Combinatorial Administration of Molecules That Simultaneously Inhibit Angiogenesis and Invasion Leads to Increased Therapeutic Efficacy in Mouse Models of Malignant Glioma

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ABSTRACT

Purpose: **We investigated the ability of the combinatorial administration of different inhibitors with activities on glioma angiogenesis, migration, and proliferation to produce a prolonged inhibition of glioma growth.**

Experimental Design: **We combined inhibitors affecting solely tumor angiogenesis (PF-4/CTF, cyclo-VEGI) or inhibitors affecting both angiogenesis and invasion together (PEX, PF-4/DLR).**

Results: **When administered in combination, these drugs produced a prolonged and increased inhibition of glioma growth independently from the type of inhibitor used. The combinatory administration was more effective than the administration of a single inhibitor alone, and a strong therapeutic response was reached with a significantly lower amount of protein. The strongest inhibition was observed when human PEX and PF-4/DLR, which affect both glioma angiogenesis and invasion by separate mechanisms, were combined.**

Conclusions: **This supports the concept that prolonged glioma growth inhibition can be achieved by simultaneous delivery of molecules that target both tumor and endothelial cells and acting by separate mechanisms.**

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Note: L. Bello and V. Lucini contributed equally to this work.

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INTRODUCTION

The growth of malignant gliomas such as that of others solid tumors depends on the balance between positive and negative regulators of cell proliferation, migration, or angiogenesis (1–4). Negative regulators are directly produced by the tumor itself or depend on the activity of the tumor cells on the host microenvironment (1–6). Some of them are acting as specific regulators of angiogenesis. Others have more complex functions and may also affect invasion and/or cell proliferation (5–7).

We and others (8–13) have shown that the systemic administration of negative regulators successfully inhibited the growth of malignant gliomas and glioma recurrence in various animal models. The treatment was always very well tolerated and not associated with the occurrence of any side effects. Inhibitors have been also administered in combination with metronomic chemotherapy optimizing and prolonging the extent of the therapeutic response (14).

It has been observed that after a period of treatment with antiangiogenic agents, the treated tumor eventually relapses and may acquire resistance to the treatment (15, 16). In the presence of a redundancy of stimulating factors, treatments that target only a specific factor may lead to variants that privilege alternative growth pathways (15). In addition, it is possible that during long-term antiangiogenic treatment, a selection of subpopulations of less vascular-dependent tumor cells, characterized by an increased capacity to survive in a nutrient- and oxygen-deprived condition, may occur (15, 16). Moreover, there is evidence that hypoxia provides a selection mechanism for cells with a diminished susceptibility to apoptosis (17). Furthermore, it has been recently showed that hypoxia activates a cell motility program that helps the cells to escape the hostile hypoxic environment by invading the adjacent tissues where oxygen and nutrients are not limited, facilitating the dissemination of tumor cells in the surrounding tissue (17). It has also been reported that blocking angiogenesis with antibodies directed against the vascular endothelial growth factor receptor-2 in experimental glioma mouse models may paradoxically increase invasion, and thus, simultaneously blocking angiogenesis and invasion may contribute beneficially to tumor therapy (12, 17, 18).

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Cancer cells possess a broad spectrum of migration and invasion mechanisms (19). They can also modify their migration mechanisms in response to different conditions. This form of adaptation occurs naturally during the course of tumor progression and may also occur during treatment (19). Thus, an efficient anti-invasive therapy needs to target more than one mechanism at the same time.

These data have important therapeutic implications and suggest that combinatory strategies, which inhibit simultaneously different mechanisms of tumor invasion and angiogenesis, may significantly increase therapeutic efficacy.

We investigated herein whether the combinatorial administration of inhibitor molecules significantly increased inhibition of glioma growth. We provide evidence that maximum inhibitory activity is observed when molecules are associated that both exhibit antiangiogenesis and anti-invasive properties.

