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Simultaneous Inhibition of Glioma Angiogenesis, Cell Proliferation, and Invasion by a Naturally Occurring Fragment of Human Metalloproteinase-2[1]

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ABSTRACT

Angiogenesis, tumor cell proliferation, and migration are the hallmarks of solid tumors, such as gliomas. This study demonstrates that a fragment derived from the autocatalytic digestion of matrix metalloproteinase (MMP)-2, called PEX, acts simultaneously as an inhibitor of glioma angiogenesis, cell proliferation, and migration. PEX is detected in the cultured medium of various human glioma, endothelial, breast, and prostate carcinoma cell lines. PEX is purified from the medium of glioma cell lines by chromatography, where PEX is constitutively expressed as a free and a TIMP-2-bound form. In human glioma tissue, PEX expression correlates with histological subtype and grade and with $\alpha v\beta 3$ integrin expression to which it is bound. Systemic administration of PEX to s.c. and intracranial human glioma xenografts results in a 99% suppression of tumor growth with no signs of toxicity. Thus, PEX is a very promising candidate for the treatment of human malignant gliomas.

INTRODUCTION

The growth of solid tumors depends on the initiation of new vascularization, extensive cell proliferation, and local and distant tumor cell migration. Recent experimental evidence suggests that angiogenesis and invasion are coeval in tumor development and involve similar biological mechanisms (1). Angiogenesis can be considered as an invasive process in which activated endothelial cells proliferate, adhere to extracellular matrix components, and migrate. A similar sequence of events regulates tumor cell invasion (2–5). Integrins and metalloproteinases are involved in both the angiogenic and invasive processes. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ have been shown to be necessary for tumor-induced or cytokine-dependent angiogenesis (6, 7). Integrin $\alpha v\beta 3$ in particular, is necessary for the formation, survival, and maturation of newly formed blood vessels (8). $\alpha v\beta 3$ integrin has been shown to be an alternative signaling pathway mediating the activities of various growth factors through separate extracellular matrix components (9–11). Metalloproteinases degrade extracellular matrix molecules and create a more permissive environment for cell migration. The activation of metalloproteinases is under the control of tightly regulated mechanisms. Tumors are characterized by an imbalance of proteolysis that favors invasion (3, 4).

This study shows that a fragment of MMP[3]-2 called PEX is naturally expressed in various human tumors and acts simultaneously to inhibit angiogenesis, cell proliferation, and migration. We isolated PEX from human glioblastoma cells in culture. Gliomas are characterized by a high proliferation rate, extensive angiogenesis, and marked local invasion, which makes these tumors resistant to conventional treatment based on surgery, chemotherapy, and radiotherapy (12–14). MMP-2 and MMP-9 are the two most abundant MMPs found in gliomas (15–17). MMP-2 was found to be colocalized with integrin $\alpha v\beta 3$ on the surface of blood vessels and of glioma cells (18). In gliomas, this colocalization was particularly prominent in high-grade glioma periphery, where angiogenesis and tumor cell invasion are particularly active. The interaction between integrin $\alpha v\beta 3$ and MMP-2 is one of the critical steps controlling endothelial cell invasion and is regulated by the hemopexin fragment PEX. PEX is derived from the proteolysis of MMP-2 and was detected *in vivo* in melanoma and during retinal neovascularization (19).

Our study shows that PEX is a powerful molecule that interferes with glioma development by both angiogenic dependent and independent mechanisms, which makes PEX a powerful candidate for the treatment of malignant gliomas and possibly other human malignancies.

MATERIALS AND METHODS

Antibodies and Other Supplies. Monoclonal antibody IM3LL (anti-MMP-2; Calbiochem, La Jolla, CA); rabbit polyclonal antibody anti-MMP-2 AB 809, monoclonal anti-TIMP-2 (AB-801), monoclonal antibody anti-TIMP-1 (AB-800), monoclonal antibody anti-TIMP-4 (AB-803), from Chemicon International (Temecula, CA); antihuman FGF-2 neutralizing antibody (AB-33-NA) from R&D systems were used. The monoclonal antibody MTV88 directed against avian PEX and the monoclonal antibody LM609 directed against $\alpha v\beta 3$ integrin heterodimer have been described before (19, 20). Human recombinant FGF-2, Matrigel, and bovine collagen type I from Becton Dickinson (Bedford, MA); human vitronectin and fibronectin from Calbiochem (La Jolla, CA); human tenascin from Life Technologies, Inc. (Grand Island, NY) were also used. $\alpha v\beta 3$ integrin was from Chemicon International.

Cell Cultures. Three types of human glioma cells (U87-MG, U373-MG, and U-118-MG; ATCC, Rockville, MD) and three types of endothelial cells [HUVECs, BCE cells, and PAE cells transfected with KDR (PAE/KDR)]. U87-MG and U373-MG cells were cultured in MEMα supplemented with 10% FBS and 2 mM glutamine. U118-MG cells were cultured in Eagle's modified MEM with 2 mM L-glutamine and Earle's BSS adjusted with 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 10 mM sodium pyruvate, and 10% FBS. HUVEC cells (ATCC) were cultured in RPMI 1640 supplemented with 5% heat-inactivated FCS, 20 μg/ml endothelial cell growth factor, and 50 μg/ml heparin. PAE/KDR were cultured in F-12 mixture with 10% non-heat inactivated FCS and 10 μg/ml geneticin (G-418 sulfate; Ref. 21). BCE cells (ATCC) were cultured in DMEM, plus L-glutamine, and 10% FBS.

As a control in $\alpha v\beta 3$ integrin and MMP-2 experiments, we used M21 human melanoma cells cultured in RPMI 1640 supplemented with 10% FCS (22) and HT-1080 human fibrosarcoma cells cultured in Eagle's modified

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[3] The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; FGF, fibroblast growth factor; ATCC, American Type Culture Collection; HUVEC, human umbilical vein endothelial cell; BCE, bovine capillary endothelial; PAE, porcine aortic endothelial; CHO, Chinese hamster ovary.

