Suppression of Malignant Glioma Recurrence in a Newly Developed Animal Model by Endogenous Inhibitors

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ABSTRACT

Glioma recurrences develop at the borders of the surgical cavity and are the main cause of their poor prognosis. There are no therapeutic advances to reduce the incidence of recurrence or animal models that closely mimic the clinical scenario to evaluate novel therapeutics. This work investigates the efficacy of endogenous inhibitors, in preventing the recurrence of human malignant gliomas, in a newly developed animal model of glioma surgical resection. We developed a nude mice model in which human glioma xenografts were microsurgically removed. After surgery, small islets of tumor cells persisted in the normal brain parenchyma, grew, and formed a recurrence. As inhibitors we used PEX and a fragment of platelet factor 4 (PF-4/CTF), which were administered systemically on a daily basis or in metronomic combination with chemotherapy for 120 days. Treatment was started 1 or 15 days after tumor removal. PEX or PF-4/CTF produced a significant improvement in survival, and delayed the appearance of glioma recurrence. Survival of animals that received daily PEX or PF-4/CTF was similar to that of animals that received metronomic PEX or PF-4/CTF and chemotherapy, respectively. The effect of treatment was dependent on the time at which the treatment was initiated. The highest level of inhibition was observed when the treatment was administered 1 day after surgical resection and when PEX was used as the inhibitor (120 days versus 35 days of the control). Tumors treated with PEX or PF-4/CTF were small and well delineated, with few vessels. Postsurgical administration of PEX or PF-4/CTF significantly reduces the incidence human malignant glioma recurrences for a long period of time.

INTRODUCTION

Gliomas are highly proliferative and invasive solid tumors with marked vascularization. Present treatment consists of surgery, followed by radiotherapy and chemotherapy. Even when treated aggressively, gliomas are characterized by a high tendency to recur (1). The area of recurrence develops at the borders of the surgical cavity and is the main cause of the poor prognosis associated with these tumors (1, 2). The brain parenchyma surrounding the main tumor mass shows massive tumor cell infiltration. Infiltrating cells in the peripheral areas are not removed at the time of surgery, and continue to proliferate and recruit new vessels, leading to a recurrence (3, 4). The efficacy of the present available therapies is especially limited in these areas of infiltration (2, 5–7).

Invasion and angiogenesis are both playing a critical role in the formation of a recurrent tumor. Invasion and angiogenesis share common mechanisms of regulation, and are both influenced by a balance of stimulating and inhibiting factors (8–10). Systemic or local administration of inhibiting factors markedly reduced the growth of solid tumors in vivo in animal models (11–15). At present, there are no animal models of gliomas that closely resemble what occurs in the human clinical scenario (16). For this study, we developed a nude mice glioma surgical resection model in which we implanted human glioma tumor cells into the brain of nude mice, allowed them to form large tumors, with signs of local invasion, followed by surgical resection of the main tumor mass. In this model, small islets of tumor cells that have invaded into the normal brain parenchyma remained after surgical removal, grew, and subsequently formed a tumor recurrence. We hypothesized that the administration of endogenous inhibitors or of their fragments, after the surgical removal of the main tumor mass, can hamper the growth of the tumor cells located in the peripheral areas of the tumor that were spared from surgical resection, delaying the appearance of glioma recurrence.

We have shown previously that PEX, a naturally occurring fragment of metalloproteinases-2, inhibited glioma growth, invasion, and angiogenesis in vivo (17, 18). PF-4 inhibited glioma growth and angiogenesis in vivo when administered by

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3 The abbreviations used are: PF, platelet factor.
Malignant Glioma Recurrence and Endogenous Inhibitors

Lyophilized peptides were dissolved in sterile H₂O and stored at in vitro genic activity (19, 20). This fragment also inhibits glioma growth in nude mice.⁴

In the present study we investigated the capacity of recombinant PEX or of PF-4/CTF to inhibit the recurrence of human gliomas in the surgical resection model in nude mice. PEX or PF-4/CTF was administered daily alone, or in metronomic combination with chemotherapy. Metronomic administration of both chemotherapy and angiogenic inhibitors was shown to sustain long-term inhibition of tumor growth (21-23). Our data indicate that the systemic postsurgical administration of PEX or PF-4/CTF effectively inhibits recurrence of experimental human malignant gliomas. In addition, the model of surgical resection we have developed is an excellent model for the study of the efficacy of novel therapeutics in preventing the appearance of human malignant gliomas recurrence.

