[CANCER RESEARCH 63, 2499–2505, May 15, 2003]

Local Intracerebral Delivery of Endogenous Inhibitors by Osmotic Minipumps Effectively Suppresses Glioma Growth *in Vivo*[1]

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ABSTRACT

The systemic administration of endogenous inhibitors significantly reduced the growth of human glioma *in vivo*, but required the production of a large amount of biologically active protein. In this study we reduced the amount of protein needed and optimized the therapeutical response by delivering the endogenous inhibitors locally into the brain by osmotic minipumps. Human hemopexin fragment of MMP-2 or COOH-terminal fragment of platelet factor-4 were delivered locally and continuously into the brain of mice implanted intracranially with glioma cells, by osmotic minipumps connected to an intracranial catheter. Local delivery of human hemopexin fragment of MMP-2 and COOH-terminal fragment of platelet factor-4 significantly inhibited the growth of well-established malignant glioma in nude and BALB/C mice. When the inhibitors were given at the same concentration, the efficacy of the local delivery was much higher than that reached with the systemic administration, both when the inhibitor was administered daily or continuously by s.c. minipumps. Moreover, the local delivery reduced the amount of protein needed to reach a significant therapeutic response. Intracerebral delivery maintained a long-term control of glioma growth and inhibited glioma recurrence in a surgical resection model. Treatment showed no side effects. Histochemical analysis of tumors showed that the tumor growth inhibition was the result of a decrease in tumor vasculature and a change in tumor vessel morphology. Our data demonstrate that local intracerebral delivery of endogenous inhibitors effectively inhibits malignant glioma growth and reduces the amount of protein needed to reach a therapeutical response.

INTRODUCTION

The growth of gliomas, such as that of other solid tumors, depends on the balance between positive and negative regulators that control tumor growth directly or indirectly through an angiogenesis-dependent mechanism (1–3). Most of these factors are produced directly by the tumor itself and are portions of extracellular matrix components or large proteins with distinct functions. Alternatively, they are generated by the host cells located in the tumor microenvironment (4, 5).

Some factors that inhibit angiogenesis have been isolated recently and studied in different animal models for their effect on tumor growth (5–8). When administered systemically *in vivo*, they produced a significant decrease in tumor growth (5, 8). The highest therapeutic response was usually achieved when the inhibitors were administered daily. In addition, treatment with endogenous inhibitors required prolonged therapy with repeated protein administration. It is usually assumed that this form of therapy would have to be carried out for the rest of the life of the patient to maintain tumor inhibition (4). Therefore, systemic administration requires the production of a large amount of protein, which is a very labor-expensive procedure. Moreover, systemic daily administration is associated with a poor patient compliance (4).

Continuous release or viral-mediated delivery has been demonstrated to be effective alternative modalities of administration of endogenous inhibitors (9–12). Local therapy has been used successfully for the treatment of experimental brain tumors (11, 13–17).

In this study we reduced the amount of protein needed, and optimized the compliance and the therapeutic response by delivering human PEX[4] and PF-4/CTF into the brain of the animals in which the tumor was implanted by osmotic minipumps.

We previously demonstrated that systemic PEX inhibited the growth of human malignant gliomas *in vivo* and delayed the appearance of glioma recurrence (18–20). Furthermore, it has been demonstrated that PF-4/CTF inhibit angiogenesis *in vitro* and *in vivo* by associating directly with angiogenesis factors and altering their conformation (21, 22). In addition, systemic PF-4/CTF was able to inhibit the growth of gliomas and the appearance of glioma recurrence in animal models (20, 22).

Osmotic minipumps maintain a well-defined and consistent pattern of drug exposure for a significant period of time (12, 23). Moreover, studies in the literature confirm that this delivery system can be successfully used to deliver protein to the brain (23, 24). Direct intracentral nervous system delivery of substances via pumps has been used in many experiments and has been applied in several clinical conditions (12, 25, 26).

Our data showed that local intracranial delivery of human PEX or PF-4/CTF effectively inhibited human malignant glioma growth and reduced the amount of protein needed to reach a significant therapeutical response. Moreover, the local intracerebral delivery of human PEX or PF-4/CTF showed a higher efficacy than the daily systemic or continuous systemic administration by osmotic minipumps. In addition, local intracerebral delivery sustained a long-term control of glioma growth and delayed the appearance of glioma recurrence. Our data indicate local intracerebral delivery by osmotic minipumps as an effective modality for the treatment of malignant gliomas.

MATERIALS AND METHODS

Production of Recombinant PEX

Production of recombinant PEX was performed as described previously (19). PEX RNA was amplified from U87 glioblastoma cells. The fragment was cloned into the pRSET vector (Invitrogen, 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 and purified by Ni-charged chelating agarose (Ni-NTA agarose). Recombinant protein was refolded, dialyzed

Received 10/18/02; accepted 3/18/03.

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[1] Supported by grants from Fondazione Monzino, Milano, AIRC (Associazione Italiana Ricerca sul Cancro), Milano, Compagnia di San Paolo, Progetto Oncologia, Torino, (to L. B., R. V.), Ministere de la Recherche, Institut National de la Santé et de la Recherche Médicale, Ligue contre le Cancer and Conseil Regional d'Aquitaine (to A. B.).

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[4] The abbreviations used are: PEX hemopexin fragment of MMP-2; PF-4/CTF, COOH-terminal fragment of platelet factor-4; PAE/KDR, porcine aortic endothelial cells stably transfected with KDR.

against water, and the protein concentration was determined. The biological activity of PEX was tested *in vitro* using angiogenic and proliferation assays as reported previously (19).