MATERIALS AND METHODS

Production of Recombinant Human PEX, PF-4/CTF, PF-4/DLR, and Cyclo-VEGI

PEX RNA was amplified from U87 glioblastoma cells as described previously (14). The fragment was cloned into the pRSET vector (Invitrogen, Carlsbad, CA) and transformed in BL21 bacteria. Transformed BL21 bacteria were grown in Luria-Bertani media followed by induction with 1 mM isopropylthiolgalactoside. PEX was purified under denaturing conditions followed by extraction by nickel-charged chelating agarose. Recombinant protein was refolded and dialyzed 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 COOH-terminal peptide of human PF-4-, PF-4/CTF-, and the PF-4/CTF-modified peptide PF-4/DLR 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 H_2O and stored at −20°C before use (11, 20, 21).

Cyclo-VEGI was synthesized by Fmoc/tBu batch solid-phase synthesis on an Applied Biosystems 430°C automated peptide synthesizer as described previously (22).

Purity of the preparation was confirmed by running the molecules on a 12% SDS-PAGE gel followed by Western blot analysis (10, 11, 20–22). The biological activity of human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI was tested *in vitro* using angiogenic and proliferation assays as reported previously (10, 11, 20–22).

Cell Cultures and Other Reagents

The human glioma cell line U87-MG (American Type Culture Collection, Manassas, VA) and the murine glioma GL261 cell line (kindly provided by Dr. David Zagzag, Department of Pathology, New York University) were used in the animal experiments. U87-MG cells were cultured in α-MEM. GL261 were grown in DMEM. Both media were supplemented with 2 mM L-glutamine, 10% FBS, and 1000 units/ml penicillin/streptomycin solution.

Two endothelial cell lines were used. Porcine aortic endothelial cells stably transfected with KDR (PAE/KDR) were cultured in Ham's F-12 media with 10% nonheat-inactivated FCS and 10 μg/ml Geneticin (G418 sulfate; Refs. 17, 18). Bovine capillary endothelial (American Type Culture Collection) cells were cultured in DMEM plus L-glutamine and 10% FBS. All media were supplemented with 1000 units/ml penicillin/streptomycin solution, and the cells were cultured in a 5% CO_2 incubator at 37°C. Endothelial cell lines were used in the angiogenic and proliferation assays.

Proliferation and Apoptosis Assays

These assays were used to test the biological activity of human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI. Bovine capillary endothelial, PAE/KDR, U87, or GL261 cells were plated on a 96-well plate (20,000 cells for each well) and cultured in the presence of increasing concentrations of human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI (0.1–25 μg/ml), alone or in combination, 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.

Apoptotic cells were detected with ApopTag plus kit (Intergen, New York, NY) with 1% methyl green as a counterstain. Apoptosis index was quantified by determining the percentage of positively stained cells for all nuclei in 20 randomly chosen fields/section at ×200 magnification (8, 10, 11, 20, 21).

Tube Formation Assay

In vitro tube formation assays were performed to test the antiangiogenic activity of the inhibitors, alone or in combination (10, 11, 23, 24). PAE/KDR cells were used (10, 11, 24). PAE/KDR cells were seeded in a 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 media; and condition 3, cells incubated with U87 conditioned media plus PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI at different concentrations (1, 5, 10, and 20 μg/ml), alone or in combination. Microvessel counts were performed on microplates and scored as described previously (10, 11, 25).

Migration Studies: Matrigel and Wound Assays

Two different types of migration assays were used: a wound assay and a Matrigel invasion assay.

The wound assay was performed according to a method described earlier (11, 22). Endothelial or tumor cells were seeded in 35-mm culture plates and were allowed to grow to confluence. Complete medium was replaced with serum-free media, and incubation was continued overnight. One linear scar was drawn in the monolayer and divided into five equal fields. A set of digital photos were taken of each scar, and the denuded area was marked using digital image analysis software. The dishes were washed, and fresh medium containing 0.1% BSA, 10 ng/ml fibroblast growth factor (FGF)-2 (bovine capillary endothelial and tumor cell migration), or 10 ng/ml $VEGF_{165}$

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(PAE/KDR migration) and peptides, at increasing concentrations (1, 5, 10, and 20 μg/ml), alone or in combination, was added. After 6, 12, and 24 h, a second and additional sets of photos were taken. Photos were superimposed, and cells that migrated across the line drawn at the border of the scar in the first photo set were counted. Each condition was tested in duplicates in three independent experiments. Means for all fields of each group were calculated, background migration subtracted, and plotted as percentage of the mean of untreated stimulated control.