MATERIALS AND METHODS
Production of Recombinant PEX
PEX RNA was amplified from U87 glioblastoma cells by the PCR using the following primers: 5'CAGGATCCACGCGCTGTA(T)AGGGA3' (sense) and 5'CAGGATCCACGCGCTGTA(T)AGGGA3' (antisense). The fragment was cloned into the pRE set vector (Invirogen, Carlsbad, CA) and transformed in BL21 bacteria. Transformed BL21 bacteria were grown in Luria-Bertani medium followed by induction with 1 mM isopropylthiogalactoside. PEX was purified under denaturing conditions because the majority of the protein was found in inclusion bodies. PEX was then purified by Ni-charged chelating agarose (Ni-NTA agarose). Recombinant protein was refolded and dia lyzed against water, and the protein concentration was determined. Purity of the preparation was confirmed by running the purified protein on a 12% SDS-PAGE gel. The biological activity of PEX was tested in vitro using angiogenic and proliferation assays as reported previously (18).

Production of PF-4/CTF
The COOH-terminal peptide of human PF-4 (PF-4/CTF) ⁴⁷GRKICLDLQAPLYKIIKKLLESS (70) was synthesized using standard solid-phase methodology and purified by high-performance liquid chromatography using a C18 column and a 0-80% linear acetonitrile gradient in 0.1% trifluoroacetic acid. Lyophilized peptides were dissolved in sterile H₂O and stored at −20°C before use. The biological activity of PF-4/CTF was tested in vitro using angiogenic and proliferation assays as reported previously (20).

Cell Cultures
Two human glioma cell lines (U87-MG; American Type Culture Collection, MD, and D566, kindly provided by Dr. Darrell Bigner, Duke University, Durham, NC) were used in the animal experiments. U87-MG cells were cultured in MEM α, and D566 cells were cultured in DMEM medium. Both cultures were supplemented with 2 mm L-glutamine and 10% fetal bovine serum.

Two endothelial cell lines were used. PAE/KDR were cultured in Ham’s F-12 medium with 10% nonheat-inactivated FCS and 10 μg/ml genetinin (G-418 sulfate; Refs. 17, 18). Bovine capillary endothelial (American Type Culture Collection) cells were cultured in DMEM plus L-glutamine and fetal bovine serum. All of the medium were supplemented with 1000 units/ml penicillin-streptomycin solution, and the cells cultured in a 5% CO2 incubator at 37°C. Endothelial cell lines were used in the angiogenic and proliferation assays.

Glioma Surgical Resection Model
Pattern of Growth of the U87 or D566 Tumor in Nude Mice. Six-week-old male nude mice (Charles River) were used in all of the animal experiments. Animals were implanted intracranially with 50,000 U87 or 100,000 D566 glioma cells using an open window technique. The U87 and D566 glioblastoma cell lines were chosen because they form large, rapidly growing, local infiltrating tumors in a short period of time. A larger number of D566 cells was used because they grow slower than U87 cells, and this is the number of cells needed to form a tumor mass in the time required for the experiments. Animals were sacrificed 10, 15, 20, 25, and 35 days after tumor implantation to monitor the pattern of tumor growth. The brains were removed, fixed in OCT, sectioned, and stained with H&E, anti-fibroblast growth factor-2 (Ab-33-NA; R&D Systems, Minneapolis, MN) and α and β-1 integrin chain (Ab 1930, CD29; Chemicon, Temecula, CA) to highlight tumor cells. The pattern of vascularization, local areas of infiltration, and the shape of the tumor at its periphery were carefully examined on serial sections of the brain.