Production of Recombinant PF4/CTF

PF-4/CTF ^{47}NGRKICLDLQAPLYKKIIKKLLESS^{70} was synthesized using standard solid-phase methodology and purified by high-performance liquid chromatography (22). The biological activity of PF-4/CTF was tested *in vitro* using angiogenic and proliferation assays as reported previously (22).

Cell Cultures and Other Reagents

The human glioma cell line U87-MG (American Type Culture Collection, Rockville, MD) and the murine glioma G261 cell line (kindly provided by Dr. David Zagzag, Department of Pathology, New York University, New York, NY) were used in the animal experiments. U87-MG cells were cultured in MEM-α. G261 were grown in DMEM. Both media were supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 1000 units/ml penicillin/streptomycin solution.

Two endothelial cell lines were used. PAE/KDR were cultured in Ham's F-12 medium with 10% nonheat-inactivated FCS, 10 μg/ml geneticin (G-418 sulfate; Refs. 17, 18). Bovine capillary endothelial (American Type Culture Collection) cells were cultured in DMEM plus L-glutamine and 10% fetal bovine serum. All of the media were supplemented with 1000 units/ml penicillin/streptomycin solution, and the cells cultured in a 5% CO_2 incubator at 37°C. Endothelial cell lines were used in the angiogenic and proliferation assays.

Proliferation Assay

This assay was used to test the biological activity of human PEX or PF-4/CTF. bovine capillary endothelial and PAE/KDR cells were plated on 96-wells plate (20,000 cells for each well), and cultured in the presence of increasing concentrations of human PEX or PF-4/CTF (50 ng/ml, 100 ng/ml, 1 μg/ml, 3 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, and 100 μg/ml) in the presence of 10% serum for 24 h. The relative number of cells was calculated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay (Promega, Madison, WI). An irrelevant substance (BSA) was used as a negative control. Each experiment was run six times in triplicate.

Tube Formation Assay

In vitro tube formation assays were performed to test the biological activity of both the inhibitors. The assay was performed as described previously with slight modifications (11, 18). PAE/KDR were used (11, 18). PAE/KDR cells were seeded in 96-well plate coated with 0.5 mm-thick type I collagen gel ($4 \times 10{,}000$ cells/cm^2) and allowed to attach and spread for 3 h. The seeded cells were subjected to three different conditions: condition 1: cells grown in Ham's F-12, 10% FCS; condition 2: cells incubated with U87-conditioned medium; and condition 3: cells incubated with U87-conditioned medium plus PEX or PF-4/CTF at different concentrations. Microvessel counts were performed on microplates and scored as described previously (18, 21, 22).

Western Blotting

For Western Blot analysis, samples were boiled and analyzed by SDS-PAGE. After blotting onto Immobilon-P membranes (Millipore, Bedford, MA), blots were blocked with 5% nonfat milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 0.9% NaCl] containing 0.1% Tween 20 for 1 h, followed by incubation for 1 h at room temperature with the primary antibody (human PEX: monoclonal antibody IM3LL-anti MMP-2, 1:100 dilution in PBS; Calbiochem, La Jolla, CA; PF-4/CTF: AB 1488P, antihuman PF-4, 1:1.000 dilution in PBS; Chemicon, Temecula, CA; Ref. 18). The blots were then incubated with secondary antimouse or antirabbit horseradish peroxidase-conjugated antibody (1:1500 in PBS) for 1 h at room temperature. Detection of antibodies was performed using the ECL-Plus system (Amersham Life Science).

Animal Experiments

Short-Term Experiments. Groups of 10 6-week-old nude mice (Charles River Italia, Monzar, Italy) were implanted intracranially with 50,000 U87 glioma cells using an open window technique (18). For the syngeneic model, groups of 10 6-week-old BALB/c mice (Charles River Italia) were implanted intracranially with 50,000 G261 glioma cells using the same technique. Eleven days after tumor cell injection, animals were implanted with 2004 Alzet osmotic minipumps. Alzet minipumps maintain a well-defined, consistent pattern of drug exposure through all of the duration of the experiment. The pumps used in these experiments afford a constant period of work of 28 days. The pump reservoir was connected to an intracranial catheter, placed slightly posteriorly to the tumor cell injection site, in the same hemisphere. The pump reservoir was filled with 1, 0.5, and 0.250 mg (total amount) of human PEX or PF-4/CTF, which correspond, after the loading of the pump reservoir, to the following concentrations: 0.25 mg/kg/day, 0.5 mg/kg/day, and 1 mg/kg/day, respectively. One group of 10 animals was implanted with pumps containing PBS, and the remaining group did not receive any pumps. The last two groups served as the controls for the experiment. Animals were sacrificed when signs of any distress were evident and after 29 days from pumps implantation. Then their brains were removed.

To compare the therapeutic response of local *versus* daily systemic and continuous systemic system of delivery, six groups of 10 animals each were implanted intracranially with glioma cells. Eleven days after tumor cell injection, four groups were implanted with Alzet minipumps in the right flank of the animal. The reservoir of each pump was filled with the inhibitor at different concentrations or with PBS. In the intracranial groups, the reservoir of the pump was connected to an intracranial catheter, as described before. The remaining two groups received a daily systemic i.p. administration of the inhibitors at the same concentrations used in the local or continuous systemic groups or PBS. After 29 days of treatment, all of the animals were sacrificed and their brains removed.

