Expression of Tissue Factor Pathway Inhibitor 2 Inversely Correlates during the Progression of Human Gliomas

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ABSTRACT

Protease inhibitors regulate a variety of physiological and pathological processes including angiogenesis, embroyo implantation, intravascular fibrinolysis, wound healing, and tumor invasion. Tissue factor pathway inhibitor (TFPI) 2 is a M r 32,000 Kunitz-type serine protease inhibitor that inhibits plasmin, trypsin, chymotrypsin, cathepsin G, and plasma kallikrein but not urokinase-type plasminogen activator, tissue plasminogen activator, or thrombin. In this study, we determined the relative amounts of TFPI-2 in low-, intermediate-, and high-grade human glioma cell lines and tumor tissue samples. TFPI-2 protein and mRNA levels (measured by Western and Northern blotting) were highest in low-grade glioma cells (Hs683), lower in anaplastic astrocytoma cells (SW1088 and SW1783), and undetectable in high-grade glioma cells (SNB19). Analysis of TFPI-2 protein in human normal brain and in glioma tumor tissues for high-grade glioma cells (SNB19) revealed the highest levels in normal brain, lesser amounts in low-grade gliomas and anaplastic astrocytomas, and undetectable amounts in glioblastomas. In situ hybridization of TFPI-2 mRNA with normal brain tissues revealed the greatest positivity in neurons, with moderate positivity in both glial and endothelial cells and moderate, little, or no TFPI-2 mRNA in low-grade glioma, anaplastic astrocytoma, and glioblastoma tumor samples, respectively. We also found that recombinant TFPI-2 inhibited the invasive ness of SNB19 glioblastoma cells in a Matrigel assay in a dose-dependent manner. Collectively, these results suggest that TFPI-2 has a regulatory role in the invasiveness of gliomas in vitro and in vivo.

INTRODUCTION

Malignant brain tumors are characterized by their invasive infiltration and destruction of surrounding normal brain tissue; their invasive behavior seems to depend in part on a variety of proteolytic enzymes, including serine, metalloproteases, and cysteine proteases. Our previous work has suggested a direct correlation between the expression of uPA 4 and its receptor uPAR and the invasiveness of human gliomas (1–3). Receptor-bound uPA converts the zymogen plasminogen to plasmin, a broad-spectrum serine protease that promotes the lysis and dissolution of the ECM and promotes tumor cell invasion (4, 5). Inhibitors of plasmin probably participate in the regulation of the invasive and malignant behavior of gliomas.

TFPI-2 (also called MSPI and PP-5), a M r 32,000–33,000 Kunitz-type protease inhibitor, inhibits trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein, and factor VIIa-tissue factor complex but not uPA, tPA, or thrombin (6–8). A variety of cell types, including human foreskin keratinocytes, dermal fibroblasts, and endothelial cells derived from human umbilical vein, aorta, and dermal microvessels, synthesize and secrete mature TFPI-2 and two other underglycosylated M r 31,000 and 27,000 forms of TFPI-2 (8–10). Studies of the TFPI-2 triplet in cell-conditioned-medium, ECM, and cytoplasmic fractions showed that most of the TFPI-2 is present in the ECM (11). Within the ECM, TFPI-2 binds heparin, a glycosaminoglycan, by means of arginine-mediated ionic interactions (12). Structurally, TFPI-2 contains a short acidic amino-terminal domain, three tandem Kunitz domains, and a carboxyl-terminal domain that is highly enriched in basic amino acids. The gene coding for human TFPI-2 was mapped to chromosome 7q22 and that for the mouse was mapped to chromosome 6 (13).

We reported earlier that recombinant TFPI-2 inhibits plasmin, regardless of whether the enzyme was associated with tumor cells or with ECM (14). In the same study, we found that TFPI-2 inhibited the ability of HT-1080 fibrosarcoma cells to degrade ECM and invade Matrigel. Moreover, we also found that TFPI-2 is not expressed in HT-1080 cells, suggesting that the plasmin (enzyme)-to-TFPI-2 (inhibitor) ratio in this tumor

4 The abbreviations used are: uPA, urokinase-type plasminogen activator; ECM, extracellular matrix; uPAR, uPA receptor; TFPI, tissue factor pathway inhibitor; MSPI, matrix-associated serine protease inhibitor; PP, placental rinsin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; rTFPI, recombinant TFPI; PMA, phorbol 12-myristate 13-acetate; tPA, tissue-type plasminogen activator; MMP, matrix metalloproteases.