MEM supplemented with 10% FCS. To test the effect of PEX in other tumor systems, we used MCF-7 cells (human mammary adenocarcinoma cells; ATCC) and CRL-1435 (human prostate carcinoma; ATCC) grown in DMEM supplemented with 10 mM sodium pyruvate and 10% FBS. CHO cells (ATCC) were cultured in DMEM, supplemented with 1% of L-glutamine, 1% nonessential amino acids, and 10% FBS. All media were supplemented with 1000 units/ml penicillin/streptomycin solution, and the cells were cultured in a 5% $C0_2$ incubator at 37°C.

Glioma Patients' Samples. Forty-six patients were included in the study, 26 males and 20 females (age 33–67 years; mean, 41 years). Forty patients were submitted for the first time to craniotomy at our institution for removal of cerebral gliomas. Nonneoplastic tissue was obtained from 6 patients who underwent temporal lobectomy for cerebral contusion. In these patients, the surgical procedures were performed within 8 h from injury, and only the portion of the specimen far from the contusion area was used for this study.

Western Blot Analysis of PEX, $\alpha v\beta 3$ integrin, MMP-2, TIMP-1, TIMP-2, and TIMP-4. Frozen glioma tissue was homogenized in 3 ml of cold lysis buffer (23). Cell lysates were obtained by adding 0.5 ml of cold lysis buffer to the cells. For PEX, $\alpha v\beta 3$ integrin, and MMP-2 determination, protein lysates (400 μg) were first immunoprecipitated with monoclonal antibodies (MTV88, LM609, IM3LL, and AB809) according to standard procedure (23) and then analyzed by SDS-PAGE (7.5 and 12.5% gels). The gels were blotted to Immobilon-P (Millipore, Bedford, MA) and incubated with primary antibodies (MTV88, 1:2500; LM609, 1:2000; IM3LL, 1:300; AB309, 1:3000; TIMP-1, 1:2000; TIMP-2, 1:3000; and TIMP-4, 1:1500; Ref. 24). The blots were incubated with a secondary antimouse or rabbit horseradish peroxidase-conjugate antibody (1:1500 dilution) for 1 h at room temperature and detected by ECL plus system (Amersham England). Quantitation of Western Blot signals was performed by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). Western blot of glioma samples was also carried out after reductive alkylation, in which each sample was exposed to 1 mM DTT for 30 min, followed by 1 mM iodoacetamide for 30 min at 37°C.

Purification of PEX from Human Glioma Cells Cultured Media. Glioma cells were cultured for 36 h in AIM-V medium. The medium was collected, concentrated, and dialyzed against collagenase buffer (50 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.5). The resultant medium was diluted 1:1 in chromatography buffer (25 mM Tris, 25 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35 solution, pH 7.4) and applied onto a Gelatin Sepharose (Pharmacia Biotech, Uppsala, Sweden) column at 4°C overnight. The column was washed with binding buffer and eluted with 10% DMSO in binding buffer. Fractions containing all forms of MMP-2 were collected, pooled, and dialyzed in dialysis buffer (25 mM Tris, 25 mM NaCl, and 5 mM $CaCl_2$, pH 7.4) overnight. Fractions were then concentrated and applied on a Concavalin A-Sepharose column equilibrated in TCB buffer (25 mM Tris, 1 M NaCl, pH 8.0) at 4°C overnight. Concavalin A affinity chromatography binds TIMP-1 linked forms. Fractions containing TIMP-1 free forms were collected, pooled, dialyzed, concentrated, and applied on a Sephacryl HR-200 column in TCB buffer containing 1 M NaCl. Fractions from this step containing M_r 72,000 gelatinase were pooled, dialyzed against TCB, concentrated, and applied to a heparin-Sepharose affinity column to separate TIMP-2 free form of MMP-2 and PEX. Fractions containing PEX are collected, dialyzed, and concentrated. Purity of PEX was assessed by SDS-PAGE with silver stain (purity >95%) and Western Blot with specific anti-PEX, anti-TIMP-1, anti-TIMP-2, and TIMP-4 antibodies (25, 26). The biological activity of PEX was tested using angiogenic assays performed on PAE/KDR cells.

***In Vitro* Angiogenic Assays.** *In vitro* angiogenic assays were performed as described previously (27). Briefly, HUVEC, BCE, and PAE/KDR cells were seeded on a 0.5-mm-thick bovine collagen type I gel (4×10^4 cells/cm^2) in a 3.8-cm^2 multiwell plate dish (Becton Dickinson, Bedford, MA). In all experiments, the medium was replaced with one of the following: (*a*) U87-MG glioblastoma cell conditioned medium (95%) and endothelial cell medium with 10% serum (5%); (*b*) U87-MG glioblastoma cell conditioned medium (95%), endothelial cell medium with 10% serum (5%), and PEX dissolved in PBS at different concentrations; (*c*) endothelial cell medium. All media were replaced daily.

***In Vitro* Migration Assay.** Two different types of migration assays were used, Boyden chambers and a monolayer migration assay. Boyden chambers experiments were performed as described with slight modifications (28, 29). Biocoat cell culture inserts (Becton Dickinson) were coated with Matrigel (Becton Dickinson) at the amount of 50 μg/filter. The upper chamber was filled with 40,000 cells in serum-free medium supplemented with increasing concentrations of human PEX (100 ng/ml and 1, 5, 25, and 50 μg/ml). The lower chamber was filled with conditioned medium obtained by incubating NIH 3T3 fibroblasts with serum free medium and ascorbate for 24 h. Cells were incubated at 37°C for 24 h, the membranes were removed, stained with hematoxylin, and mounted on slides, and the cells from five adjacent diagonal fields, each covering 0.25-mm^2 area, were counted at ×200. All experiments were run in quadruplicate, and all specimens were counted twice by independent reviewers.