Glioma Surgical Resection Model. Nude mice were implanted intracranially with human U87 and D566 glioma cells. Twenty days after implantation, cells formed well-established tumors. At this time the animals were submitted to the surgical resection of the tumor. The tumor mass was macroscopically completely removed using a microsurgical technique. The same principles used for human tumor removal surgery were followed to perform the surgical resection of glioma xenografts. Surgical removal was pursued until white walls corresponding to microscopically normal brain were evident in the surgical cavity. Hemostasis was achieved with gelofom, surgical, and prolonged irrigation. Coagulation was never used. The average time required to perform this operation was 35 min. The mortality rate was <5% and occurred within the first 2 days from the operation. Animals were also examined for the appearance of any surgical related morbidity.

Histological analysis of brain sections from animals the day after surgery showed the persistence of numerous islands of infiltrating cells in close proximity to the surgical cavity, in an area that at the time of surgery was thought to be normal brain parenchyma. A glioma recurrence consisting of small tumor masses was observed in these areas within 10 days from operation, and all of the animals had to be sacrificed within 40 days from the tumor removal surgery. At sacrifice, histological analysis of the brains from these animals showed the presence of a large tumor mass in the site of the surgical cavity.

⁴ Unpublished observations.
Animal Experiments

Two inhibitors, PEX or a PF-4/CTF, were administered by systemic injection, daily alone (first regimen) or in metronomic combination with chemotherapy (second regimen). To investigate the effect of the delay of the treatment on the appearance of the recurrence, in half of the animals treatment was started the day after surgery. In the remaining half, treatment was delayed until 15 days after surgery.

Daily Administration of PEX or PF-4/CTF Alone. For each inhibitor, 45 animals were implanted intracranially with U87 or D566 cells. Twenty days later, two-thirds of the animals (30 animals) were operated on, and the main tumor mass was removed. These animals were then additionally randomly divided into two groups: treatment with PEX or PF-4/CTF, and surgery alone. PEX or PF-4/CTF was given i.p. at the dose of 2 mg/kg/day. The animals assigned to the surgery group received a daily injection of vehicle only. The remaining 15 animals were not operated on and were used as an additional control.

Metronomic PEX or PF-4/CTF and Low Dose Chemotherapy. As chemothapeutic drugs we used carboplatin and etoposide (18). Chemotherapy is based on the scheme in use in our hospitals for the treatment of glioblastomas and anaplastic astrocytomas (2). In this study we used a metronomic chemotherapy regimen, as reported previously (18). Carboplatin and etoposide were administered at lower doses than those given in the traditional high dose chemotherapy regimen (2, 24, 25). Chemotherapy and the inhibitor were both administered in a metronomic regimen (21). This consisted of: day 1, bolus with 6 mg/kg of carboplatin i.p. and 4 mg/kg of etoposide i.p.; and every 3 days a cycle composed of 2 mg/kg of carboplatin plus 2 mg/kg of etoposide and 2 mg/kg of PEX or PF-4/CTF i.p. for 2 days (18).

For each inhibitor, 75 animals were implanted intracranially with U87 or D566 cells. Twenty days later, 60 animals had their main tumor mass removed. These animals were then randomly divided in four groups of 15 animal each: (a) metronomic low dose chemotherapy plus metronomic PEX or PF-4/CTF according to the scheme described above (Surg.+metr.PEX or PF-4/CTF and CHT); (b) metronomic low dose chemotherapy administered as described above with no inhibitor (Surg.+metr.CHT); (c) metronomic PEX or PF-4/CTF alone without any chemothapeutic drugs as described in treatment group (Surg.+Metr.PEX or PF-4/CTF); and (d) vehicle substance only (PBS) administered i.p (surgery alone). The remaining 15 animals that were not operated on were used as an additional control (control).

Treatment was either started the day after surgery or 15 days later. In all of the experiments, treatment was continued for a total of 120 days. The overall health of the animals including body weight was recorded every 2 days. Animals were carefully monitored for the occurrence of any signs of discomfort or any neurological signs. Animals were sacrificed at the onset of neurological deficits, and their brains removed and fixed. At the end of each experiment, the remaining animals were sacrificed and the brains removed, embedded in OCT, and stored at −70°C. The brains were sectioned and a portion submitted to routine histological examination with H&E staining. Tumor volume was calculated and expressed as a mean ± SE. The remaining slides were stained with anti-CD31, anti-Ki67, and with terminal deoxynucleotidyl transferase-mediated nick end labeling assay as described below. Tumor volume was estimated using the formula for ellipsoid (width² × length)/2. Kaplan-Meier survival curves were statistically analyzed using ANOVA with repeated measures.