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cell line heavily favored the enzyme (14). These observations imply that TFPI-2 expression is not conducive to tumor invasiveness and malignant behavior. In the present study, we further evaluated the role of TFPI-2 in tumor cell invasiveness by measuring TFPI-2 mRNA and protein levels in human glioma cell lines and in tissue samples of low-, intermediate-, and high-grade tumors. We also assessed the effect of recombinant TFPI-2 on the invasiveness of a high-grade glioblastoma cell line through Matrigel. Our results suggest that the presence of TFPI-2 is inversely correlated during the progression of gliomas.

**MATERIALS AND METHODS**

**Cell Culture.** Low-grade glioma cells (Hs683), intermediate-grade anaplastic astrocytoma cells (SW1088 and SW1786), and high-grade glioblastoma cells (SNB19) were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were grown in DMEM supplemented with 1.0% glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37°C and subcultured every 3–5 days.

**Measurement of TFPI-2 in ECM, Conditioned-Medium, and Cell-Lysate Samples.** Glioma cell lines were grown to 80–90% confluence in six-well tissue culture plates, after which the medium was replaced with serum-free medium, and 24 h later conditioned-medium, cell-lysate, and ECM fractions were prepared as previously described (8–11). The TFPI-2 was quantified in the conditioned-medium and cell-lysate fractions by trypsin-affinity chromatography followed by Western blotting as follows. Briefly, trypsin (2 mg/ml) was coupled to Reactigel agarose beads according to the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL). The conditioned-medium and cell-lysate fractions were incubated for 1 h at room temperature with 50 μl of trypsin-Reactigel agarose beads that had previously been equilibrated with 15 mm Tris-HCl (pH 7.4; equilibration buffer). Trypsin-bound proteins were extracted into 100 μl of SDS-PAGE sample buffer, and the TFPI-2 triplet levels were measured in 25-μl aliquots by Western blotting. The ECM proteins were extracted with 300 μl of the SDS-PAGE sample buffer, and 25-μl aliquots of this extract were also assayed for TFPI-2 triplet levels by Western blotting.

**Measurement of TFPI-2 in Tissue Extracts.** Normal brain tissues (4) and brain tumor tissue samples (five samples from each group, obtained through the Department of Neurosurgery at The University of Texas M. D. Anderson Cancer Center) were stored frozen at −75°C until use. The frozen tissue was homogenized in acidic buffer [0.1 m glycine-HCl (pH 3.0)] with a Polytron homogenizer and centrifuged at 15,000 × g for 30 min at 4°C. The pellet was discarded, and the supernatant was collected and immediately neutralized with 1 m Tris base. The tissue proteins (3 mg) were then incubated at room temperature for 1 h with 50 μl of trypsin-Reactigel beads in 1 ml of 15 mm Tris-HCl (pH 7.4) with 0.15 m NaCl. After incubation, unbound proteins were removed, and bound proteins were extracted into 100 μl of SDS-PAGE sample buffer. A 25-μl aliquot of the extract was assayed for TFPI-2 by Western blotting.

**Western Blotting.** Proteins were boiled for 3 min, separated by SDS-PAGE using 12% polyacrylamide gels, and electroblotted onto nitrocellulose membranes (15). After electroblotting, the membranes were blocked with 4% nonfat dry milk in 10 mm Tris-HCl with 150 mm NaCl (pH 7.4) and 0.1% Tween 20 (TTBS) for 2 h at 23°C. The membranes were then incubated overnight at 4°C with anti-TFPI-2 antibody (diluted 1:2000 in TTBS with 1% BSA). After several washes, the membranes were incubated for 1 h with peroxidase-conjugated secondary antibody (diluted 1:3000 in TTBS with 1% BSA). The immunoreactive proteins were identified by using an enhanced chemiluminescence reagent system, according to the manufacturer’s instructions (enhanced chemiluminescence; Amersham).

