Expression and Cellular Localization of Messenger RNA for Plasminogen Activator Inhibitor Type 1 in Human Astrocytomas in Vivo


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Abstract

We investigated the expression and cellular localization of plasminogen activator inhibitor type 1 (PAI-1) in human astrocytoma in vivo. Northern blot and densitometric quantitation of PAI-1 mRNA indicated that PAI-1 transcripts were significantly higher in human malignant astrocytomas and especially in glioblastomas than in low-grade gliomas and normal brain tissues in vivo. Using in situ hybridization with paraffin-embedded surgical specimens of human gliomas and normal brain tissues, PAI-1 mRNA was abundantly expressed in glioblastomas. PAI-1 mRNA was localized mainly in tumor cells and endothelial cells. The distribution of PAI-1 mRNA expression was particularly abundant around areas of vascular proliferation and in remnant tumor cells surrounding necrotic foci. PAI-1 mRNA was also expressed in both the tumor and endothelial cells of anaplastic astrocytomas, whereas it was not expressed or only weakly expressed in low-grade astrocytomas or normal brain tissues. These results suggest that high expression of PAI-1 is associated with the malignant progression of astrocytic tumors and that excessive PAI-1 expression might be associated with intratumoral necrosis in glioblastomas.

Introduction

PAs are serine proteases that catalyze the cleavage of a single peptide bond of plasminogen, resulting in the formation of the trypsin-like protease plasin (1). There are two types of plasminogen activators: tissue-type PA, a key enzyme in thrombolysis (1); and uPA, which is synthesized by a variety of tissues, including those in the central nervous system. PAs may play a pivotal role in the tissue remodeling process in several physiological and pathological conditions, such as cellular migration, malignant tumor invasion, and angiogenesis of endothelial cells, that require localized degradation of extracellular matrix (1, 2).

It is known that most cells that synthesize PAs also secrete their specific inhibitors, the fast-acting high-affinity plasminogen activator inhibitors types 1 and 2 (PAI-1 and PAI-2) and protease nexin, all of which are members of the serpin (serine proteinase inhibitor) family (3). PAI-1 has been shown to be deposited within the extracellular matrix of cultured cells where it may play a role in preventing extracellular proteolysis mediated by uPA during the migration of tumor and endothelial cells (4, 5). Cell surface fibrinolytic activity depends on the degree of regulation of proteases as well as protease inhibitors that are secreted into the cellular microenvironment.

The main function of the fibrinolytic system is to maintain blood vessel patency by dissolving blood clots, and endothelial cells play an important role in this process (6). Endothelial cells negatively regulate thrombosis by secreting potent antifibrinolytic agents such as PAI-1 that block tissue-type PA activity (7). Abnormal elevations of PAI-1 have been reported in a number of clinical conditions associated with thrombosis, including myocardial infarction, deep venous thrombosis, and disseminated intravascular coagulation (8). Elevated levels of plasma PAI-1 may contribute to the increased risk of thrombotic diseases (9).

Glioblastoma, the most common and most malignant brain tumor, is distinguished from astrocytoma by the presence of necrosis and vascular proliferation in the tumor tissue (10). A hallmark of malignant tumors is their ability to induce neovascularization in vivo. During the formation of new vessels, the endothelial cells degrade basement membrane and migrate into the surrounding tissue, activities that require increased production of serine proteinases from the endothelial cells (11). Similar to fibrinolysis described above, tumor invasion and angiogenesis are complex phenomena that depend upon a balance between degradative enzymes and their inhibitors. Several tumors of glial or neuroectodermal origin have been shown to produce inhibitors of serine proteases, such as PAIs (12). In addition, our recent immunohistochemical (13) and biochemical (14) studies demonstrated that PAI-1 was expressed at higher levels in malignant brain tumors in vivo than in benign brain tumors or normal brain tissue where it was barely detectable. The expressed PAI-1 protein was localized to the vascular basement membrane, the perivascular connective tissue, and also to regions with endothelial cell proliferation and necrosis, suggesting that PAI-1 may play a role in preventing tumor cell invasion and in promoting intratumoral necrosis. Despite its potential importance in the pathogenesis of these phenomena, the cellular origin of PAI-1 has not yet been determined. Using Northern blot analysis and in situ hybridization to investigate the source of PAI-1, we examined the quantitative expression and distribution of PAI-1 mRNA in human astrocytomas in vivo. Our studies demonstrated high levels of PAI-1 mRNA in glioblastomas with abundant PAI-1 expression in the tumor cells and weaker expression in the tumor-associated endothelial cells.