Long-Term Experiments. For the long-term experiments, three groups of 10 animals each were implanted intracranially with 50,000 U87 or G261 glioma cells. Eleven days later, animals received Alzet minipumps, connected with an intracranial catheter, that were placed slightly posteriorly to the tumor injection site, in the same hemisphere, as described before. The reservoir of the each pump was filled with an inhibitor or with PBS. The lowest concentration of the inhibitor that showed the highest efficacy in the short-term experiment was used in these experiments. The remaining group of animals did not receive pumps and served as an additional control. After 28 days, the pump reservoir was changed and reconnected to the intracranial catheter. This procedure was done three times, to afford a 94-day period of treatment. Animals were sacrificed at the onset of neurological signs. After 105 days from tumor cell implantation, the remaining animals were sacrificed and the brains removed.

Glioma Surgical Resection Model. In this group of experiments, five groups of 10 nude mice each were implanted intracranially with 50,000 U87 glioma cells. Twenty days after tumor cell implantation, 40 animals were submitted to surgery for tumor removal. The glioma surgical resection model was performed as described previously (20). Briefly, the tumor was removed using a microsurgical technique, and the resection was pursued until white walls corresponding to apparently normal brain were documented in the surgical cavity. One day after surgery, two groups of 10 animals were implanted intracranially with Alzet osmotic minipumps, of which the reservoirs were filled with 0.5 and 0.250 mg (total amount) of human PEX or PF-4/CTF. The remaining group of animals received intracranial Alzet minipumps, in which the reservoirs were filled with PBS. Animals were sacrificed 29 days after pumps implantation.

All of the animal experiments were repeated at least twice.

All of the brains were fixed in 5% paraformaldehyde in PBS for 24 h at 4°C, dehydrated in 30% sucrose in PBS for 24 h at 4°C, embedded in OCT, and stored at −70°C. The brains were then sectioned, and a portion of them submitted to routine histological examination with H&E staining. Tumor volume was calculated and expressed as a mean ± SD Tumor volume was estimated using the formula for ellipsoid (width^2 × length)/2. The remaining slides were used for the immunohistochemistry analysis as described below. Kaplan-Meier survival curves were statistically analyzed using repeated measures of ANOVA.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on 5- and 20-μm sections. Immunohistochemistry on 5-μm sections was carried using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Primary antibodies include anti-CD31 (1:100 dilution; BD PharMingen), 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 (Genenco International, New York, NY MS) with 1% methyl green as a counterstain. Apoptosis and proliferative indices were quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields per section at ×200 magnification (11, 18).

Twenty 100-μm sections were used for immunofluorescence staining for CD31 to study the vascular network and vessel morphological changes. A donkey antirat IgG FITC (AP189F; Chemicon) was used as a secondary antibody. Analysis was performed by confocal microscope (Zeiss) followed by three-dimensional reconstruction.

RESULTS

Human PEX or PF-4/CTF Released by Osmotic Minipumps Are Biologically Active. To evaluate the capacity of osmotic minipumps to release biologically active human PEX or PF-4/CTF for a prolonged period of time, pump reservoirs were filled with the inhibitor at the same concentrations used in the *in vivo* experiments, and were placed in a Petri dish containing serum-free medium. The medium was changed and collected every 2 days. The release of the inhibitor by the minipump was assessed by performing a Silver Stain followed by Western Blot with specific anti-inhibitor antibodies. Human PEX appeared as a single M_r 28,000 band and PF-4/CTF as a single M_r 4,000 band (Fig. 1, *A* and B*)*. The biological activity of the inhibitor released by the minipump was assessed by performing proliferation and angiogenic assays with the serum-free medium collected from the Petri dishes containing the pump reservoir (Fig. 1, *C* and *D*).

Local Intracerebral Delivery of PEX or PF-4/CTF by Osmotic Minipumps Reduces the Growth of Well-established Human Malignant Glioma *in Vivo*. To evaluate the capacity of PEX or PF-4/CTF, released intracerebrally by osmotic minipumps to inhibit human malignant glioma growth in nude mice, osmotic minipumps containing 1, 0.5, and 0.25 mg/kg of human PEX or PF-4/CTF, were implanted in nude mice 11 days after tumor cell injection. The pump reservoir was connected to an intracranial catheter and placed posteriorly to the tumor cell injection site in the same hemisphere. Animals were sacrificed 29 days after pump implantation. A dose-dependent inhibition of glioma growth was observed. Local intracerebral delivery of 1, 0.5, and 0.25 mg/kg/day of human PEX resulted in a 99%, 97.2%, and 93% inhibition of glioma growth, respectively. Local intracerebral delivery of 1, 0.5, and 0.25 mg/kg/day of PF-4/CTF produced a 90%, 87%, and 80% reduction in tumor volume, respectively (Fig. 2*A*).

Local Intracerebral Delivery of PEX or PF-4/CTF by Osmotic Minipumps Reduces the Growth of Well-established Murine Malignant Glioma *in Vivo*. The ability of human PEX or PF-4/CTF to inhibit the growth of a murine glioma in a syngeneic model was performed by using the same experimental design described in the previous section, with nude mice. A dose-dependent inhibition of murine glioma growth was observed. Local intracerebral delivery of 1, 0.5, and 0.25 mg/kg/day of human PEX in this model resulted in a 99%, 97%, and 95% inhibition of glioma growth, respectively. Local intracerebral delivery of 1, 0.5, and 0.25 mg/kg/day of PF-4/CTF