For the Matrigel invasion assay, chamber Permanox slides (Miles Scientific, Naperville, IL) were coated with Matrigel and incubated with media containing 10% FBS (8). A sterile sedimentation cylinder was placed into the lumen of the cylinder and allowed to attach. The cylinder was removed, and the endothelial or glioma 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. Human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI were added alone or in combination to the cultured medium, at increasing concentrations (1, 5, 10, and 20 μg/ml), and the medium was changed daily. An irrelevant substance (BSA) was added at the same concentration and used as a negative control. Migration was quantified in comparison to unstimulated controls.

Statistical analysis was performed by ANOVA followed by Student's Newman-Keul pairwise comparison (Statview for Macintosh).

Animal Studies

Short-Term Combinations Experiments. In this set of experiments, groups of 10 6-week-old male nude or BALB/c mice were implanted with 50,000 U87 or GL261 glioblastoma cells. Twelve days after tumor cell injection, animals were implanted with Alzet 2004 osmotic minipumps, which the reservoir was filled with human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI alone or in combination, at different concentrations, according to the following groups: group 1, 0.5 mg of human PEX + 0.5 mg of PF-4/DLR; group 2, 0.5 mg of human PEX + 0.5 mg of PF-4/CTF; group 3, 0.5 mg of PF-4/CTF + 0.5 mg of cyclo-VEGI; group 4, 0.5 mg of human PEX; group 5, 0.5 mg of PF-4/DLR; group 6, 0.5 mg of PF-4/CTF; group 7, 0.5 mg of cyclo-VEGI; group 8, controls; group 1, 0.25 mg of human PEX + 0.25 mg of PF-4/DLR; group 2, 0.25 mg of human PEX + 0.25 mg of PF-4/CTF; group 3, 0.25 mg of PF-4/CTF + 0.25 mg of cyclo-VEGI; group 4, 0.25 mg of human PEX; group 5, 0.25 mg of PF-4/DLR; group 6, 0.25 mg of PF-4/CTF; group 7, 0.25 mg of cyclo-VEGI; group 8, controls; and group 1, 0.5 mg of human PEX + 0.5 mg of PF-4/DLR; group 2, 0.5 mg of human PEX + 0.5 mg of PF-4/CTF; group 3, 0.5 mg of PF-4/CTF + 0.5 mg of cyclo-VEGI; group 4, 0.25 mg of human PEX + 0.25 mg of PF-4/DLR; group 5, 0.25 mg of human PEX + 0.25 mg of PF-4/CTF; group 6, 0.25 mg of PF-4/CTF + 0.25 mg of cyclo-VEGI; group 7: controls.

In each experiment, treatment was continued for 28 days, corresponding to the working period of the pumps. Animals were sacrificed at the occurrence of any side effect or neurological distress and, in any case, 29 days after pump implantation. At sacrifice, brains were removed, 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 ornithine carbamyl transferase, 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 ± SE. 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. Statistical analysis of tumor volumes was performed with a two-way ANOVA. Pairwise comparison between treatment groups at each dose was performed by the Newman-Keuls posttest.

Long-Term Combinations Experiments. In this set of experiments, groups of 10 6-week-old male nude or BALB/c mice were implanted with 50,000 U87 or GL261 glioblastoma cells. Twelve days after tumor cell injection, animals were implanted with Alzet 2004 osmotic minipumps, which the reservoir was filled with human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI, alone or in combination, at the following concentrations: 0.25, 0.5, and 1 mg/kg/day. The pump reservoirs were changed four times to afford a treatment period of 140 days. Animals were sacrificed at the occurrence of any side effects or neurological distress and, in any case, 152 days after tumor cell implantation.

At sacrifice, the brains were removed and processed as described previously. Kaplan-Meier survival curves were designed, and survival for treated and untreated mice were compared with log-rank test.