For the monolayer migration assay, chamber Permanox slides (Miles Scientific, Naperville, IL) were coated with Matrigel and incubated with MEMα containing 10% FBS. A sterile sedimentation cylinder was placed onto the center of the chamber. Glioma cells in suspension were placed into the lumen of the cylinder and allowed to attach. The cylinder was removed, and the cells were allowed to spread. The area occupied by the attached cells was imaged with a camera (Olympus) placed on an inverted microscope. The migration was calculated as the increase of the radius beyond the initial radius and expressed as the mean ± SE (29). PEX at increasing concentrations (50 and 100 ng/ml and 1, 25, and 100 μg/ml) was added to the cultured medium, and the medium was changed daily. An irrelevant substance was added at the same concentration and used as a negative control. Migration was quantified in comparison to unstimulated controls.

Proliferation, Cytotoxicity, and Apoptosis Assays. U87-MG, U373-MG, and U118-MG glioma cells and HUVEC, BCE, PAE/KDR, and MCF-7 cells were plated on 96-well plates (20,000 cells for each well) and cultured in the presence of increasing concentrations of PEX (50 and 100 ng/ml and 1, 3, 5, 10, 25, 50, and 100 μg/ml) for 24, 48, and 72 h. The relative number of cells was calculated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay (Promega Corp., Madison, WI). An irrelevant substance was used as a negative control. Each experiment was run six times in triplicate. The number of apoptotic cells was measured using Apoptag peroxidase kit (Genenco International, New York, NY) and expressed as relative number of positive cells from 100 cells counted in five randomly chosen fields.

Adhesion Assays. The adhesion assay was performed as described with slight modifications (30). Briefly, FGF-2, vitronectin, or $\alpha v\beta 3$ integrin (10 μg/ml) were suspended in 100 mM sodium bicarbonate (pH 9.6) and incubated in a 96-well plate at 4°C for 16 h. The solution was removed, and the wells were washed three times with cold PBS and blocked with BSA. Cells (30,000/well) were suspended in serum-free medium or medium containing 1% FBS and plated. This serum concentration is required for optimal adhesion of cells to FGF-2-coated plates (30). After 2 h at 37°C, the wells were washed three times with PBS containing 2 mM EDTA and serum-free medium. In a second set of experiments, cells were preincubated with different concentrations of PEX or LM609, antihuman FGF-2 neutralizing antibody, vitronectin, tenascin, or fibronectin and shaken for 2 h at 37°C. Cells were centrifuged and resuspended in MEMα containing 1% FBS and plated. All of the experiments were performed in quadruplicate.

To test the cell-free interaction of PEX to $\alpha v\beta 3$ integrin, aliquots of carbonate buffer containing free $\alpha v\beta 3$ integrin and BSA (each at 20 μg/ml/well) were added to 24-well plates (30). After 16 h of incubation, the solutions were removed, washed three times with cold PBS, and incubated for 30 min at 37°C with 1 mg/ml of BSA. Increasing concentrations of LM609 were added to the dishes and incubated for 2 h at 37°C. Aliquots of PEX (6 μg/ml/well) were added to each dish and incubated for 4 h at 37°C on an orbital shaker. The solution was removed, and the dishes were washed three times with PBS containing 2 mM EDTA. Non-reducing SDS sample buffer was added and incubated for 1 h at 50°C. Dishes were scraped, and samples were recovered and analyzed by Western blot for PEX.

***In Vivo* Inhibition of Glioma Growth.** Two groups of 10 Swiss nude male mice, 6 weeks of age, received 2×10^6 cells s.c. in the right flank. Two groups of 10 Swiss male nude mice, 6 weeks of age, were implanted intracranially with 50,000 U87-MG and U373-MG cells. The tumors were allowed to grow for 5 days. Half of the animals of each group were treated with PEX at a dose of 1 or 5 mg/kg/day administered i.p. The remaining half of the animals were treated with a control substance (PBS). All animals were sacrificed 30 days after treatment, and tumor volumes were measured. Tumors were embedded in OCT and stored at −70°C. Tumor sections from treated and control animals were stained with hematoxylin and CD-31 antigen to determine angiogenic

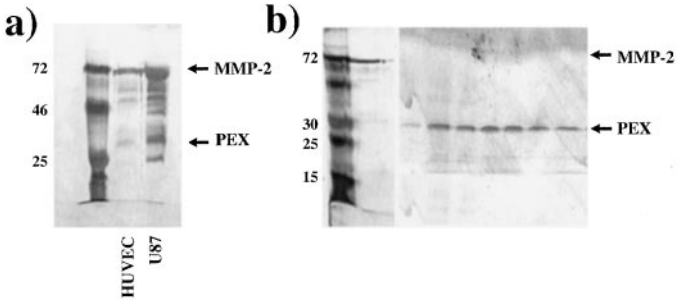


Fig. 1. *a*, silver stain of fractions collected from a gelatin Sepharose column on which medium from HUVEC and U87-MG cells was applied and recirculated overnight. Medium from these cells contains both MMP-2 in all its activated forms and PEX (*arrows*). Most of MMP-2 is present as a M_r 72,000 form. *b*, silver stain of fractions containing free PEX and free MMP-2 collected from a heparin-Sepharose column. MMP-2 appears as a single band of M_r 72,000, PEX as a single band of M_r 29,000.

indices and stained with Apotag kit and Ki-67 to measured the apoptotic and proliferation indices, respectively. Immunohistochemistry was carried out using standard immunohistochemical techniques. Microvessel count and density were scored according to the method described by Leon *et al.* (31) and Weidner *et al.* (31, 32). The apoptotic and proliferative indices were defined as the percentage of positively stained cells/100 nuclei from 10 randomly chosen fields/section, scored under light microscopy at ×200. All animal experiments were performed at least twice.

RESULTS

Detection and Purification of PEX in Glioma Cell Culture Medium. PEX was detected in the culture medium of U87-MG, U373-MG, U118-MG, HUVEC, PAE/KDR, BCE, MCF-7, and CRL-1435 cells. After immunoprecipitation with MTV88, two bands of M_r 29,000 and M_r 46,000 were detected by Western blot under reducing conditions. The M_r 29,000 band had the expected size of PEX. The second band may result from the cross-reactivity of the anti-PEX antibody with a cleavage product of MMP-2.