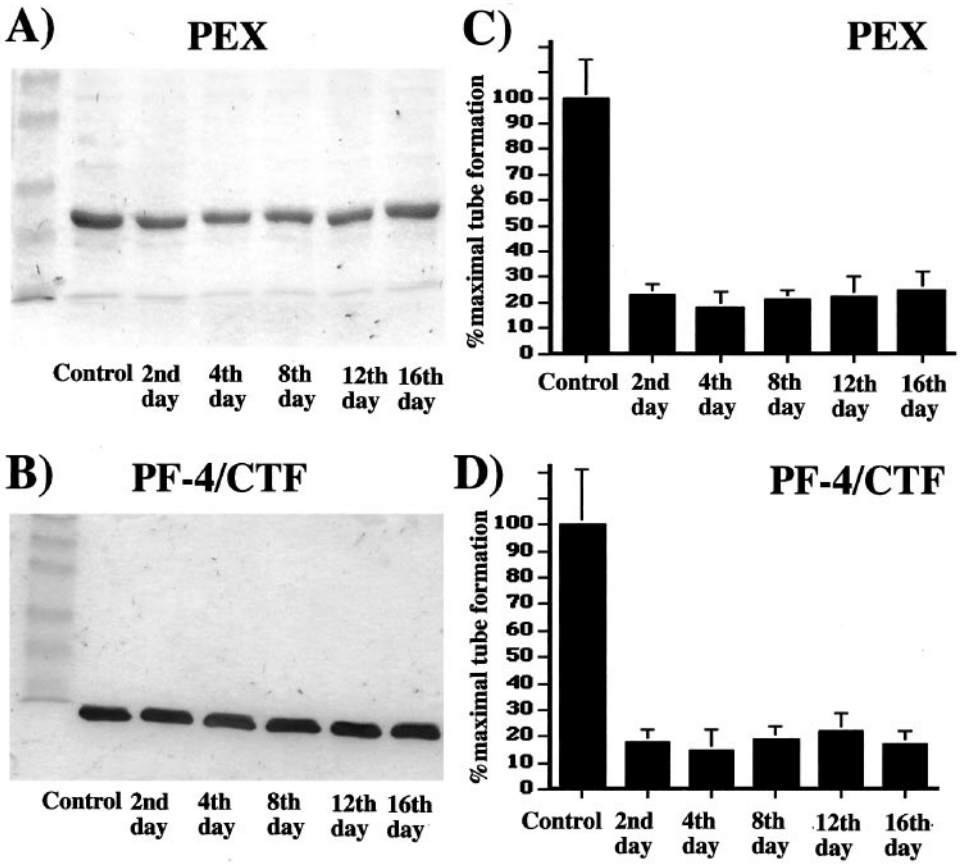

Fig. 1. Osmotic minipumps release biologically active human PEX and PF-4/CTF. The pump reservoir was filled with the inhibitor at the same concentrations used in the *in vivo* experiments, and put in a Petri dish with medium. The medium was replaced every 2 days. The release of human PEX (*A*) and PF-4/CTF (*C*) was demonstrated by Silver Stain. Purified human PEX or PF-4/CTF were used as a control. The biological activity of the inhibitor was shown by tube formation assay (*B* and *C*). No change in release or inhibitory activity was documented in the medium collected from day 2 to day 16. Free serum medium was used as a negative control; *bars*, ±SD.

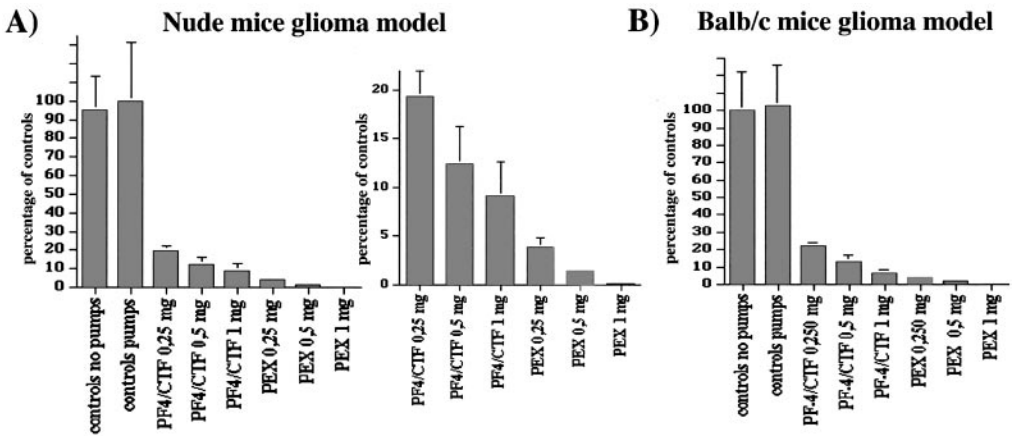

Fig. 2. *A*, local intracerebral delivery of human PEX or PF-4/CTF inhibited the growth of human glioma in nude mice. Pumps filled with the inhibitor (0.25, 0.5, and 1 mg/kg/day) were implanted 11 days after glioma cell injection. The intracranial catheter was placed posteriorly to the tumor cell injection site in the same hemisphere. The pumps afforded a working period of 28 days. Animals were sacrificed 29 days after pump implantation and the tumor volume measured. A potent dose-dependent inhibition of glioma growth was documented. The efficacy of human PEX was higher than that of PF-4/CTF. *B*, a similar dose-dependent inhibition of tumor growth was observed when the same experiments were repeated with a murine glioma cell line (G261) implanted intracranially in BALB/c mice. Data are presented as a percentage of controls; *bars*, ±SD. *Controls no pumps*, animals in which no pumps were implanted after tumor cell injection. *Controls pumps*, animals in which pumps containing PBS were implanted 11 days after tumor cell injection. The total amount of human PEX or PF-4/CTF contained in the pump reservoir is reported.

produced a 93%, 86%, and 78% reduction in tumor volume, respectively (Fig. 2*B*). The inhibitors showed a similar activity both in the orthotopic and syngeneic model.