All animal experiments were repeated at least two times.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on 5- and 100-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 (8–10, 14). Microvessel count and density were scored as reported previously (8–10, 14, 25). Apoptotic cells were detected with ApopTag plus kit (Intergen) with 1% methyl green as a counterstain. Apoptosis and proliferative indices were quantified by determining the percentage of positively stained cells for all nuclei in 20 randomly chosen fields/section at ×200 magnification (8–10, 14, 22). One-hundred-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, Temecula, CA) was used as a secondary antibody. Analysis was performed by co focal microscope (Zeiss), followed by three-dimensional reconstruction (10). Serial H&E slides were investigated for the pattern of glioma cell invasion. The interface between tumor mass and normal brain parenchyma was carefully examined for the pres-

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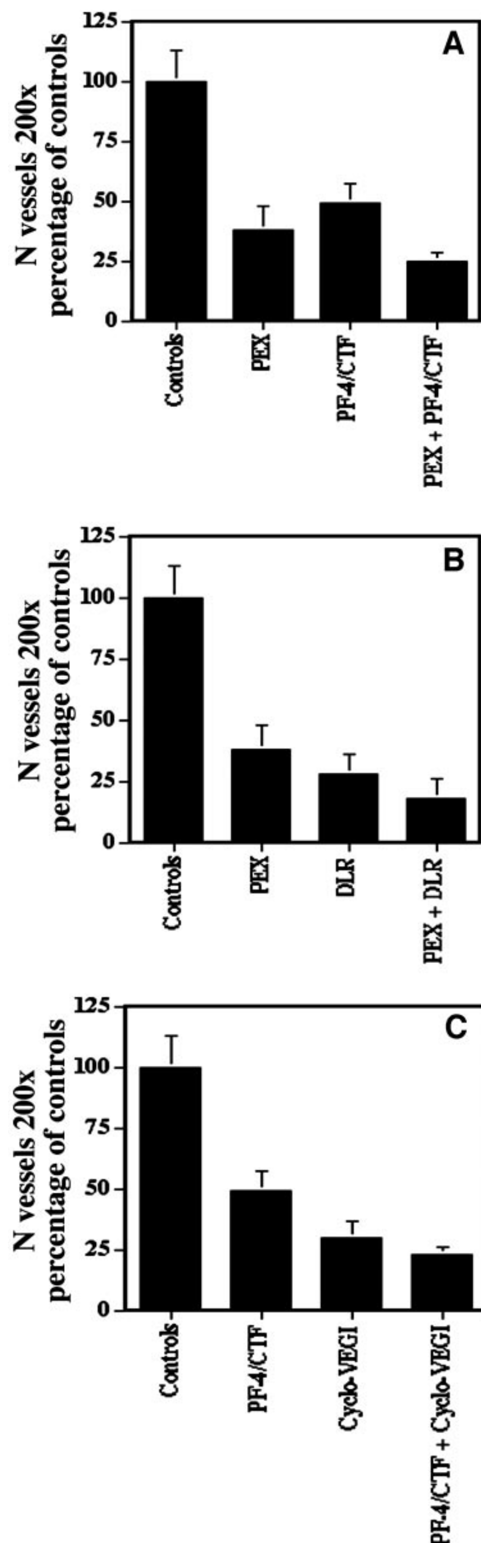
ence of signs of local infiltration consisting of trails of tumor cells invading the normal brain parenchyma and tumor satellites distant from the main tumor mass (9). Signs of tumor infiltration were scored as described previously (12).



RESULTS

Biological Activities of Each Inhibitor. We combined in this study four different inhibitors, which include PF-4/CTF, cyclo-VEGI, human PEX, and PF-4/DLR. We have chosen two compounds that only inhibit angiogenesis and two compounds that simultaneously inhibit angiogenesis and invasion. PF-4/CTF inhibits only endothelial cell proliferation, migration, and angiogenesis (Fig. 1; Refs. 11, 21 and data not shown). Glioma cell migration in the wound and Matrigel assays is not inhibited by PF-4/CTF (Fig. 2 and data not shown). Cyclo-VEGI only inhibits angiogenesis as well (Fig. 1; Ref. 22 and data not shown). Human PEX inhibits simultaneously glioma cell proliferation, migration, and angiogenesis (Figs. 1 and 2; Ref. 8). Similarly, PF-4/DLR also targets endothelial and tumor cells (Figs. 1 and 2; Ref. 11 and data not shown). PF-4/DLR not only inhibits angiogenesis but also impairs potently glioma cell migration in the wound assay, invasion in the Matrigel assay, apoptosis *in vitro*, and glioma cell proliferation to a smaller extent (Figs. 1 and 3 and data not shown). Although human PEX and PF-4/DLR are both inhibiting angiogenesis and tumor cell invasion, they have different mechanisms of action (see "Discussion").