Materials and Methods

Surgical Specimens. Fresh human brain tumor tissue and normal brain tissue samples were collected in the operating room from patients undergoing craniotomy and used for Northern blotting and in situ hybridization. Some samples were flash frozen in liquid nitrogen immediately after surgical removal and kept at −80°C. The tissue samples included nine glioblastomas, eight anaplastic astrocytomas, seven low-grade astrocytomas, and eight normal
brain tissue samples. Histological diagnosis was confirmed for each individual tissue block by standard light microscopic evaluation of sections stained with hematoxylin and eosin.

**Northern Blot Analysis.** Total RNA from human gliomas and normal brain tissues was extracted by use of a standard guanidinium thiocyanate-phenol-chloroform technique (15). As determined by A260 measurement and ethidium bromide staining, 20 μg of total RNA was electrophoresed in 1.5% agarose gels and transferred to Nytran-modified nylon filters by capillary action in 10X SSC (1X SSC = 150 mM NaCl-15 mM sodium citrate). Cloned cDNA probes were labeled with a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN) using [32P]dCTP as the radioactive label. The filters were then hybridized overnight at 42°C with the prehybridization solution containing the radiolabeled and denatured 3.0-ko DNA specific for the PAI-1 mRNA. Stringency washes in 1X SSC-0.75% sodium dodecyl sulfate at 50°C were undertaken. X-ray films (Kodak XAR) were exposed to the filters for 1-4 days at —70°C using intensifying screens. Loading of RNA was checked by reprobing the blot with a cDNA probe to GAPDH.

**Oligonucleotide Probes.** PAI-1-specific oligonucleotide DNA probes were designed complementary to the 5'-end of the human mRNA transcript based on the published cDNA sequence (16). The 30-base oligonucleotide of sequence 5'-ATA GGG TGA GAA CAC CTT GGT CTC TTT-3' was unique to the PAI-1 mRNA as determined by a GenBank database search. The oligonucleotide DNA probes were labeled by digoxigenin-labeled-dUTP, and to optimize the labeling conditions, the probes were tested on positive control cell lines that expressed PAI-1 mRNA.

**In Situ Hybridization.** All steps were performed at room temperature unless otherwise specified. Sections (4-μm thick) of formalin-fixed, paraffin-embedded tissues were mounted on silane-coated slides. The slides were dewaxed, and sections were pretreated with 0.2 n HCl and 5 μg/ml proteinase K (Boehringer Mannheim) at 37°C before hybridization. The samples were rinsed with 0.2% glycine in 0.1 M Tris-HCl (pH 7.5) and were acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0) for 10 min. After prehybridization for 2 h at 37°C, the slides were incubated with hybridization buffer containing 0.3 μg/ml of the digoxigenin-labeled oligonucleotide probe in a humidified chamber at 37°C overnight. Slides were washed and incubated in 1% blocking reagent (Boehringer Mannheim) for 60 min. The slides were placed in a humidified chamber and incubated with a 1:1000 dilution of mouse anti-digoxigenin monoclonal antibody (Boehringer Mannheim) for 60 min at 37°C. The sections were washed three times for 30 min each and incubated with secondary biotinylated goat antimiouse antibody and then with an avidin-biotinylated horseradish peroxidase conjugate for 30 min each at room temperature. The sections were rinsed with Tris-buffered saline, and tissue-bound horseradish peroxidase activity was visualized by incubation with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) for 2 h at 37°C, according to the manufacturer’s protocol. The reaction was stopped by rinsing the sections with an EDTA solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The slides were counterstained with methylgreen, air dried, and fixed with Permount mounting medium. Appearance of a brown or a blue-brown color was considered to be a positive reaction. Tumor cells positive for PAI-1 mRNA and blood vessel density were assessed by light microscopy. To confirm the specificity of the hybridization signal, we performed various control experiments, such as substituting the antisense probe with a sense-oriented oligonucleotide probe (Fig. 3c).