PEX was purified from glioma cell culture medium by a complex chromatographic procedure. The total amount of 0.5 mg of free protein was purified from 5 liters of U87-MG or U373-MG cell conditioned medium (Fig. 1). Western Blot analysis performed on the fractions collected from heparin-Sepharose chromatography showed that PEX appeared as a TIMP-2 complexed form (the first high peak represents 75% of the total protein) and a free form (the second low peak represents 25% of the total protein; data not shown). Purity of these forms was assessed by silver stain analysis. When similar studies were performed with anti-TIMP-4 antibodies, no bands were detected.

PEX Regulates Tumor-induced Angiogenesis. A tube formation assay was used to determine the capability of purified human PEX to regulate angiogenesis in tumors. When increasing concentrations of human purified PEX were added to HUVEC, BCE, and PAE/KDR cells cultured in the presence of glioma cell culture medium, a dose-dependent inhibition of tube formation was documented (Fig. 2*a*). Inhibition was observed at a concentration of 1 μg/ml, and no tube formation was documented at a concentration of 10 μg/ml. The inhibiting effect of PEX was reversible because 4 days after the withdrawal of PEX, capillaries reappeared spontaneously. PEX also inhibited the limited basal tube formation seen when PAE/KDR cells were cultured only in F-12 medium.

PEX Regulates Glioma and Endothelial Cell Migration *in Vitro*. We used a Boyden chamber model and a monolayer migration assay to determine the effect of human purified PEX on glioma and endothelial cell migration. We investigated the inhibition of glioma and endothelial cell migration *in vitro* by human purified PEX, performing two sets of experiments. Glioma and endothelial cells were plated in Boyden chambers and exposed to an increasing concentration of PEX. We observed a dose-dependent decrease in the number of migrating cells with increasing concentrations of PEX (the maximum inhibition was reached at 25 μg/ml of PEX; Fig. 2, *b* and *c*). In a second set of experiments, glioma and endothelial cells were plated in the center of a single Matrigel-coated dish and exposed to increasing concentrations of PEX (Fig. 2*d*). The effect of PEX was dose dependent and was first observed at a concentration of 100 ng/ml. At a concentration of 1 μg/ml, a 52% inhibition was observed, which reached 82% of inhibition at 10 μg/ml. The effect of PEX on cell migration was $\alpha v\beta 3$ integrin independent as demonstrated by its inhibitory effect on migration of CHO cells in Boyden chambers (Fig. 2*c*).

PEX Inhibits Proliferation and Induces Apoptosis of Glioma and Endothelial Cells *in Vitro*. We studied the effect of PEX on glioma and endothelial cell proliferation by exposing the cells to increasing concentrations of PEX for 24, 48, and 72 h, both in normal serum (10%) and in low serum (1%) conditions (Fig. 3, *a* and *d*). We documented a dose-dependent inhibition of glioma cell proliferation

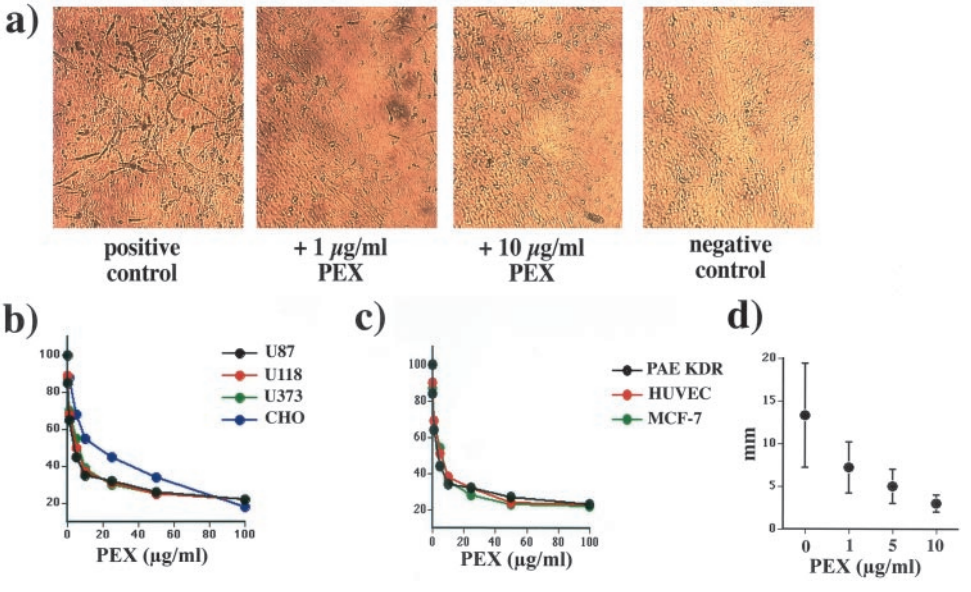


Fig. 2. Purified human PEX regulates glioma-associated angiogenesis and glioma and endothelial cell migration. *a*, purified human PEX inhibits glioma-induced angiogenesis *in vitro*. HUVECs cultured in the presence of glioma cell medium form tubes after 4 days of treatment (*positive control*). Increasing concentrations of PEX are able to inhibit tube formation activity of HUVECs grown in the same conditions. Negative controls indicate the same cells grown in the presence of endothelial cell medium do not form tubes. Purified human PEX inhibits glioma (*b*) and endothelial cell (*c*) migration in a Boyden chamber model. Increasing concentrations of PEX are added to glioma or endothelial cells plated in the upper chamber. After 24 h, a dose-dependent decrease in the number of migrating cells is observed. The effect is also seen on MCF-7 cells as well as on $\alpha v\beta 3$-deficient cells (CHO). Data are presented as a percentage of controls. *d*, purified PEX inhibits glioma cell migration in a monolayer migration assay. Cells are plated in the center of a single Matrigel-coated dish and exposed to increasing concentrations of PEX. PEX reduces cell migration in a dose-dependent manner. *Bars*, SE.