At sacrifice, internal organs of all of the animals were macroscopically carefully examined for the occurrence of any pathological changes. Histological sections of lungs, kidney, heart, and of the skin at the site of injection were performed on randomly chosen animals. Each animal experiment was repeated two times.

Immunohistochemistry

Immunohistochemistry was carried using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Primary antibodies include anti-CD31 (1:100 dilution; R&D System, Minneapolis, MN) and anti-Ki-67 (1:100 dilution; Dako, Carpinteria, CA). Detection was carried out using 3,3′-diaminobenzidine chromogen. Sections were counterstained with Hematoxylin. Negative control slides were obtained by omitting the primary antibody. Ki-67 staining was quantified by counting the number of positively stained cells of 100 nuclei in 20 randomly chosen fields (17, 18). Microvessel count and density were scored as reported previously (15, 17, 18). Apoptotic cells were detected with ApopTag plus kit (Intergen, MS) with 1% methyl green as a counterstain. Apoptosis was quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields per section at ×200 magnification (17, 18).

RESULTS

Animal Model of Surgical Resection

Animals implanted with U87 and D566 cells were sacrificed 10, 15, 20, 25, and 35 days after tumor cell implantation. Brain sections were evaluated for tumor size, shape, pattern of vascularization, and infiltration.

Ten days after implantation, U87 tumors were apparent (approximate diameter 2 mm), round in shape, and showed no focal local infiltration and some vessels. At 15 days, tumors were larger (approximate diameter 3 mm), showed increased vascularization and initial focal peripheral infiltration consisting of short projections of cells invading the normal brain parenchyma. After 20 days tumors had an approximate diameter of 4 mm. The pattern of infiltration became prominent, with wide, short projections of invading tumor cells along with many islands of tumor cells documented around the main tumor mass, inside the surrounding normal brain parenchyma (Fig. 1A).

D566 cells 10 days after tumor cell implantation formed small islets of tumor cells with initial signs of local invasion, consisting of short projections of cells invading the normal brain and very few vessels. At 15 days, a more organized tumor mass was visible (approximate diameter 3 mm), with an increase in vascularization and little change in invasion. At 20 days from implantation, a large tumor mass was evident (approximate diameter 4 mm). At this stage, tumors showed their own vascularization and presented longer projections of cells invading...
Fig. 1  Nude mice model of glioma surgical resection. A, pattern of growth of U87 tumors. Nude mice were sacrificed at 10, 15, 20, 25, and 35 days after tumor cell implantation. Ten days after implantation tumors were round in shape and showed no local infiltration. After 20 days, tumors were larger and showed wide projection of invading cells along with islands of tumor cells inside normal brain parenchyma (×200 magnification; B1 integrin staining). Surgery was performed at 20 days. 1, photomicroscopy of U87 tumor at 20 days (arrow); 2, microsurgical removal of the tumor (arrow); 3, surgical cavity documenting a “surgically” complete tumor removal and white walls indicating “normal” brain (arrow). B, pattern of growth of U87 glioma recurrence in nude mice. Two days after surgery small islets of cells (arrows) were evident in the apparently normal brain parenchyma, surrounding the surgical cavity. Eight days after surgery, larger islets of tumor cells were evident in close proximity to the surgical cavity. Eighteen days after surgery, a large tumor inside the surgical cavity was evident (×200 magnification; B1 integrin staining).
the surrounding normal tissue than those observed in the U87 tumors.

The time point of 20 days was chosen as the appropriate time for performing the tumor removal surgery (Fig. 1A). At 20 days, tumors showed a well-established vascularization and signs of local infiltration. Animals were also able to tolerate the surgical procedure, as demonstrated by the absence of neurological morbidity and by the very low mortality rate after the surgery.

Histological analysis of the brains 2 days after surgery showed that the main tumor mass was always removed, but small islets of cells in the apparently normal brain parenchyma surrounding the surgical cavity persisted. Tumor cells were identified by histology, and immunoreactivity for β1, ov integrins and fibroblast growth factor-2. Eight days after surgery, small tumors were evident all around the surgical cavity. At 18 days after surgery, histological analysis showed that animals had a recurrence in the surgical cavity. The mean volume of the recurrence (n = 30) at 18 days was 11 ± 2.3 mm³. Animals presented neurological deficits within 20 days from surgery and had to be sacrificed within 36 days from the operation. Histological analysis of the brains of these animals showed large tumors in the site of the surgical cavity (Fig. 1B).