**Northern Blotting.** Total cellular RNA was extracted from confluent cultures as previously described (16), and 10 μg of RNA were electrophoresed in agarose-formaldehyde gel, transferred to a nylon membrane by capillary action overnight, and cross-linked with UV irradiation. The filters were hybridized at 65°C overnight with a 0.68-kb TFPI-2 cDNA probe labeled with 32P-deoxycytidine triphosphate by random primer labeling. The filters were then washed twice in 0.5× SSC and 0.1% SDS for 20 min and then incubated for 15 min at 65°C and exposed to X-ray film at −70°C. The membranes were then stripped and rehybridized with GAPDH cDNA. TFPI-2 mRNA in the cell lines was quantified by densitometry.

**Matrigel Invasion Assay.** Cell invasiveness in the presence or absence of recombinant TFPI-2 was quantified in a Matrigel assay as follows. Polycarbonate filters (8-μm pores) from invasion chambers were coated with Matrigel (150 μg/fil; Collaborative Research, Inc., Bedford, MA). SNB19 cells were detached (from the tissue culture plates?), suspended in RPMI 1640 with 0.1% BSA. Then, 400 μl of the tumor cell suspension (5 × 10⁵ cells in RPMI 1640, 0.1% BSA, and 0.01% gentamicin), with or without 30, 60, or 120 nm recombinant TFPI-2, was placed in the upper chamber (14). Serum-free conditioned medium was placed in the lower chamber as a chemotactrant. After incubation for 36 h at 37°C, the filters were removed and stained with Hema 3, and invasive cells adhering to the lower surface of the filter were quantified under a light microscope (×400). Cells from 10 nonoverlapping fields from triplicate filters were counted, and the mean was used for analysis (3, 14).

**In Situ Hybridization.** Tissue sections were prepared for in situ hybridization with TFPI-2 as follows. Serial 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissues and mounted on silane-coated slides. The slides were then dewaxed with a 3:5 mixture of xylene and acetone. Before hybridization, the sections were treated with 0.2 N HCl and 5 μg/ml of proteinase K (Boehringer Mannheim) at 37°C and postfixed with 4% paraformaldehyde in PBS. Samples were then rinsed with 0.2% glycine in 0.1 m Tris-HCl (pH 7.5) and acetylated with 0.25% acetic anhydride in 0.1 m triethanolamine (pH 8.0) for 10 min. The samples were then rinsed with 2× SSC and incubated for 2 h at 37°C in prehybridization buffer [40% formamide, 10% dextran sulfate, 1% Denhardt’s solution, 2× SSC, 10 mm Tris-HCl (pH 7.5), 1 mm EDTA (pH 8.0), 100 μg/ml denatured salmon sperm DNA, 0.1 μg/ml polyadenylcy
acids, and 5 μg/ml polydeoxyadenylic acid]. Next, the sections were hybridized in prehybridization buffer supplemented with 0.1 μg/ml of digoxigenin-labeled, 1.2-kb antisense TFPI-2 probe overnight in a humidified chamber at 37°C. After hybridization, the slides were washed three times in 50% formamide in 2× SSC (60 min each wash) at 37°C, twice in 0.5× SSC (60 min each) at 37°C, rinsed with 2× SSC for 15 min, and incubated in 1% blocking reagent (Boehringer Mannheim) for 60 min. At that time, the slides were placed in a humidified chamber and incubated with a 1:1000 dilution of monoclonal mouse antidigoxigenin antibody (Boehringer Mannheim) for 60 min and then washed three times (for 30 min each time) in Tris-buffered saline [0.1 M Tris-HCl and 0.15 M NaCl (pH 7.5)] and incubated with biotinylated antimouse IgG at room temperature. The sections were then 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, humidified chamber. The enzyme reaction was stopped by rinsing the sections with EDTA solution [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. The sections were counterstained with methyl green, air dried, and covered with Permount mounting medium. Numbers of cells stained brown or blue (indicating the presence of TFPI-2 mRNA) were assessed by light microscopy.

RESULTS

TFPI-2 mRNA in Glioma Cell Lines. The constitutive expression of TFPI-2 mRNA in low-grade (Hs683), intermediate-grade (SW1083 and SW1783 anaplastic astrocytoma), and high-grade (SNB19 glioblastoma) glioma cell lines, as determined by Northern blotting, is shown in Fig. 1. Autoradiography of the hybridization signals indicated that the Hs683, SW1083, and SW1786 cell lines expressed 1.8- and 1.2-kb fragments of TFPI-2 mRNA (Fig. 1). Quantitative evaluation after the TFPI-2 signal was normalized to that of GAPDH revealed that the greatest amounts of TFPI-2 mRNA were found in the Hs683 cell line (P < 0.001) compared with SW1083 and SW1786 cell lines. TFPI-2 mRNA was not present in detectable amounts in SNB19 cells.