Local Intracranial Delivery of PEX or PF-4/CTF Is More Effective Than Systemic Daily i.p. or Systemic Continuous Administration by s.c. Minipumps. In this group of experiments, the efficacy of the intracerebral local delivery of the inhibitor was compared with those observed with the systemic daily i.p. or continuous systemic administration of the inhibitor given at the same concentration used in the local intracerebral delivery. In the local delivery group, 11 days after tumor cell injection, animals were implanted with osmotic minipumps in which the reservoir was connected with an intracranial catheter. In the continuous systemic delivery group, the pump was placed s.c. in the right flank of the animal. In both groups, the pump reservoir was filled with 0.5 or 0.25 mg (total amount) of human PEX or PF-4/CTF. Animals belonging to the daily systemic group were treated with a daily i.p. injection of 0.5 mg/kg/day or 0.25 mg/kg/day of the inhibitor, which corresponds to the same amount delivered daily by the minipumps in the previous two groups.

The local intracerebral delivery of 0.5 mg/kg of human PEX resulted in a 98.5% inhibition of tumor growth. When the same concentration was delivered by s.c. pumps, a 83.7% inhibition was observed. Daily systemic i.p. delivery of the same amount of human PEX produced a 67% inhibition of glioma growth (Fig. 3*A*). Intracerebral and s.c. continuous delivery of 0.5 mg/kg PF-4/CTF was associated with a 89% and 67% decrease in tumor volume, respectively. When the same amount of PF-4/CTF was delivered daily by i.p. injection, it resulted in a 28% reduction of glioma growth (Fig. 3*A*).

The local intracerebral delivery of 0.25 mg/kg of human PEX produced a 95.5% inhibition of glioma growth, compared with a 62% and a 27% inhibition observed with the systemic continuous and the daily systemic administration, respectively (Fig. 3*B*). When PF-4/CTF was used, a 78% reduction of tumor growth was documented with the local intracerebral delivery, *versus* a 38.5% and a 12% inhibition showed with the systemic continuous and daily systemic delivery, respectively (Fig. 3*B*).

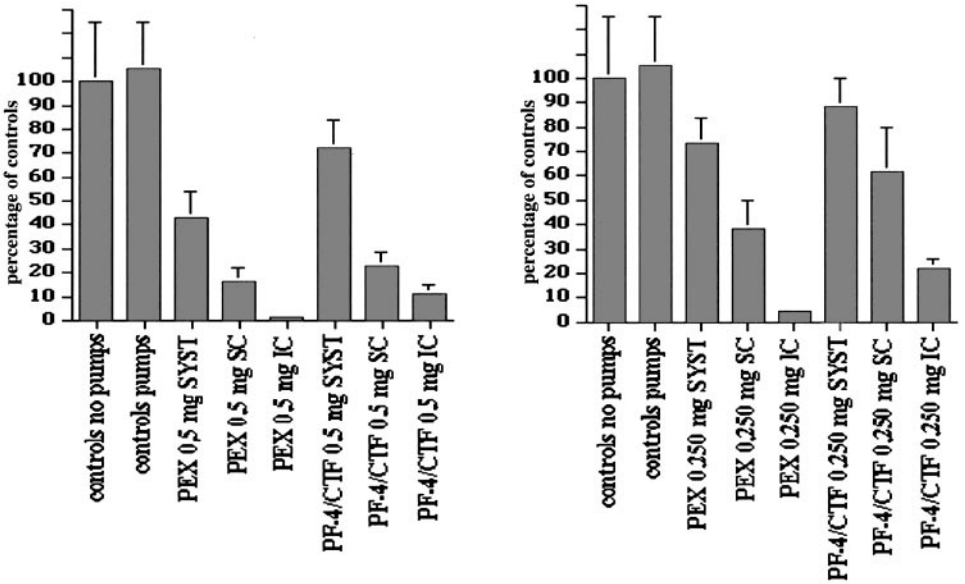

Fig. 3. Comparison of the efficacy of local intracerebral delivery, systemic continuous delivery by s.c. minipumps, and systemic daily i.p. delivery of human PEX or PF-4/CTF. Eleven days after tumor cell injection, animals were implanted with osmotic minipumps connected to an intracranial catheter, or with s.c. minipumps, or they received daily i.p. injection of the inhibitor or PBS. Human PEX or PF-4/CTF were given at the concentrations of 0.25 and 0.5 mg/kg/day. Twenty-eight days later animals were sacrificed and the tumor volumes measured. Local intracerebral delivery by osmotic minipumps afforded the highest inhibition at both concentrations. Data are expressed as percentage of controls. *SYST*, daily i.p. injection; *SC*, continuous systemic administration by s.c. minipumps; *IC*, continuous local intracerebral administration by osmotic minipumps connected to an intracranial catheter. Data are presented as a percentage of controls; *bars*, ±SD. *Controls no pumps*, animals in which no pumps were implanted after tumor cell injection. *Controls pumps*, animals in which pumps containing PBS were implanted 11 days after tumor cell injection. The total amount of human PEX or PF-4/CTF contained in the pump reservoir is reported.

Local Intracranial Administration of Human PEX and PF-4/CTF Sustains a Long-Term Control of Glioma Growth *in Vivo*. To evaluate the capacity of human PEX or PF-4/CTF delivered intracerebrally to sustain a long-term control of glioma growth, 11 days after tumor cell injection, animals were implanted with osmotic minipumps connected to an intracranial catheter. The pump reservoir was filled with 0.5 mg (total amount) of human PEX, PF-4/CTF, or PBS. The pump reservoir was replaced three times, to afford a 94-day period of treatment. Animals belonging to the control group (PBS) had a 50% survival of 28 days. Local delivery of PEX or PF-4/CTF maintained a long-term inhibition of glioma growth. Eighty-one percent of the animals treated with PEX were alive after 105 days from tumor cell implantation. Histological analysis of the brains of these animals showed small tumors, with a mean volume of 2.3 ± 1.5 mm^3, corresponding to a 85% inhibition of tumor growth. Fifty-nine percent of the animals treated with PF-4/CTF were alive at 105 days from tumor cell injection. The mean tumor volumes of these animals were 4.5 ± 2 mm^3, corresponding to a 72% inhibition of tumor growth (Fig. 4, *A* and *B*).