The Postoperative Administration of Endogenous Inhibitors Delayed the Appearance of Glioma Recurrence

Systemic Administration of Daily PEX or PF-4/CTF Alone. Animals in which the tumors were not removed and did not receive any inhibitor (control) showed a 50% survival rate of 23 days. Animals had to be sacrificed within 36 days from tumor cell implantation. The mean tumor volume of these animals (n = 30) was 19 ± 2.6 mm³ at sacrifice (Fig. 2A).

Animals submitted only to tumor removal surgery (no inhibitors, surgery alone) increased their 50% survival to 35 days (Fig. 2A). All of the animals of this group were sacrificed within 47 days from implantation. Histology of the brains from these animals showed that the recurrence developed in the area of the residual surgical cavity. The mean tumor volume measured in the histological sections of the brains of these animals (n = 30) was 18 ± 4.5 mm³ at sacrifice.

The longest survival was observed in the group of animals with tumor removal surgery and treated with daily PEX alone. These animals had a 50% survival of 120 days when the treatment was initiated the day after surgery and of 63 days, when treatment was started 15 days from the operation (Fig. 2A). Eleven of 30 animals in which the treatment with daily PEX was started the day after tumor removal were still alive 140 days after tumor cell implantation. At sacrifice, histological analysis of the brains from these animals showed small tumors that were located in the site of the previous surgical cavity. The mean tumor volume was 2.4 ± 1.1 mm³.

Animals that received daily systemic PF-4/CTF the day after tumor removal had a 50% survival of 72 days (Fig. 2B). In contrast, those in which this treatment was initiated 15 days after the operation had a 50% survival of 55 days.

Animals submitted to treatment with inhibitors immediately after surgery did not experience delays in the healing of the surgical incision or any side effects during the entire duration of the treatment. At sacrifice, the macroscopic and histological analysis of organs as well as of the skin at the site of injection showed no pathological changes.

No significant difference in survival was observed between animals implanted with U87 or D566 cells, and treated with PEX or PF-4/CTF 1 day or 15 days after tumor removal surgery (Fig. 2C).

Systemic Administration of Metronomic PEX or PF-4/CTF and Chemotherapy. We then investigated the effect of the metronomic combination of PEX or PF-4/CTF and chemo-
therapy. We demonstrated previously that PEX given metronomically in combination with carboplatin and etoposide administered at lower doses than those given in the traditional regimen significantly improved survival in intracranial human glioma cell nude mice models, in absence of side effects (18). In the metronomic regimen used in this work, PEX or PF-4/CTF was given for 2 days every 3 days, alone or in combination with the chemotherapeutics drugs. Treatment was initiated 1 day or 15 days after surgery.

When treated with metronomic PEX alone at 1 day or 15 days after tumor removal, the 50% survival was 65 and 45 days, respectively (Fig. 3, A and B). For PF-4/CTF alone, survival rates at 1 and 15 days after surgery were 55 and 40 days, respectively (Fig. 3, C and D). Animals submitted to treatment with metronomic chemotherapy alone had a 50% survival rate of 53 days when the treatment was started the day after the tumor removal surgery. The 50% survival was 35 days when this was started 15 days after surgery. Metronomic PEX associated with metronomic chemotherapy significantly increased survival. When the combined treatment was initiated 1 day after surgery, a 50% survival of 80 days was observed (Fig. 3A). Fifty percent survival was 54 days when treatment was started 15 days after tumor resection (Fig. 3B). Animals submitted to metronomic PF-4/CTF and metronomic chemotherapy had a 50% survival of 63 days when the treatment was initiated the day after surgery (Fig. 3C), and of 50 days when this was started 15 days after surgery (Fig. 3D).