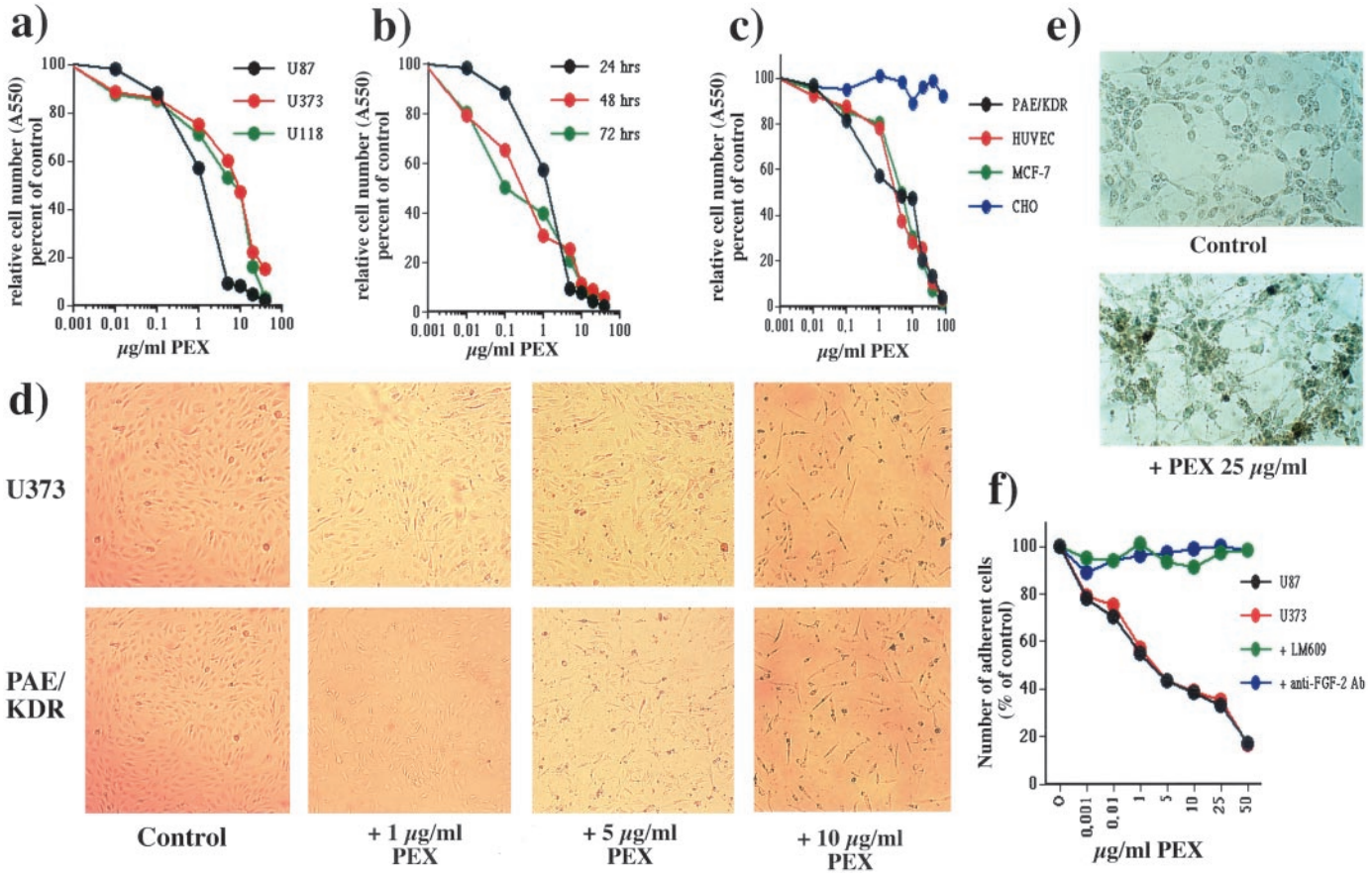


Fig. 3. Effect of purified human PEX on glioma and endothelial cell proliferation. *a*, increasing concentrations of PEX inhibits the growth of U87, U373, and U118 glioblastoma cells in a dose-dependent manner. Glioma cells are exposed to increasing concentrations of PEX for 24 h in 10% serum condition. Relative cell number is calculated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and expressed as a percentage of control. SE are not reported because they are <5%. *b*, effect of increasing concentration of PEX on U87 cells cultured for 24, 48, and 72 h in 10% serum condition. *c*, the inhibitory effect of PEX is also seen on endothelial cells (HUVEC and PAE/KDR) and on human mammary carcinoma cells (MCF-7) but not on $\alpha v\beta 3$-deficient cells (CHO). Cells are exposed to increasing concentrations of PEX for 24 h. The relative number of cells is calculated as described above. *d*, a reduction of the number of U373 and PAE/KDR cells exposed to increasing concentrations of PEX for 24 h. The concentrations of the molecule increases from *left* to *right*. *e*, the effect of PEX on glioma cells is accompanied by an increase in apoptosis. Apoptotic nuclei are evident by the brown staining (terminal deoxynucleotidyl transferase-mediated nick end labeling assay). *f*, PEX inhibits the adhesion of U87 and U373 cells on FGF-2-coated plates. Glioma cells are incubated in suspension for 2 h at 37°C with increasing concentrations of PEX. The cells were centrifuged to remove unbound molecule and seeded onto a 96-well plate coated with 10 μg/ml of FGF-2 and saturated with BSA. The number of adherent cells is evaluated after 2 h of incubation at 37°C, corrected for nonspecific cell adhesion measured onto BSA-coated plates, and expressed as a percentage of cells adherent to FGF-2 in the absence of PEX. The inhibitory adhesion effect of PEX is prevented by pretreating the cells with an anti-$\alpha v\beta 3$ blocking antibody (LM609) or with an anti-FGF-2 antibody. SEs are not reported because they are <5%.

in both conditions. The activity of PEX was not restricted to glioma cells, as documented by its inhibitory effect on endothelial cells and on MCF-7 and CRL-1435 cell proliferation (Fig. 3*c*). PEX was not able to inhibit proliferation of CHO cells, suggesting that expression of $\alpha v\beta 3$ integrin is one of the factors required for exerting its antiproliferative effect (Fig. 3*c*). Under the same conditions, PEX induced a dose-dependent increase of apoptosis (Fig. 3*e*).