Animals were carefully examined for the occurrence of any distress or side effects, related both to the treatment or the system of delivery. Both were well tolerated, without any side effects.

Local Intracerebral Delivery of Human PEX and PF-4/CTF Suppressed Glioma Recurrence in the Glioma Surgical Resection Model. In this group of experiments we studied the ability of human PEX or PF-4/CTF delivered locally into the brain of the animals to delay the appearance of glioma recurrence in the glioma surgical resection model. Twenty days after tumor cell implantation, the tumor was removed by a microsurgical technique, followed the day after surgery by the implantation of the osmotic minipumps connected to a catheter positioned slightly posteriorly to the surgical cavity. The pump reservoirs were filled with 0.25 and 0.5 mg (total amount) of human PEX or PF-4/CTF delivered for 28 days. After that period, the animals were sacrificed, their brains removed, and the tumor volumes measured. A dose-dependent inhibition of glioma recurrence was observed. The local delivery of 0.25 and 0.5 mg/kg/day of human PEX resulted in a 85% and 75% inhibition of the growth of the recurrent tumors. The local administration of the same amount of PF-4/CTF produced a 77% and a 66% decrease in the volume of the recurrent tumors (Fig. 4*C*).

Immunohistochemical Analysis. Local intracerebral treatment with endogenous inhibitors was associated with a decrease in microvessel density, a change in tumor vasculature, an increase in apoptosis rate, and in the tumors treated with human PEX, also a decrease in proliferation rate. In treated tumors, vasculature formed a regular network of small vessels, composed by a unilayer of endothelial cells. On the contrary, in tumors from animals belonging to the control groups, vessels formed a very complex network, characterized by large vessels and glomeruloid structures.

The higher therapeutic efficacy of the local intracerebral delivery *versus* the continuous systemic and daily systemic delivery was confirmed by the analysis in the tumors belonging to these groups of treatment of microvessel density, apoptosis, and proliferation indices. Specifically, at the same concentration, tumors from animals to which human PEX or PF-4/CTF were delivered locally intracerebrally by osmotic minipumps showed the lowest microvessel counts, the highest apoptotic rate, and for those treated with human PEX also the lowest proliferation rate. Tumors from animals treated with PF-4/CTF had no change in proliferation rate independently from the modality of delivery that was used (Fig. 5). Tumors from animals in which the inhibitor was delivered by s.c. minipumps showed intermediate values, whereas the highest microvessel counts and proliferation rates, and the lowest apoptosis indices were observed in tumors belonging to the control groups.

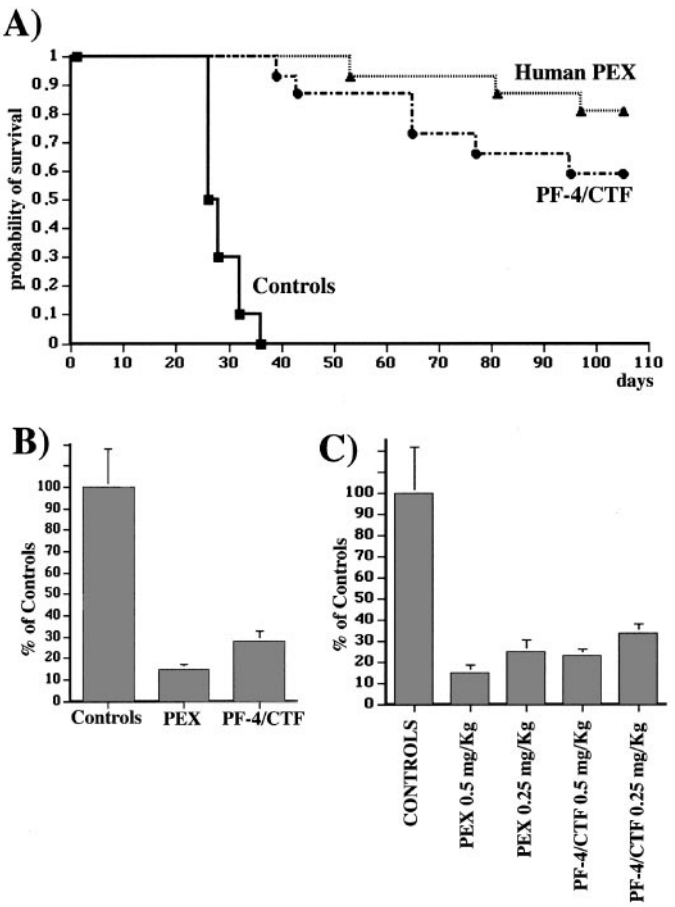

Fig. 4. *A*, local intracerebral delivery of human PEX or PF-4/CTF maintained a long-term control of glioma growth. Eleven days after tumor cell injection, animals were implanted with osmotic minipumps connected to an intracranial catheter, of which the reservoir was filled with 0.5 mg/kg/day of human PEX or PF-4/CTF or PBS. The reservoir of the pumps was replaced twice to afford 94 days of treatment, then the animals were sacrificed. At 105 days from tumor cell implantation, 81% of animals treated locally with human PEX were alive *versus* 59% of those treated locally with PF-4/CTF. *B*, at 105 days these animals were sacrificed, the brains removed, and the tumor volumes measured. Locally administered human PEX or PF-4/CTF produced a potent long-term inhibition of tumor growth. Data are presented as a percentage of controls; *bars*, ±SD. *C*, local intracerebral delivery of human PEX or PF-4/CTF reduced the volume of glioma recurrence. Twenty days after tumor cell implantation, animals were submitted to surgery for removal of the xenografts. The day after surgery, animals were implanted with osmotic minipumps connected to an intracranial catheter, of which the reservoir was filled with 0.25 or 0.5 mg/kg/day of human PEX or PF-4/CTF or with PBS (*controls*). Twenty-eight days later the animals were sacrificed, and the volumes of the recurrent tumors measured and compared. Data are presented as a percentage of controls; *bars*, ±SD.