A small portion of the animals (9 of 30) that received the combined metronomic treatment of PEX and chemotherapy were still alive at 140 days from implantation (Fig. 3, A and B). At sacrifice, histological analysis of their brains showed only a small recurrent tumor around the surgical cavity. There was a significant difference in tumor volume in relationship to the time the treatment was initiated. In animals in which the treatment was started 15 days after the operation, the mean tumor volume reached $7 \pm 0.11004 \text{ mm}^3$ (4 animals); when the treatment was started the first day after surgery, the mean tumor volume reached $4 \pm 1.6 \text{ mm}^3$ (5 animals).

Treatments were well tolerated and not associated with any side effects. Macroscopic and histological analysis of organs as well as of the skin at the injection site from treated animals showed no abnormalities. No significant difference in survival was observed for animals harboring U87 or D566 xenografts (data not shown).

Fig. 3 Kaplan-Meier survival curves of animals submitted to metronomic PEX and metronomic chemotherapy, starting 1 day (A) or 15 days (B) after surgery. Control, no treatment; surgery, surgery only; Surgery + Metr. CHT, surgery followed by metronomic chemotherapy (CHT) only; Surg + Metr. PEX, surgery followed by metronomic PEX only; Surg + Metr. PEX and CHT, surgery followed by metronomic PEX and metronomic CHT. Kaplan-Meier survival curves of animals submitted to metronomic PF-4/CTF and metronomic chemotherapy, starting 1 day (C) or 15 days (D) after surgery. Control, no treatment; surgery, surgery only; Surgery + Metr. CHT, surgery followed by metronomic chemotherapy (CHT) only; Surg + Metr. PF-4, surgery followed by metronomic PF-4/CTF only; Surg + Metr. PF-4/CTF and CHT, surgery followed by metronomic PF-4/CTF and metronomic CHT. Day 0 corresponds to the day of tumor cell implantation. Surgery was performed on day 20.
Microvessel count, apoptosis index, and proliferation index in U87 tumors treated with daily systemic PEX, PF-4/CTF alone, or metronomically combined with metronomic chemotherapy the day after surgery. All analysis was done at the time of sacrifice. Data are reported as mean and expressed as a percentage of untreated control; bars, ± SE. Control, no treatment; surgery, surgery alone; Surg. + daily PEX, animals submitted to treatment with daily PEX alone; Surg. + daily PF-4/CTF, animals submitted to treatment with daily PF-4/CTF alone; Surg. + Metr. PEX, animals submitted to treatment with PEX administered according to the metronomic scheme; Surg. + Metr. PF-4/CTF, animals submitted to treatment with PF-4/CTF administered according to the metronomic scheme; Surg. + Metr. CHT, animals submitted to treatment with metronomic chemotherapy; Surg. + Metr. PEX+CHT, animals submitted to treatment with metronomic chemotherapy and metronomic PEX; Surg. + Metr. PF-4/CTF+CHT, animals submitted to treatment with metronomic chemotherapy and metronomic PF-4/CTF.

**Statistical Analysis.** The daily administration of PEX or PF-4/CTF, and the combination of metronomic inhibitor and chemotherapy, respectively, produced a similar survival. Treatment with PEX alone or in combination with metronomic chemotherapy was more effective than treatment with PF-4/CTF alone or metronomically combined with chemotherapy.

**Histological Analysis of the Tumors.** Sections of brain removed from each group of treatment were stained with H&E for routine histological examinations, and with anti-CD31 and anti-Ki67 antibodies to determine microvessel counts and proliferation rate, respectively. Other sections were stained with terminal deoxynucleotidyl transferase-mediated nick end labeling assay to determine the apoptotic rate. Sections were also stained for human β1 integrin to highlight tumor cells.

Histological analysis of sections from recurrent tumors showed in those treated with PEX or PF-4/CTF alone or metronomically associated with chemotherapy, a decrease in microvessel counts (Fig. 4). Tumors belonging to animals submitted to treatment with daily PEX or PF-4/CTF or with PEX or PF-4/CTF metronomically associated with chemotherapy had the lowest count. In these tumors, vessels were small, composed by unilayer of endothelial cells, and formed a well-defined and regular vascular network. There were no teleangiectatic vessels nor glomeruloid structures. Tumors from animals submitted to metronomic chemotherapy only had intermediate counts. The highest microvessel counts were measured in animals belonging to the control and surgery alone groups. In these tumors, vessels were irregular in shape and formed a complex network with many teleangiectatic vessels and some glomerular structures.