PEX Inhibits Adhesion of Glioma and Endothelial Cells to FGF-2 and Vitronectin and Binds to $\alpha v\beta 3$ Integrin. We used adhesion assays to determine the effect of PEX on the $\alpha v\beta 3$ integrin-dependent adhesion of glioma and endothelial cells onto FGF-2 and vitronectin-coated plates. PEX reduced glioma and endothelial cell adhesion on FGF-2-coated plates in a dose-dependent manner (Fig. 3*f*). Total and 50% inhibition were reached at 50 and 3 μg/ml, respectively. Adhesion was prevented by treating the cells with LM609 or the plates with an antihuman FGF-2 or anti-vitronectin antibodies. Similarly, PEX reduced the adhesion of glioma cells to vitronectin-coated plates (data not shown). Its effect was dose dependent and started at concentrations over 5 μg/ml. Total and 50% inhibition were reached at 80 and 10 μg/ml, respectively.

A cell-free interaction assay was used to determine the capability of PEX to bind to a surface-bound $\alpha v\beta 3$ integrin. Increasing concentrations of LM609 inhibited the binding of PEX to $\alpha v\beta 3$ integrin (data not shown).

PEX Expression in Human Glioma Tissue Correlates with $\alpha v\beta 3$ Integrin Expression. We studied the expression of PEX in 40 surgical specimens of human glioma. Histopathological analysis revealed 13 glioblastomas, 10 anaplastic gliomas (4 anaplastic astrocytomas, 3 mixed anaplastic gliomas, and 3 anaplastic oligodendrogliomas), and 17 low-grade gliomas (4 mixed low-grade gliomas, 5 fibrillary astrocytomas, and 8 oligodendrogliomas). PEX protein was detected by immunoprecipitation analysis in all tumor samples, regardless of tumor grade, but not in the normal tissue. After immunoprecipitation with MTV88, two bands of M_r 29,000 and M_r 46,000 were detected, by Western Blot under reducing conditions (Fig. 4*a*). The first has the expected size of the peptide, whereas the second may still result from a cross-reactivity of the anti-PEX antibodies with a cleavage product of MMP-2. The same experiments were repeated with anti-TIMP-2 and anti-TIMP-4 antibodies, but no signal was detected.

In human glioma tissues, PEX expression correlated with tumor grade and type. PEX was strongly expressed by astrocytic tumors and only weakly detected in oligodendroglial tumors (Fig. 4*b*).

The expression of $\alpha v\beta 3$ integrin was examined by immunoprecipi-

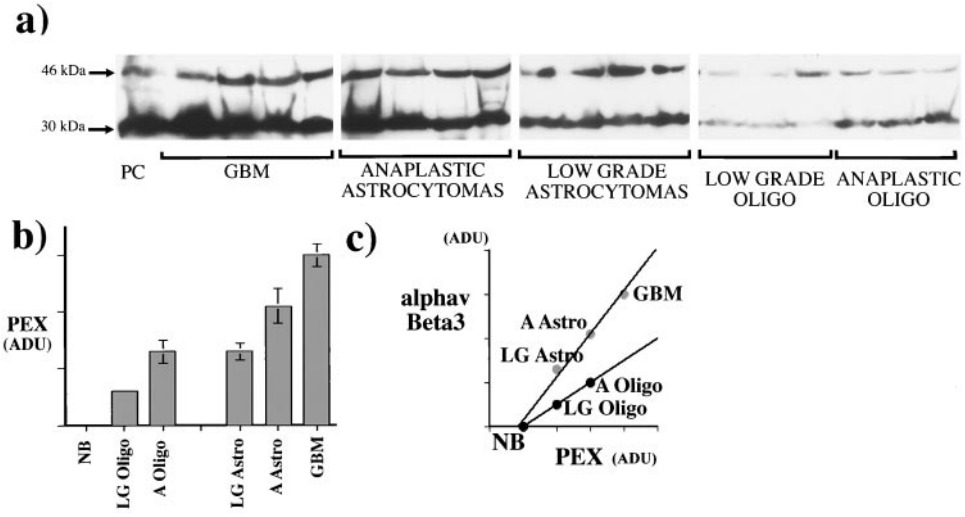


Fig. 4. Expression of PEX in human glioma specimens. *a*, Western blot analysis of PEX in representative cases of human gliomas. After immunoprecipitation with specific anti-PEX antibody, two bands of M_r 29,000 and M_r 46,000 were detected. The M_r 29,000 band corresponds to PEX. PEX expression is correlated with tumor grade (*b*) and $\alpha v\beta 3$ integrin expression (*c*). *Bars*, SE. *NB*, normal brain; *LG oligo*, low-grade oligodendrogliomas; *A oligo*, anaplastic oligodendrogliomas; *LG astro*, low-grade astrocytomas; *A astro*, anaplastic astrocytomas; *GBM*, glioblastomas. PEX and $\alpha v\beta 3$ integrin expression are analyzed by Western blot. Quantitation of Western blot signals is performed by laser scanning densitometry and expressed as arbitrary density units (*ADU*).

tation in the same samples analyzed previously for PEX. $\alpha v\beta 3$ integrin, under nonreducing conditions, appeared as three bands of M_r 140,000, M_r 130,000, and M_r 85,000. $\alpha v\beta 3$ integrin was expressed more prominently in astrocytic than in oligodendroglial tumors, and its expression correlated with the histological grade of tumors (Fig. 4*c*). A strong correlation was demonstrated between $\alpha v\beta 3$ integrin and PEX expression ($r = 0.942$; $P < 0.001$; Fig. 4*c*). Moreover, we showed the ability of $\alpha v\beta 3$ integrin to bind PEX in samples of human gliomas, detecting PEX in samples of gliomas that were previously immunoprecipitated with an anti-$\alpha v\beta 3$ integrin antibody (data not shown). PEX expression correlated weakly with MMP-2 expression in the same samples ($r = 0.633$; $P < 0.05$).