Analysis of recurrent tumors from treated *versus* control animals showed that tumors from animals treated with human PEX or PF-4/CTF were small, round in shape, and had well-delineated margins.

DISCUSSION

Treatment of aggressive tumors such as human malignant gliomas requires prolonged therapy with repeated protein administration (8, 18, 19). In addition, the highest therapeutic response in most of the cases is reached when systemically administered endogenous inhibitors are given daily (4, 6–8). Therefore, the systemic administration requires the production of a large amount of protein that is a very laborious and expensive procedure. Daily administration is associated with poor patient compliance (4).

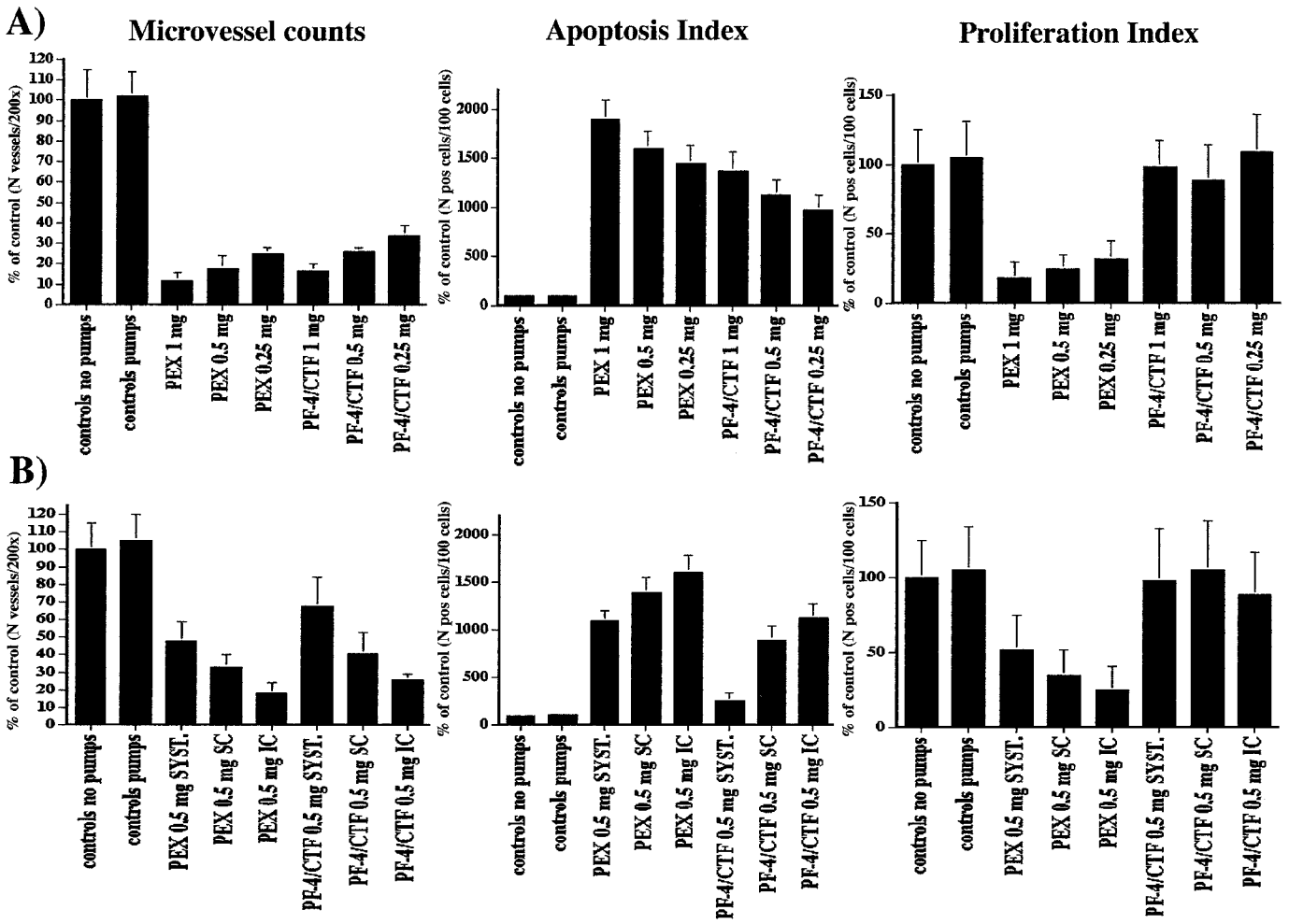

Fig. 5. *A*, microvessel counts, apoptosis, and proliferation indices measured in tumors from nude mice implanted with osmotic minipumps connected with an intracranial catheter, of which reservoirs were filled with different concentrations of human PEX or PF-4/CTF, or PBS. Pumps were implanted 11 days after tumor cell injection. Treatment continued for 28 days. Data are presented as a percentage of controls; *bars*, ±SD. *B*, comparison of microvessel counts, apoptosis, and proliferation indices measured in tumors from animals submitted to systemic i.p. daily delivery (*PEX* or *PF-4/CTF SYST.*), to systemic continuous delivery (*PEX* or *PF-4/CTF SC*), or to local intracerebral delivery (*PEX* or *PF-4/CTF IC*) of 0.5 mg/kg human PEX or PF-4/CTF. Pumps were implanted 11 days after tumor cell injection. i.p. injection was started after the same interval of time. Each treatment continued for 28 days. Data are presented as a percentage of controls; *bars*, ±SD. *Controls no pumps*, animals in which no pumps were implanted after tumor cell injection. *Controls pumps*, animals in which pumps containing PBS were implanted 11 days after tumor cell injection. The total amount of human PEX or PF-4/CTF contained in the pump reservoir is reported.