Tumors from animals treated with daily PEX or PEX metronomically associated with chemotherapy were also characterized by an increase in apoptotic rate and a decrease in proliferative index (17). Recurrent tumors from animals treated with PF-4 alone or metronomically combined with chemotherapy were only associated with an increase in apoptotic rate. In fact, the proliferation rate in these tumors did not show significant changes in comparison with the tumors from the control or surgery alone groups (Fig. 4).

We also carefully looked at the shape and margins of the recurrent tumors. Recurrent tumors from animals treated with the inhibitors alone or metronomically combined with chemotherapy were round in shape, and showed well-delineated margins with no (U87 xenografts) or very limited (D566 xenografts) signs of local infiltrations. These findings were particularly evident in tumors from animals treated with PEX (Fig. 5). Recurrent tumors from animals belonging to the control or surgery alone groups showed an irregular shape and margins with many cells invading and islets of cells inside the normal brain parenchyma.

**DISCUSSION**

Recurrence of human gliomas develops at the borders of a previous surgical cavity even when surgery is considered radical and adjuvant therapies have been implemented (1, 2, 26, 27). Presently there are no significant therapeutic advances to reduce the incidence of recurrence in gliomas (1, 2, 7). In addition, there are no animal models that closely mimic the clinical scenario to evaluate novel therapeutics (16).

For the present study we developed a model of glioma surgical resection in nude mice. We observed that when U87 and D566 cells were implanted in the hemisphere of nude mice using a microscopic technique, they formed tumors that 20 days from cell implantation showed signs of local infiltration, and had their own established vasculature and a substantial volume. A larger number of D566 cells compared with U87 cells was needed to form a tumor mass with the histological features described above. Twenty days was chosen as the most appro-
Appropriate time to perform the tumor removal surgery. Tumors had achieved some local infiltration and were of substantial volume. In addition, animals exhibited no signs of distress and were capable of tolerating the procedure. The surgical procedure was performed using a microsurgical technique after the same principles reserved for human gliomas. The procedure was reproducible and associated with a low mortality and morbidity rates. Immediately after surgery, histological analysis of the borders of the surgical cavity showed the persistence of some islets of tumor cells infiltrating in the normal parenchyma. Labeling tumor cells with green fluorescent protein could more readily identify postresection residual disease in the resected animals (26). The recurrence developed from these cells within 10 days from the operation, and a large recurrent tumor mass was evident 20 days from surgery. These findings show that this model closely resembles the clinical setting. In addition, it is an excellent model for the study of the efficacy of novel therapeutics.

Invasion and angiogenesis are both playing a critical role in the formation of a recurrent tumor (4, 27). They depend on the balance between stimulating and inhibiting factors (8–10). The administration of endogenous inhibitors in animal models reduced the growth of several human tumors in vivo (11–15).

In this work, we hypothesized that the administration of endogenous inhibitors after tumor removal surgery could hamper the growth of glioma cells located in the peripheral areas of the tumor that were spared from surgical resection and delay the appearance of the recurrence. To prevent recurrence, treatment should be chronic (27, 28). Endogenous inhibitors are suitable for chronic administration because of the absence of side effects associated with their administration. We decided to test the efficacy of the daily administration of inhibitors alone or metronomically combined with chemotherapy. The last paradigm was chosen because metronomic administration of chemotherapy and inhibitors achieved eradication of resistant tumors for a long period of time (18, 21–23, 29). In addition, intraoperative chemotherapy reduces metastases and recurrences in the novel nude mice human hepatocellular carcinoma resection model (30). We used PEX and a fragment of PF-4 as inhibitors. PEX is a fragment of human metalloproteinases-2 (31, 32), which we showed recently to simultaneously inhibit glioma angiogenesis, invasion, and proliferation in vitro, and to potently suppress glioma growth in vivo (17). When metronomically combined with traditional chemotherapeutic drugs, PEX sustained a long-term inhibition of glioma growth (18). PF-4 is an antiangiogenic molecule, and for these studies we used a COOH-terminal fragment (PF-4/CTF) that contains the critical sequence for its angiogenic activity. This fragment potently suppressed angiogenesis in vitro and in vivo when administered systemically (20), and glioma growth in nude mice not submitted to surgery.