PEX Reduces Glioma Growth *in Vivo*. The effect of PEX on glioma growth *in vivo* was tested on s.c. and intracranial glioma models. i.p. administration of PEX decreased glioma growth in both models. s.c. U87 and U373 glioma xenografts treated with PEX reached a size of 3.1 ± 1.5 (U87) and 4.1 ± 1.4 (U373) mm^3 by 30 days after treatment, and the tumors were difficult to separate from overlying skin. During the same period, control s.c. U87 and U373 glioma xenografts reached a size of 1050 ± 245 (U87) and 990 ± 155 (U373) mm^3, and the skin overlying the tumor began to ulcerate (Fig. 5*a*).

Thirty days after implantation, control intracranial glioma xenografts reached a size of 19.245 ± 6.395 (U87) and 16.334 ± 5.44 (U373) mm^3. At the same time, in the group of animals treated with 1 mg/kg/day of PEX, tumor xenografts reached a size of 0.23 ± 0.32 (U87) and 0.18 ± 0.32 (U373) mm^3, corresponding to a 99% of growth suppression. In the groups of animals treated with 5 mg/kg/day of PEX, tumor xenografts reached a size of 0.088 ± 0.32 (U87) and 0.093 ± 0.45 (U373) mm^3, leading to a higher suppression of tumor growth (Fig. 5*b*). Most of the tumors were composed of microscopic tumor foci (Fig. 5*b*).

In both intracranial and s.c. groups, no signs of toxicity such as weight loss, inactivity, opportunistic infections, or reduced appetite were observed. At sacrifice, major organs were examined for the occurrence of any gross pathological changes. Sections from lungs, heart, kidney, and bowel, together with the skin at the injection site of randomly chosen intracranially and s.c. implanted animals, were also examined. We did not document any signs of disease.

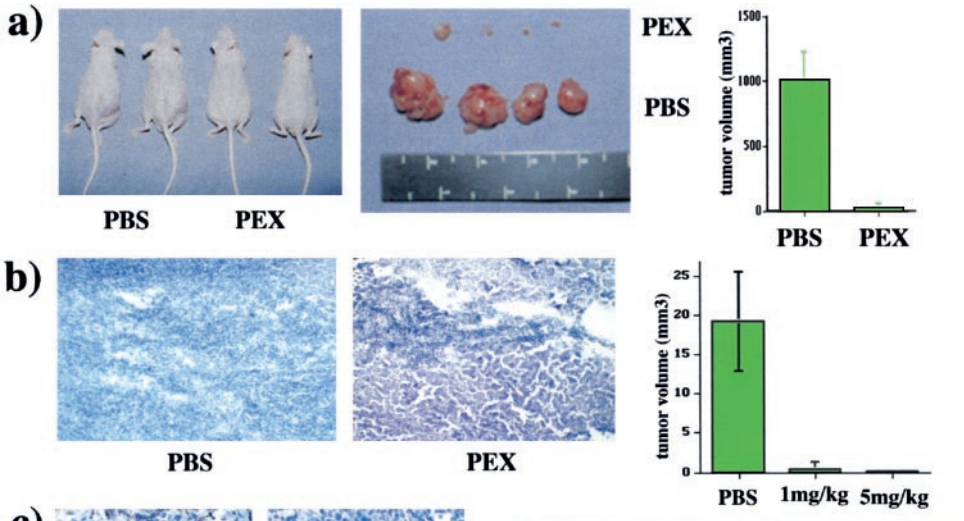
s.c. or intracranial tumors from PBS-treated mice were characterized by high microvessel density and count, presence of glomeruloid structures, particularly evident in U87 tumors, and polymorphic capillaries of varying calibers. s.c. or intracranial tumors treated with PEX were characterized by a decreased vascularity, mainly composed of uniform, few branches capillaries, an increased apoptosis, and a decreased proliferative index (Fig. 5, *c* and *e*). Apoptotic cells as well as those stained with Ki-67 antigen were dispersed inside the tumor parenchyma (Fig. 5, *d* and *e*). Histological analysis of serial sections of brain from PEX-treated and PBS-treated animals showed that in the PEX-treated mice, a reduced number of islands of tumor cells surrounded the main tumor mass, and in two animals, there was only a limited vascular subpial dissemination.

DISCUSSION

The hallmarks of malignant tumors are angiogenesis, tumor cell proliferation, and migration. Inhibition of angiogenesis has been shown to block tumor growth in a number of animal models (33–35). Angiogenesis has been shown to be permissive for invasion. The acquisition of the angiogenic phenotype occurs at the same time the cells are able to migrate away from the main tumor mass (7). Gliomas are the most frequent tumors in the central nervous system. They are characterized by marked angiogenesis, tumor cell proliferation, and local invasion (1). These processes are particularly active in the same region at the tumor periphery, which has limited the therapeutic effect of present available therapies. Therefore, gliomas are an excellent model to study the interaction among these biological processes in tumors.

The present study demonstrates the isolation and characterization of a fragment of MMP-2 called PEX that is naturally expressed in various tumors *in vivo* and simultaneously regulates angiogenesis, cell proliferation, and migration. PEX was detected in the medium of glioma, endothelial, breast, and prostate cell cultures. PEX was purified from the glioma culture medium by a complex chromatographic procedure, combining both affinity and size exclusion chromatography. In glioma cell medium, PEX was isolated as a free and a TIMP-2-bound form, the latter being more prevalent. Western blot analysis of gliomas of different histological grade and subtype showed that in human gliomas, PEX expression correlated with tumor grade and histological subtype, being highly expressed in more aggressive, vascularized, and proliferative astrocytic tumors, such as glioblastomas. Furthermore, in the same tumors, PEX expression correlated with $\alpha v\beta 3$ integrin expression, to which it was bound.