In this work we demonstrated that local intracerebral administration of endogenous inhibitors by osmotic minipumps: (*a*) reduced the amount of protein needed to reach a significant therapeutic response; (*b*) was more effective than the continuous systemic delivery; (*c*) was able to sustain a long-term control of tumor growth in the absence of toxicity or side effects; and (*d*) efficiently inhibited glioma recurrence.

We have used human PEX and PF-4/CTF for these studies because both factors have a known mechanism of action and are active *in vitro* and *in vivo* (19, 21, 27, 28). Furthermore both proteins inhibit potently glioma growth when given systemically and are able to inhibit tumor recurrence (18–22).

Osmotic minipumps could be easily and successfully used to deliver proteins or other molecules into the brain (23, 24, 29). It has been shown previously that the systemic continuous administration of endogenous inhibitors by minipumps reduces the amount of protein needed to reach a tumor growth inhibition (5). Studies performed on large animals demonstrated that the molecule released by the pumps entirely distributed into the hemisphere where the pump was implanted (24). A previous study showed the higher efficacy on tumor growth of systemic continuous administration of endogenous inhibitors by s.c. osmotic minipumps *versus* daily systemic administration by i.p. injection (5).

In the present work, we tested the ability of osmotic minipumps to deliver biologically active inhibitors *in vitro*. We demonstrated that human PEX and PF-4/CTF released by osmotic minipumps were able to inhibit tube formation or endothelial cell proliferation even after a prolonged interval of time from when the reservoir was filled. In our experiments, intracerebral delivery of human PEX or PF-4/CTF was an effective modality of treatment for gliomas. A significant tumor inhibition was reached with a considerable low amount of protein, and in both nude mice and syngeneic glioma models. In both models, pumps were implanted 11 days after tumor cell injection, to start the treatment when the tumors were well established, and with their own vascularization. In addition, local intracerebral delivery was also effective in delaying the appearance of recurrence in the glioma surgical resection model in nude mice. All of the recurrent tumors from treated animals showed small volumes indicating an effective suppression of the growth of the recurrent tumors. The comparison of the therapeutic response reached with local intracerebral delivery and continuous or daily systemic administration demonstrated that the intracerebral delivery afforded the highest gliomas growth inhibition with the lowest amount of protein. Moreover, local intracerebral delivery maintained a long-term control of tumor growth. Treatment was always very well tolerated without the occurrence of any side

effects. Taken together, these data indicate that local intracerebral delivery by osmotic minipumps is a very effective modality for the treatment of gliomas, and it inhibits glioma growth also for a prolonged period of time. In addition, a significant therapeutic response is reached with a very low amount of protein and in absence of any distress or side effects.

The higher therapeutic efficacy of the local intracerebral delivery is probably related to the fact that the inhibitor is more easily available at the tumor site and at the same time less susceptible to the degradative action of the proteases. The activity of endogenous proteases significantly limits the effect of the systemic modality of administration even when the inhibitor is delivered continuously by both s.c. or i.p. devices (4, 23, 24). In addition, local delivery of molecule to the brain overcomes many of the limitations associated to the presence of the blood-brain barrier (24). When an osmotic minipump is chosen as a device, the intracranial catheter should be placed in the same hemisphere where the tumor is located. Data from the literature on convection-enhanced deliveries, as well as our experience with intracranial osmotic minipumps and gliomas, show that when the catheter is positioned in the contralateral hemisphere, the efficacy reached by this system of delivery is very limited (20, 24, 29).

The higher efficacy of the local intracerebral delivery is confirmed by the histological and immunohistochemical analysis of tumors. At the same concentrations, local intracerebral delivery is accompanied by the highest decrease and change in tumor vascularity, increase in apoptosis, and when human PEX is used as an inhibitor, by a decrease in tumor cell proliferation. In accordance with previous observations, human PEX produced a more pronounced inhibition of glioma growth than PF-4/CTF when used at the same concentrations (20). Human PEX is a simultaneous inhibitor of angiogenesis, and of tumor and endothelial cell proliferation and migration (18, 19). On the contrary, PF-4/CTF inhibits angiogenesis and endothelial cell proliferation but has no effect on tumor cell (21, 22). Analysis of recurrent tumors from treated *versus* control animals showed that treated tumors were small, round in shape, and with well-delineated margins. In accordance with previous observations, the effect on invasion was more pronounced in the tumors from animals treated with human PEX (20).

In conclusion, our data demonstrated that local intracerebral delivery of endogenous inhibitors by osmotic minipumps is a very effective modality for the treatment of aggressive tumors such as malignant gliomas. Local intracerebral delivery is more effective than the systemic continuous system and is also able to control tumor growth for a prolonged period of time.

ACKNOWLEDGMENTS

We thank Rita Paglino and Antonio Ladislao, for technical assistance, Cinzia Marcias for surgical assistance, and Daniela Santoni for animal care.

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Cancer Res 2003;63:2499-2505.