solution and hematoxylin counterstain. The primary antibody was omitted in the controls.

For immunohistolocalization of uPA in these sample tissues, paraffin sections were incubated with mouse monoclonal antibody for uPA (394; American Diagnostica) and used at a protein concentration of 40 μg/ml for 1 h of incubation. Staining was done with biotin-streptavidin alkaline phosphatase (Biogenes Laboratorys, San Ramon, CA) and alkaline phosphatase activity was visualized by the addition of a substrate solution consisting of naphthol AS-BI phosphate, levamisole, and fast red TR, which forms an intense red color. Control study was performed by using a nonimmune serum as the primary antibody instead of a mouse monoclonal antibody for uPA.

Northern Blot Analysis. Total RNA from human astrocytomas and normal brain tissues was extracted with the use of a standard guanidinium thiocyanate-phenol chloroform technique (25). Twenty μg of total RNA, as determined by absorbance measurement and ethidium bromide staining, were electrophoresed in 1.5% agarose gels and transferred to Nytran-modified nylon filters by capillary action using 10× SSC buffer (1× SSC is 150 mM NaCl-15 mM sodium citrate). Cloned cDNA probes were labeled with a random primed labeling kit (Boehringer Mannheim) using [³²P]dCTP as radioactive label. The filters were then hybridized overnight at 42°C with the prehybridization solution containing the radiolabeled and denatured 1.5-kilobase DNA specific for uPA mRNA as described (26, 27). The filters were then washed in 1× SSC-0.75% sodium dodecyl sulfate at 50°C and dried. X-ray films (Kodak XAR) were exposed to the film for 1 to 3 days at −70°C using intensifying screens. RNA loading equalities were checked by reproping the blot with a cDNA corresponding to glyceraldehyde phosphate dehydrogenase. The levels of uPA mRNA were determined by densitometry and normalized to the control probe.

Oligonucleotide Probes. uPA-specific oligonucleotide DNA probes were designed complementary to the 5′ end of the human mRNA transcript based on published reports of the genomic DNA sequences (28, 29). Oligonucleotide probes were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer and purified using a PD-10 Sephadex G-25 column (Pharmacia Fine Chemicals, Piscatway, NJ). The 30-base oligonucleotide of sequence 5′-CGA TGG AAC ATG AAG TTA TTC ATT GCT GCC 3′ was unique to the uPA mRNA as determined by a GenBank database search. The sequence of the corresponding control sense oligonucleotide was 5′-GCG AGC AAT GAA CTT CAT CAA GTT CCA TCG 3′.

The oligonucleotide DNA probes were labeled with digoxigenin-labeled dUTP using a DIG Oligonucleotide Labeling Kit (Boehringer Mannheim) according to the manufacturer’s instructions. Labeled probes were then purified on Sephadex G-50 columns (Boehringer Mannheim). To optimize the labeling conditions, the probes were tested on positive control cell lines that were known to express uPA. The specificity of the antisense oligonucleotide was also confirmed by Northern blot analysis in uPA mRNA-positive glioblastoma cell lines (27).

Preparation of Samples for in situ Hybridization. Serial 4-μm-thick histological sections were cut from formalin-fixed, paraffin-embedded tumor tissues and mounted on silane-coated slides. All steps were performed at room temperature unless otherwise specified. The slides were dewaxed with a solution containing 3 parts of xylene and 5 parts of acetone. After hybridization, the sections were pretreated with 0.2 N HCl and 5 μg/ml of proteinase K (Boehringer Mannheim) at 37°C and postfixed with 4% paraformaldehyde phosphate-buffered saline. The samples were rinsed with 0.2% glycine in 0.1 M Tris-HCl, pH 7.5, and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0) for 10 min. After a rinse with 2× SSC, prehybridization was performed for 2 h at 37°C in buffer containing 40% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 2× SSC, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0), 125 μg/ml of yeast RNA, 100 μg/ml of denatured salmon sperm DNA, 0.1 mg/ml polyacrylamide, and 5 μg/ml of polydeoxyadenylic acid. Hybridization was done in a prehybridization buffer supplemented with 0.3 μg/ml of digoxigenin-labeled oligonucleotide probe. Sections were hybridized overnight in a humidified chamber at 37°C. After hybridization, the slides were washed three times in 50% formamide/2× SSC (60 min each) at 37°C and twice in 0.5× SSC (60 min) at 37°C and then rinsed with 2× SSC for 15 min. The slides were incubated in 1% blocking reagent (Boehringer Mannheim) for 60 min and then placed in a humidified chamber and incubated with a 1:1000 dilution of monoclonal mouse anti-digoxigenin antibody (Boehringer Mannheim) for 60 min. The sections were then subjected to three 30-min washes in Tris-buffered saline (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and incubated with biotinylated anti-mouse immunoglobulin G for 30 min at room temperature. The sections were incubated with avidin-alkaline phosphatase conjugate for 30 min at room temperature and rinsed in Tris-buffered saline. Tissue-bound alkaline phosphatase activity was visualized by incubation with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as substrate for 1–2 h at 37°C, according to the Boehringer Mannheim protocol, and the slides were placed in a dark, humid chamber. The enzyme reaction was stopped by rinsing the sections with EDTA solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The sections were counterstained with methyl green, air dried, and covered with Permount mounting medium. A brown or blue-brown color was considered a positive reaction. uPA mRNA-positive tumor cell and blood vessel density was assessed by light microscopy.