Effect of the Combinatorial Administration of Human PEX, PF-4/DLR, PF-4/CTF, and Cyclo-VEGI on Glioma Angiogenesis *in Vitro*. The effect of the combinatorial administration of human PEX, PF-4/DLR, PF-4/CTF, and cyclo-VEGI on tumor angiogenesis *in vitro* was investigated by performing *in vitro* angiogenic assays. In these experiments, we evaluated the ability of endothelial cells to form tubes when grown in the presence of media from glioblastoma cells and exposed to different concentrations of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI, administered alone or in combination. The combinatorial administration of endogenous inhibitors resulted in all of the cases in an increase in inhibition of tube formation. This effect was particularly evident when the inhibitors were used at low concentration and when human PEX (1 μg/ml, 40 nM) and PF-4/DLR (1 μg/ml, 370 nM) were combined (Fig. 1).

Fig. 1 Effect of the combinatorial administration of inhibitors on angiogenesis *in vitro*. The combinatorial administration of human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI produced an increase in the inhibition of angiogenesis *in vitro*. PAE/KDR endothelial cells seeded on a collagen gel were grown in the presence of U87 glioblastoma cell cultured media and exposed to 1 μg/ml human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI, supplemented alone or in combination. Media were replaced every day for 4 days. When grown in the presence of glioblastoma-cultured media, PAE/KDR cells formed tubes after 6 h. When the media were supplemented with the inhibitors, given alone or in combination, a decrease in the tube formation activity was observed. When given in combination, a significant increase in the inhibition of tube formation was observed in comparison when the inhibitors were administered alone (human PEX + PF-4/CTF *versus* human PEX or PF-4/CTF = $P < 0.01$ or $P < 0.001$; human PEX + PF-4/DLR *versus* human PEX or PF-4/DLR = $P < 0.001$ or $P < 0.05$; PF-4/CTF + cyclo-VEGI *versus* PF-4/CTF or cyclo-VEGI = $P < 0.001$ or $P < 0.05$). The number of vessels in each condition was quantified as previously described (9, 24) and expressed as a percentage of the controls ± SE. The data are representative for six experiments done in triplicates.

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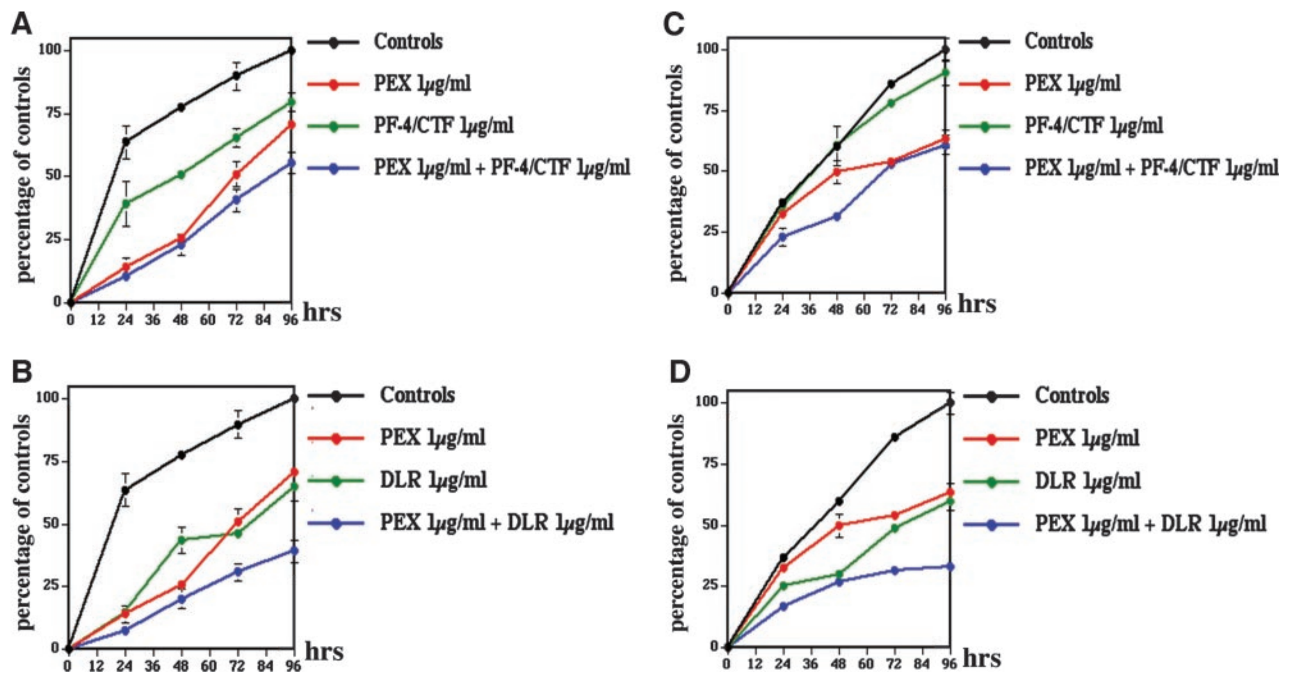