**Results**

**Northern Blot Analysis.** Total RNA was extracted, and steady-state levels of the PAI-1 transcript were compared in various types of astrocytomas and normal brain tissues by Northern blot analysis using a 3.0-kilobase cDNA PAI-1 probe. Representative autoradiographs of relevant hybridization signals confirmed that anaplastic astrocytomas and glioblastomas expressed PAI-1 mRNA of the same sizes (3.0 and 2.2 kilobases; Fig. 1). Detectable levels of PAI-1 mRNA were not found in low-grade gliomas or in normal brain tissues. High levels of PAI-1 mRNA were detected in glioblastomas and moderate levels in anaplastic astrocytomas. The hybridization signal of PAI-1 mRNA was used for quantitative evaluation after normalization to the GAPDH hybridization signal. Detectable levels of PAI-1 mRNA were not found in low-grade gliomas and normal brain tissues (Fig. 2). PAI-1 mRNA levels in glioblastomas were six times higher than those found in anaplastic astrocytomas (Fig. 2).

**mRNA Localization.** Fig. 3 shows that glioblastoma cells contained the most abundant and heterogeneously distributed PAI-1 mRNA (Fig. 3, a and b). The hybridization signal in the glioblastoma cells was particularly strong around sites of vascular proliferation, in remnants of tumor cells surrounding necrotic foci, and within areas undergoing necrosis. Endothelial cells in the tumor tissue also expressed PAI-1 mRNA, but this expression was weaker than that seen in the tumor cells (Fig. 3a). The PAI-1 mRNA signal in endothelial cells was predominantly found at sites of vascular proliferation surrounding necrotic foci. Specific hybridization signals were not seen in controls using a sense-oriented oligonucleotide probe (Fig. 3c). Control hybridization with tissue sections pretreated with RNase also showed no staining (data not shown). PAI-1 mRNA was also expressed in both the tumor and endothelial cells of anaplastic astrocytomas (Fig. 3d). PAI-1 mRNA-expressing cells of the vascular wall were identical to the endothelial cells that were positive with the von Willebrand factor.

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**Fig. 1.** Northern blot analysis of PAI-1 mRNA in human astrocytomas and normal brain tissues. Total RNA (20 μg) was electrophoresed in 1.5% agarose gels and transferred to Nytran-modified nylon filters by capillary action. The membrane was probed with a radiolabeled 3.0-kilobase cDNA specific for the PAI-1 mRNA and, after stripping, it was rehybridized with a GAPDH probe to check mRNA loading amounts. NB, normal brain; LGG, low-grade; AA, anaplastic astrocytoma; GBM, glioblastoma.

**Fig. 2.** Quantitated levels of PAI-1 mRNA from basic densitometric scanned autoradiograms. The relative hybridization signal was calculated by ascribing an arbitrary value of 1 to the least intense signal seen on the Northern blot after calculating loading equalities based on the GAPDH probing. In each group, PAI-1 mRNA bands at three positions were scanned with a laser densitometer at different exposures, and the peak areas were averaged to give the values presented. The data are shown as the mean of five different patient tissue samples from each group, bars, SD.
Fig. 3. In situ hybridization of PAI-1 mRNA expression in human gliomas and normal brain tissues. a and b, hybridization with antisense oligonucleotide probe for PAI-1 mRNA in glioblastoma. c, control hybridization in serial sections with sense-oriented oligonucleotide probe. d, hybridization with antisense oligonucleotide probe for PAI-1 mRNA in anaplastic astrocytoma. e and f, hybridization with antisense oligonucleotide probe for PAI-1 mRNA in low-grade astrocytoma and normal brain tissue, respectively. × 415.

Willebrand factor (data not shown). There were almost no signals for PAI-1 mRNA expression in low-grade astrocytoma (Fig. 3e) and normal brain tissue (Fig. 3f).