As a positive control in the in situ hybridization study, we generated formalin-fixed and paraffin-embedded cell pellets of UWR3, a glioblastoma cell line that expresses uPA (27). To confirm the specificity of the hybridization signal, we performed various types of control experiments, which indicated that the staining pattern was abrogated under the following conditions: substitution of the antisense probe with a digoxigenin-labeled sense probe; RNase pretreatment of tissue section (100 μg/ml for 37°C at 1 h); or use of 50-fold excess amount of unlabeled antisense oligonucleotide. As a control for endogenous alkaline phosphatase, we included treatment of the samples in the absence of the digoxigenin-labeled probe.

Results

Quantitation of Tumor-associated uPA. To determine the level of endogenous uPA in human astrocytomas and normal brain tissues, we performed zymography and quantitated the intensity of the clear bands by densitometry. Fig. 1A shows that the levels of Mr 55,000 uPA were significantly higher in anaplastic astrocytomas and glioblastomas than they were in normal brain tissues or low-grade gliomas. Significantly higher levels of uPA activity were found in anaplastic astrocytomas (P < 0.001) and glioblastoma (P < 0.0001) compared to low-grade gliomas and normal brain tissues (Fig. 1B).
UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN ASTROCYTOMAS IN VIVO

low-grade gliomas and normal brain tissues (Fig. 3, c and d). No staining was seen when nonimmune serum was substituted for the anti-uPA antibody.

In Situ Hybridization for uPA mRNA. In situ hybridization was performed to localize uPA mRNA in brain tumor and normal brain tissue (Fig. 4). In glioblastomas, uPA mRNA was heterogeneously and abundantly expressed in the tumor cells (Fig. 4, a and b). Endothelial cells also were strongly stained but the staining was weaker than that in astrocytoma cells (Fig. 4a). uPA mRNA-expressing cells of the vascular wall coexpressed von Willebrand factor (data not shown). The expression and distribution of uPA mRNA were related to the structural components of the tumor, such as the viable tumor cells, vascular structures, and leading edge of the tumor. A general feature of hybridization with uPA mRNA was the strong expression of the cells that were near sites of vascular proliferation.

Northern Blot Analysis. Total RNA was extracted from various types of gliomas and normal brain tissue, and the steady-state levels of uPA transcript were compared by Northern analysis using a 1.5-kilobase cDNA probe. uPA mRNA was present in all samples but only at very low levels in normal brain tissues and low-grade gliomas (Fig. 2A). Quantitative evaluation of hybridization signals after normalization to the glyceraldehyde phosphate dehydrogenase hybridization signal indicated that uPA mRNA levels were 6-fold higher in anaplastic astrocytomas ($P < 0.001$) and 20-fold higher in glioblastomas ($P < 0.0001$) than they were in normal tissues (Fig. 2B). Anaplastic astrocytomas and glioblastomas with high levels of uPA activity had proportionately higher levels of uPA mRNA than did normal brain tissues and low-grade gliomas. Thus, we found a correlation between uPA mRNA levels and endogenous uPA activity in these tissues.

Immunohistochemical Detection of uPA. We determined the distribution of the uPA in the tumor and normal tissue by immunohistochemical staining using paraffin-embedded sections. Antibodies against uPA showed strong immunoreactivity in the tumor cells and the vasculature of glioblastomas and anaplastic astrocytomas (Fig. 3, a and b). uPA was almost undetectable or very faintly stained in
Plasminogen activators have an important regulatory role in both the normal and malignant processes (1, 3–6). The increased activities of the plasminogen activator pathway in malignant cell phenotypes of the central nervous system have been well described in in vitro studies (19–21) and the differing expressions of the plasminogen activators are thought to reflect significant differences in the biological behavior of intracranial malignant tumors. Our previous studies of various types of human intracranial tumors showed that there was higher PA activity in glioblastomas and metastatic brain tumors than in normal brain tissues, low-grade gliomas, and meningiomas (22) but significantly decreased levels of tissue-type plasminogen activator in glioblastomas compared to those in normal brain tissues or low-grade gliomas (23).