We demonstrated here that the systemic administration of PEX or PF-4/CTF after tumor removal surgery significantly improved animal survival and delayed glioma recurrence. Moreover, the significance of the effect was dependent on the time at which the treatment was initiated. The most significant effect was documented when the treatment was initiated the day after surgery, when small islets of tumor cells were documented around the surgical cavity. The administration of PEX or PF-4/CTF was still able to increase survival even when treatment was started 15 days after surgery, when a tumor mass with a substantial volume was already present in the surgical cavity.

Survival of animals receiving daily PEX or PF-4/CTF was

![Fig. 5](https://example.com/fig5.jpg) Histological analysis of recurrent and control tumors (×200 magnification; β1 staining). Tumors belonging to the control and surgery only groups showed signs of local infiltration, consisting in trails and islets of tumor cells invading normal brain parenchyma. The same pattern of local invasion was evident in tumors from animal belonging to the metronomic chemotherapy only group. Recurrent tumors treated with daily PEX alone, metronomic PEX alone, or metronomic PEX combined with metronomic chemotherapy showed well-delineated margins. Tumors from animals treated with PF-4/CTF alone or metronomically combined with chemotherapy had delineated margins, but this pattern was less pronounced than in the tumors treated with PEX.
similar to that of animals that were treated with the metronomic regimen. This indicates that the combination of metronomic inhibitor with metronomic chemotherapy produced a synergistic effect (18). This is additionally supported by the fact that the metronomic administration of PEX or PF-4/CTF alone was not as effective as the daily administration. Furthermore, metronomic chemotherapy alone also resulted in a shorter survival than the combined treatment. Low dose metronomic chemotherapy produces an antivascular effect (23, 33). The concurrent administration of PEX or PF-4/CTF amplifies this effect and enhances the inhibitory effect on endothelial cell motility, invasion, and vessel remodeling produced by the metronomic chemotherapy itself. Furthermore, it might make endothelial cells more sensitive to cytotoxic therapies and improves tumor response (21–23). The combination of metronomic chemotherapy and daily administration of the inhibitor may produce an additional improvement in survival.

Treatment with PEX was always associated with the longest survival. Specifically, the longest 50% survival was observed in the group of animals treated with daily PEX 1 day after tumor removal surgery. This may result from the unique properties of PEX. It has antiangiogenic activity, and antiinvasive and antimitotic properties on both endothelial and glioma cells (17). This hypothesis is also supported by the histological analysis of the tumors from animals treated with PEX. These tumors showed a decrease and change in vasculature, an increase in apoptotic index, and a decrease in proliferation index (17, 18). In contrast, PF-4 acts as an antiangiogenic molecule, and its activity is restricted to endothelial cells. Tumors from animals treated with PF-4/CTF showed a decrease and change in vascularity and an increase in apoptosis but no change in proliferation index (13). In agreement with these data, glioma cell proliferation in vitro is not inhibited by PF-4/CTF.4

Histological analysis of the brains of the animals that survived the longest showed a small tumor mass in the area of the surgical cavity, with well-delineated margins and very little sign of local infiltration. These findings were particularly evident in tumors from animals that were treated with PEX. The ideal treatment for high-grade gliomas should prevent more tumor cells from migrating into the normal brain parenchyma, and inhibit neoangiogenesis and cell proliferation. Recurrences should present themselves as well-delineated nodules that are curable by surgery or radiotherapy (28). In addition, management of recurrence necessitates chronic treatment. Treatment with PEX or PF-4/CTF produced small, well-delineated tumors without the occurrence of any side effects also after prolonged time of administration.

In summary, the administration of PEX or PF-4/CTF significantly delayed the appearance of glioma recurrence for a long period of time. Our data open new perspectives for the concurrent administration of PEX or PF-4/CTF to delay glioma recurrence for a long period of time. The combination of metronomic chemotherapy and daily administration of the inhibitor may produce an additional improvement in survival.

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Suppression of Malignant Glioma Recurrence in a Newly Developed Animal Model by Endogenous Inhibitors

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