Discussion

Serine proteases and their inhibitors (serpins) play important roles in regulating fibrinolysis during physiological and pathological conditions requiring tissue remodeling (1, 2, 4, 5, 17). This has led us to study their participation in the biology of malignant brain tumors (18). Increased PAI-1 activity in glioblastoma cell lines in vitro has been described (12). In addition, previous in vivo studies suggested that higher levels of PAI-1 are found in glioblastomas and in necrotic areas than in normal brain tissues (13, 14).

In the present study, we show that the expression of PAI-1 mRNA was prominent in both the tumor and endothelial cells of malignant astrocytomas, especially in glioblastomas, but was not detectable in...
low-grade gliomas or in normal brain tissues. PAI-1 levels of brain tumors determined by Western blotting and reverse zymography (14) demonstrated a similar pattern to that observed in Northern blots, indicating that the presence of high levels of PAI-1 mRNA results in high levels of PAI-1 protein. The expression of PAI-1 mRNA, particularly in glioblastoma, was higher in palissading cells and in endothelial cells adjacent to necrotic areas, and the results of in situ hybridization correlated with those obtained by Northern blot analysis (Fig. 2). Thus, the results of the present study and those of a previous report using immunohistochemical procedures (13) indicate that PAI-1 is synthesized in both tumor and endothelial cells in malignant astrocytoma tissue. This molecule is probably then secreted in an active form and then binds to the extracellular matrix, consistent with previous reports dealing with non-central nervous system tumors (4, 19).

High levels of PAI-1 activity in tumor tissue might be important in preventing tumor cells from destroying surrounding extracellular matrix, which is secreted, in part, by the tumor cells themselves (20). The expression of PAI-1 mRNA by proliferating endothelial cells might have a similar purpose, i.e., to limit the degradation of surrounding extracellular matrix and to maintain blood vessel integrity.

The role of PAI-1 in facilitating cell migration was recently proposed (21), suggesting that receptor bound uPA is sensitive to PAI-1 (4). After PAI-1 binds to receptor-bound uPA, receptor-uPA-PAI-1 complexes are internalized and degraded in lysosomes, whereas internalized uPA receptors are recycled and relocalized at the cell surface adjacent to a different adhesive surface (22). This suggests that the proteolytic enzyme and its inhibitor are both required for cell migration, adhesion, and invasion (21).

PAI-1 also possesses potent antifibrinolytic properties. Excessive amounts of PAI-1 activity in the tumor microenvironment might cause fibrin thrombi to form within dilated and hyperplastic blood vessels (9), resulting in tissue ischemia and necrosis in glioblastoma in vivo. Sprengers and Kluft (23) suggested that increased amounts of PAI-1 in the blood circulation increases the likelihood of thrombosis. Glioblastoma is the most common primary malignant intracranial neoplasm, and it is often associated with thrombosis of dilated and hyperplastic blood vessels (10). Thus, increased expression of PAI-1 mRNA in glioblastomas, especially near necrotic foci, suggests that PAI-1 is closely associated with the pathogenesis of intratumoral necrosis.

A variety of hormones, cytokines, and soluble growth factors, such as tumor necrosis factor-α, transforming growth factor-β, and basic fibroblast growth factors, may influence the synthesis and secretion of both plasminogen activators and their inhibitors, resulting in modulation of the invasive behaviors of cancer cells and angiogenesis of tumor tissue (17, 24–26). The levels of PAI-1 were increased in cultured endothelial cells with the addition of these factors (17). Thus, the association of growth factors and cytokines and the regulation of PAI-1 activity is likely to be an important determinant of tumor cell growth, motility, and microvascular patency. Since we have presented evidence that PAI-1 is produced in tumor cells and endothelial cells of high-grade malignant astrocytomas, this evidence suggests that PAI-1 also has a potential important role in the pathogenesis of intratumoral necrosis.

Acknowledgments

We thank Midori Sugihara, Pamela P. Pyle, Janet E. Quinones, and Alan Rayford for their technical help; Norma Adams for preparation of the manuscript; and Kimberly J. T. Herrick for manuscript review.

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