TFPI-2 Protein in Glioma Cell Lines. Expression of TFPI-2 protein in the serum-free conditioned medium of glioma cell lines. ECM extracts (A) and conditioned medium (B) were collected from the various glioma cell lines, and equal amounts of protein from each group were run on 12% SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes and probed with an anti-TFPI-2 antibody in an enhanced chemiluminescence protocol as described in “Materials and Methods.” Numbers below kDa, Mr in thousands.
induces the synthesis and secretion of TFPI proteins in several cell types (9, 10, 14), and analyzed mRNA by Northern blotting and protein by Western blotting. Hs683 cells were used as a positive control. PMA treatment increased the expression of TFPI-2 mRNA in Hs683 cells (P < 0.001) but not in SNB19 glioblastoma cells (Fig. 3A). Similarly, PMA treatment increased TFPI-2 protein levels in the ECM of Hs683 cells but not in the ECM of SNB19 glioblastoma cells (Fig. 3B). Collectively, these results suggest that the SNB19 high-grade glioma cell line is deficient in TFPI-2 mRNA and protein.

TFPI-2 Protein in Normal Brain and Tumor Samples. Amounts of TFPI-2 protein in normal human brain and tumor tissue, measured by trypsin-affinity chromatography and Western blotting, are shown in Fig. 4. TFPI-2 appeared as a broad, diffuse Mr 30,000 band. Amounts of this protein in normal brain tissue were twice as high as in the low-grade gliomas and six to eight times higher than in the anaplastic astrocytomas (P < 0.001). No TFPI-2 protein was detected in glioblastoma tissue extracts.

Inhibition of Invasion by SNB19 Cells. The effects of recombinant TFPI-2 on the invasiveness of SNB19 glioblastoma cells through a reconstituted basement membrane (Matrigel) are shown in Fig. 5. Setting the cell-invasiveness index at 100% in the absence of the recombinant inhibitor, 30 nM rTFPI-2 produced a 35% inhibition, 60 nM produced a 55% inhibition, and 120 nM produced a 80% inhibition in cell invasiveness (P < 0.001).

Localization of TFPI-2 Expression. In situ hybridization of normal brain and brain tumor tissue sections with an anti-TFPI-2 mRNA probe showed that neurons and to a lesser extent glial and endothelial cells in normal brain tissue contained TFPI-2 mRNA (Fig. 6). In comparison, low-grade glioma, anaplastic astrocytoma, and glioblastoma tumor tissue sections showed that moderate, little, or no TFPI-2 mRNA, respectively. Use of a sense TFPI-2/MSPI cDNA, pretreatment with RNase, or use of an unlabeled probe confirmed the absence of a specific hybridization signal (data not shown).
Human TFPI-2 is a M₄, 32,000 Kunitz-type inhibitor present in the ECM of endothelial cells (6) and fibroblasts (9). TFPI-2 transcripts are abundant in the full-term placenta and are widely expressed in various adult human tissues, such as liver, skeletal muscle, heart, kidney, and pancreas (13). In the present study we determined the levels of TFPI-2 in various grades of glioma cell lines and tumor tissue samples. Proteolytic enzymes have been implicated in the invasive behavior of malignant tumors, including glioblastomas (1, 3, 17, 18). Protease inhibitors that can protect the ECM from degradation by malignant cells may represent a new therapeutic strategy for blocking the invasion and spread of malignant tumors. Many types of tumors secrete polypeptides with a wide range of biological activities. In vivo, the activities of proteinases are regulated by their natural inhibitors (19–21). Some human cancer cell lines secrete serine proteinases, including trypsin (22, 23) and multiple forms of trypsin inhibitors. These proteinase inhibitors seem to be essential in curbing the activity of secreted matrix proteinases in vivo (22, 23). Cancer is often associated with coagulopathies of unknown etiology; possibly tumors in such cases may be producing TFPI-2. Although the biological significance of TFPI-2 production by cancer cells remains to be clarified, this protease inhibitor seems to have a role in tumor invasion and metastasis and perhaps tumor-induced coagulopathy.