We performed tube formation assays to determine the biological effect of PEX on glioma-associated angiogenesis *in vitro*. PEX potently inhibited glioma angiogenesis starting from a concentration of



e)

	U87-MG		U373-MG	
	PBS	PEX	PBS	PEX
Tumor volume	19.245 ± 6.395	0.088 ± 0.32	16.334 ± 5.44	0.093 ± 0.45
Microvessel Count	79.25 ± 4.787	9 ± 5.05	74 ± 6.897	7 ± 6.98
Apoptosis index	0.4 ± 0.5	5.6 ± 1.14	0.2 ± 0.6	5.8 ± 2.14
Proliferation Index	1.9 ± 0.5	0.3 ± 0.8	1.5 ± 0.8	0.4 ± 0.98

Fig. 5. Purified human PEX reduces glioma growth *in vivo*. *a*, PEX reduced the growth of s.c. U87 glioma xenografts. PBS-treated animals developed large tumors; those treated with PEX had small, hardly visible tumors (*left panel*). At surgery, s.c. tumors from PEX-treated animals were small and difficult to isolate from the skin (*central panel*). Comparison of tumor volumes between PBS and PEX-treated animals (*right panel*) showed that treatment with 5 mg/kg/day of PEX produced a 99% growth suppression. *b*, PEX reduced the growth of intracranial U87 glioma xenografts. PBS-treated animals developed large tumors (*left panel*; hematoxylin, ×10); those treated with PEX had small tumors, visible at the site of injection (*central panel*; hematoxylin, ×10). Comparison of tumor volumes (*right panel*) among PBS-treated and PEX-treated animals. Treatment with PEX resulted in 99% suppression of tumor growth. *Bars*, SE. *c*, intracranial tumors treated with PBS (control) were characterized by a high microvessel count (vessels are stained with CD-31; ×200); those treated with PEX had few capillaries (×200). *d*, intracranial tumors treated with PBS (control) were characterized by a low apoptotic index (×100); in those treated with PEX, the apoptotic index increased (×100). *e*, tumor volume, microvessel count, apoptotic, and proliferative index in intracranial glioma xenografts. Treated animals received 5 mg/kg/day of PEX.

1 μg/ml, and its antiangiogenic activity was displayed against various endothelial cell lines.

Boyden chambers and a monolayer migration assay were used to determine the effect of PEX on cell migration *in vitro*. In both models, PEX decreased cell migration in a dose-dependent manner, and its activity was displayed against several glioma and endothelial cell lines. The antimigratory activity *in vitro* was evident at higher concentrations than those required to inhibit angiogenesis, but at concentrations of 10 μg/ml and above, the migration of various cell lines was significantly suppressed. PEX reduced the migration of αvβ3-negative cells, such as CHO, indicating that its effect on cell migration is αvβ3 integrin independent.

We cultured glioma and endothelial cells in the presence of increasing concentrations of PEX to determine its effect on cell proliferation. Both in low and normal serum conditions, PEX inhibited glioma and endothelial cell proliferation in a dose-dependent manner, starting from concentrations similar to those required to inhibit cell migration. The antiproliferative activity against tumor cells was not specific for glioma cells but was also observed on human mammary carcinoma and prostate cells. The inhibitory activity was accompanied by increased apoptosis. The lack of activity in αvβ3-deficient cells suggests that the expression of αvβ3 integrin is one of the factors required for its antiproliferative activity.

The ability of human purified PEX to bind to αvβ3 integrin was determined by performing separate experiments. In adhesion assays, PEX was able to reduce the αvβ3 integrin-dependent adhesion of glioma and endothelial cells to FGF-2 and vitronectin. In a binding assay, PEX was shown to be bound to a surface-bound αvβ3 integrin. PEX was also bound to αvβ3 integrin in human glioma specimens.

Our data indicated that PEX was able to simultaneously regulate angiogenesis, tumor cell proliferation, and migration. Regulation of angiogenesis may combine inhibition of endothelial cell migration, proliferation, and increased cell apoptosis. These characteristics make PEX a powerful molecule that is able to control tumor growth very efficiently. This significantly differentiates PEX from other tumor natural inhibitors, such as angiostatin and endostatin, that mainly act on endothelial cells (33, 34).

The powerful properties of PEX were confirmed by *in vivo* experiments. When administered i.p. at 1 and at 5 mg/kg concentrations, PEX potently inhibited glioma growth in s.c. and intracranial glioma models. At these concentrations, PEX produced a 99% suppression of tumor growth. With the same concentration, angiostatin achieved a 35% suppression, whereas endostatin produced a 65% inhibition (33, 34). Tumor growth suppression by PEX was accompanied by a decrease of vascularity, an increased apoptosis, and a decreased proliferative index. Apoptotic cells were homogeneously dispersed in the tumor parenchyma and not surrounding tumor vessels as documented in angiostatin or endostatin-treated tumors (33–35). The decrease of the proliferative rate was a unique feature of PEX-treated tumors and confirmed its ability to reduce tumor cell proliferation. The systemic administration of PEX was not associated with any detectable signs of local or systemic toxicity (33–35). Previous observations with recombinant avian PEX demonstrated that this form inhibited tumor growth in a chorioallantoic membrane assay and s.c. melanoma model (19, 36).

The antitumor activity of PEX is not in contradiction with the finding that its level of expression increases with tumor grade in human gliomas. This finding is not unprecedented. The generation of

endogenous inhibitors *in vivo* from large precursor proteins with distinct functions is a recurrent theme in the inhibition of angiogenesis (37). Elevated levels of endostatin or antiendostatin antibodies have been found in serum of patients with various malignant tumors, including glioblastoma (38, 39). In addition, proapoptotic ligands such as Fas ligand are highly expressed in tumors (40). Tumor angiogenesis is controlled by a balance of angiogenesis inhibitors and proangiogenic molecules. Although PEX is expressed in high-grade gliomas, they are highly invasive and angiogenic because proangiogenic factors override PEX and other inhibitor molecules (3, 4).

In conclusion, our results indicate that purified human PEX potently inhibits malignant tumor growth *in vitro* and *in vivo* by both angiogenesis-dependent and -independent mechanisms. This makes PEX a powerful candidate for the treatment of malignant tumors.

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