Fig. 2 Effect of combinatorial administration of inhibitors on endothelial and glioma cell migration in the Matrigel invasion assay. PAE/KDR (*A* and *B*) and U87 (*C* and *D*) cells were seeded in a Matrigel gel and grew in the presence of 1 μg/ml human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI, given alone or in combination. The migration of the cells from the initial radius was measured after 24, 48, and 72 h and compared with the controls. The combinatorial administration of the inhibitors increased the inhibition of endothelial cell migration (*A* and *B*; human PEX + PF-4/CTF, $P = 0.05$; human PEX + PF-4/CTF, $P < 0.001$). The combinatorial administration of human PEX and PF-4/CTF did not increase the antimigratory effect on glioma cell, which was similar to that exerted by human PEX given alone (*C*). On the contrary, the combinatorial administration of human PEX and PF-4/DLR produced an increase in the inhibition of glioma cell migration (*D*; $P < 0.0001$). The data are representative for six experiments done in triplicates and are expressed as a percentage of controls ± SE.

Effect of the Combinatorial Administration of Human PEX, PF-4/DLR, PF-4/CTF, and Cyclo-VEGI on Glioma and Endothelial Cell Invasion and Migration. The effect of the combinatorial administration of the inhibitors on glioma and endothelial cells invasion and migration was investigated by performing Matrigel invasion and monolayer wound assays. In the Matrigel invasion assay, we evaluated the effect of different concentrations of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI, used alone or in combination, on tumor and endothelial cells invasion inside a Matrigel gel. In this experiment, the combinatorial administration of different concentrations of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI produced an increase in the inhibition of endothelial cell migration in comparison when the inhibitors were used alone (Fig. 2, *A* and *B*, and data not shown). The effect was more prominent when human PEX and PF-4/DLR were combined and particularly evident when the inhibitors were used at low concentration (40 nM for human PEX and 370 nM for PF-4/DLR). The inhibitory effect on glioma cell migration was dependent on the type of the inhibitor used. The combinatorial administration of human PEX and PF-4/CTF did not significantly increase the antimigratory effect, which was similar to that observed when human PEX was administered alone (Fig. 2*C*). On the contrary, when human PEX and PF-4/DLR were combined, a significant increase in the antimigratory activity was documented, particularly when the inhibitors were used at low concentration (1 μg/ml; 40 nM for human PEX and 370 nM for PF-4/CTF; Fig. 2*D*). Similar results were observed when a monolayer wound assay was performed (data not shown).

Effect of the Combinatorial Administration of Human PEX, PF-4/DLR, PF-4/CTF, and Cyclo-VEGI on Endothelial and Glioma Cell Apoptosis and Proliferation *in Vitro*. To investigate the effect of the combinatorial administration on endothelial and glioma cell apoptosis, endothelial and glioma cells were exposed to increasing concentrations of the inhibitors, alone or in combination, for 24 h. The presence of apoptotic cells was then determined by terminal deoxynucleotidyl transferase-mediated nick end labeling staining. The combinatorial administration of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI was associated with a significant increase in endothelial cell apoptosis. The effect was particularly evident when the inhibitors were used at low concentration and human PEX and PF-4/DLR were combined (Fig. 3*A* and data not shown). On the contrary, the combination of PF-4/CTF and cyclo-VEGI did not exert any effect on glioma cell apoptosis (data not shown). Similarly, the combination of human PEX and PF-4/CTF did not result in a significant increase in the apoptotic rate of glioma cells, which was similar to that measured when human PEX was used alone (data not shown). On the contrary, the apoptotic rate of

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glioma cells was significantly increased when human PEX and PF-4/DLR were supplemented in combination (Fig. 3*B*).