Control hybridization with a sense oligonucleotide probe showed the absence of a specific hybridization signal in glioblastoma tissues (Fig. 4c); control hybridization with RNase pretreatment of the tissue section or use of an unlabeled antisense probe also exhibited no staining (data not shown). Tumor cells at the leading edge of the invasively growing tumors also showed a positive hybridization signal to uPA mRNA in glioblastoma (Fig. 4d). In anaplastic astrocytomas, both tumor cells and endothelial cells demonstrated weak to strong hybridization signal with the uPA mRNA (Fig. 4e). Using a sense oligonucleotide probe we did not observe specific hybridization signals in anaplastic astrocytomas (Fig. 4f). uPA mRNA was absent or barely detectable in low-grade astrocytomas and normal brain tissues (Fig. 4, g and h, respectively). Only a few scattered astrocytes in low-grade gliomas showed weak hybridization with the uPA mRNA (Fig. 4g).

Discussion

We have examined the endogenous levels and cellular sources of uPA in human astrocytomas growing in vivo to ascertain whether the expression of uPA is associated with malignant progression in astrocytomas and whether overexpression of uPA is associated with the invasive properties of malignant primary brain tumors. We found significantly (P < 0.001) increased levels of uPA activity and uPA mRNA in malignant astrocytomas, especially in glioblastomas, compared to levels in low-grade gliomas and normal brain tissues (Figs. 1 and 2). Immunohistochemical and in situ hybridization studies indicated that uPA protein and uPA mRNA are localized within astrocytoma cells and endothelial cells and are heterogeneously distributed within glioblastomas, preferentially localized near vascular proliferation zones and at the leading edges of tumors (Figs. 3 and 4). Similar to previous studies (30, 31), we found that PA activity and PA antigen expression are associated with the expression of uPA mRNA. Our results suggest that the uPA expression is increased in tumor cells and in endothelial cells during the development and malignant progression of astrocytomas; these results suggest further that uPA may contribute to the invasion of malignant astrocytomas into adjacent normal brain tissue and to tumor angiogenesis. An enhanced plasminogen activation system at the surface of tumor cells could lead to a proteolytic cascade that results in pericellular degradation of the surrounding extracellular matrix. In particular, we observed high uPA mRNA expression at the leading edges of tumors, suggesting a role for uPA in the invasive process.

Cell-surface-associated dissociation and degradation of the extracellular matrix are important features of tumor cell invasion (4, 32). Clinically, uPA activity has been reported to be significantly associated with an increased relapse rate in patients with breast cancer (12,
Fig. 4. uPA mRNA in situ hybridization in various types of human gliomas and normal brain tissue observed with antisense and sense probes. (a, b, d, e, g, and h) Hybridization with antisense oligonucleotide probe. (c and f) Hybridization with sense oligonucleotide probe that was used as a control showed no specific hybridization signals in glioblastoma and anaplastic astrocytoma, respectively. (a and b) uPA mRNA expression in glioblastoma tissue samples from two different patients. (d) Specific expression of uPA mRNA at the leading edge of tumor in glioblastoma. (e, g, and h) In situ hybridization of tissue samples from anaplastic astrocytoma, low-grade glioma, and normal brain tissue, respectively. × 315.
Moreover, the expression of uPA increases during the progression of malignant disease in patients with brain tumors (22, 34). However, ours is the first report to show a direct correlation between expression of uPA mRNA in tumor cells and malignant progression in brain tumors in vivo and to localize uPA mRNA and antigen in tumor and normal cells.

High expression of uPA is not always found in tumors. Pyke et al. (35) studied the localization of uPA mRNA in sections of human colon carcinomas and could not detect uPA mRNA expression in the colon cancer cells. They concluded that the malignant colon epithelial cells recruit fibroblast-like cells and endothelial cells into the tumor stroma to produce uPA. We have previously demonstrated that several cell types of glioblastomas that express uPA possess specific high-affinity cell surface receptors that bind both the single- and two-chain forms of the enzyme and that the uPA receptor contributes significantly to the invasive capacity of the cells by facilitating uPA activity (27). A variety of hormones, cytokines, and soluble growth factors may influence the synthesis and secretion of plasminogen activators and their inhibitors to modulate the invasive and metastatic behavior of cancer cells (3, 11). These actions may provide new therapeutic targets for intervention to limit the invasiveness of glioblastoma.

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