TFPI-2, an ECM-associated serine protease inhibitor (11), plays a major role in ECM degradation during tumor cell invasion and metastasis, wound healing, and angiogenesis. A previous study of recombinant TFPI-2 showed that TFPI-2 inhibited the generation of plasmin at the surfaces of the ECM and HT-1080 fibrosarcoma cells and inhibited the degradation and invasion of the matrix by these cells (14). Findings from studies of plasminogen activators suggested that the direct inhibition of plasmin may be more effective for blocking tumor invasion and metastasis than the inhibition of uPA or tPA. Other molecules such as uPAR, uPA, tPA, PAI-1, and PAI-2 were overproduced in malignant tumors (i.e., colon, stomach, brain, breast and skin cancers; Refs. 24 and 25). Thus, the presence of PAI-1 and PAI-2 correlated positively with the malignancy of the disease. Indeed, in a recent report, endothelial cell PAI-1 stimulated rather than inhibited metastasis in neuroblastoma (26). Our own reports indicated that PAI-1 increases with the extent of malignancy in human gliomas (27). We found here that TFPI-2, a newly described broad-spectrum serine protease inhibitor, is expressed differentially in human glioma cell lines and tissues of differing invasive potential. Specifically, mRNA and protein for TFPI-2 is expressed in the greatest amounts in normal tissue, followed in decreasing order by low-grade gliomas and intermediate-grade gliomas, and was undetectable in high-grade gliomas.

TFPI-2 and PAIs act at different steps of the plasminogen-activation system. Specifically, PAI-1 binds the uPA-uPAR complex with plasminogen, and TFPI-2 directly inhibits plasmin, thereby releasing growth factors from the ECM and activating transforming growth factor β and certain pro-MMPs by tumor cells, which causes degradation of the ECM followed by invasion (28, 29). Eitzman et al. (30) raised the possibility that interfering with the plasminogen-activation system at the level of plasmin rather than at the level of uPA and tPA may be a more effective strategy for blocking tumor cell invasion and metastasis. However, a major obstacle in testing this concept is the lack of an identified natural plasmin inhibitor that inhibits cell-surface plasmin. Plasmin bound to the tumor cells is not accessible to inhibition by α₂-antiplasmin, a known plasma plasmin inhibitor (30–34).

Among other natural serine protease inhibitors, protease nexin I primarily inhibits α-thrombin and does not inhibit uPA or plasmin on the cell surface (35). Maspin (mammary serine protease inhibitor), a tumor suppressor protein (36), is highly homologous to serpins, a family of proteins that either is able (e.g., α₂-antitrypsin, PAI-1, and PAI-2) or unable (e.g., ovalbumin and angiotensiongen) to inhibit proteases (37). Moreover, the report that revealed protease inhibition activity to maspin showed that it inhibits tPA but not uPA or plasmin (38). Interestingly, Sektor et al. (39) showed that maspin suppressed the invasive phenotype in MDA-MB-435 cells by altering their integrin profiles.

Nevertheless, TFPI-2 is different from PAI-1 and PAI-2, protease nexin I, and maspin proteins. Unlike these proteins (all are serpins), TFPI-2, a Kunitz-type inhibitor, directly inhibits plasmin (derived from plasminogen by uPA or tPA, regardless of whether the enzyme is cell or matrix associated), thereby inhibiting ECM degradation and invasion and the activation of pro-MMP-1 and pro-MMP-3 by tumor cells. Interestingly, TFPI-2, in addition to inhibiting plasmin and five other related proteases, inhibits tissue factor-factor VIIa complex, the cellular initiator of the extrinsic coagulation pathway (6, 40). For example, TFPI-2 was observed to inhibit the activation of human factor X by the tissue factor-factor VIIa complex on J82 bladder carcinoma cells (40). Recent studies have demonstrated that tissue factor-factor VIIa complex promotes tumor progression (41–43). Tissue factor also up-regulated the production of vascular endothelial cell growth factor (44) and uPAR (45) in tumor cells, and its levels perfectly correlated with the degree of tumor angiogenesis and progression in gliomas, fibrosarcomas, and melanomas and in breast and pancreatic tumors (46–49). The
present study suggests an inverse correlation between the TFPI-2 expression and the progression of human gliomas and their invasive behavior.

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REFERENCES