We also performed proliferation assays with the different inhibitors administered alone or in combination. In all of the cases, a dose-dependent inhibition of endothelial cells proliferation was observed (data not shown). Under similar experimental conditions, an increase in the inhibition of glioma cell proliferation was only documented when human PEX and PF-4/DLR were combined. The combination of human PEX and PF-4/CTF did not significantly reduce glioma cell proliferation, and the inhibition was similar to that observed when human PEX was used alone (data not shown).

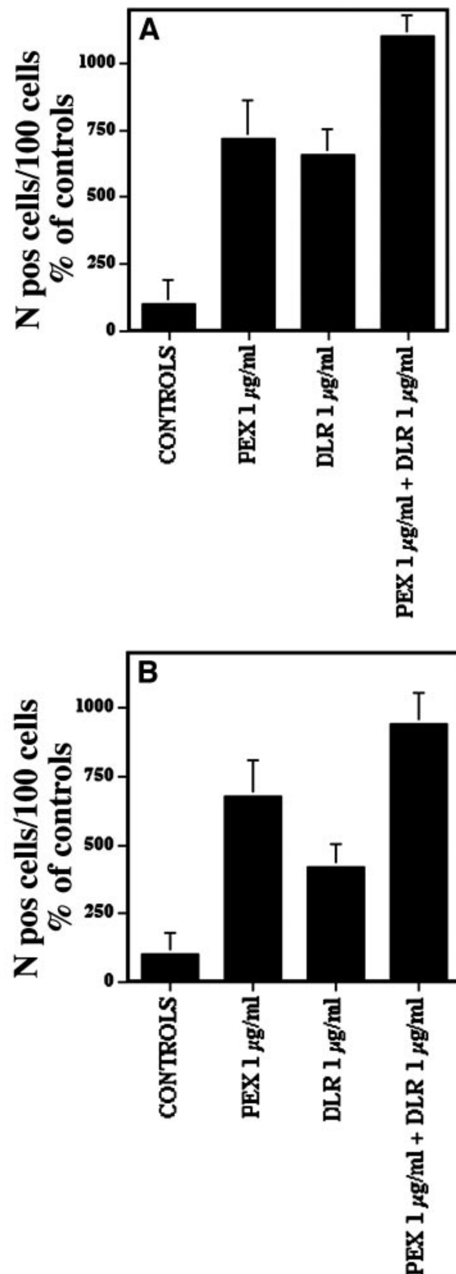

Fig. 3 Effect of the combinatorial administration of human PEX and PF-4/DLR on endothelial and glioma cell apoptosis. PAE/KDR (*A*) and U87 (*B*) cells were grown in the presence of 1 μg/ml of the inhibitors, given alone or in combination, for 24 h. The number of apoptotic cells was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling assay, counted as number of positive cells on 100 nuclei, and expressed as a percentage of controls ± SE. The combinatorial administration of human PEX and DLR increased both PAE/KDR and U87 cells apoptosis ($P < 0.001$ and $P < 0.001$). Data are representative for six experiments done in triplicates.

The Combinatorial Administration of Human PEX, PF-4/DLR, PF-4/CTF, and Cyclo-VEGI in Nude and Syngeneic Mice Glioma Models Reduces Glioma Growth *in Vivo*. The effect of the combinatorial administration of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI on glioma growth *in vivo* was initially investigated by performing short-term studies. In these studies, the inhibitors were administered alone or in combination, continuously and systemically, by the use of osmotic minipumps implanted s.c. in the right flank of the animals. The pumps were implanted 12 days after tumor cell injection. Animals were sacrificed after 29 days from the implantation of the pumps, at the end of their working period. After the sacrifice, the brains were removed, fixed, and sectioned, and the tumor volumes were calculated. The combinatorial administration of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI resulted in a potent inhibition of glioma growth. The highest inhibition was observed when human PEX and PF-4/DLR were combined (98% tumor volume inhibition at 0.5 mg/kg/day) and the lowest when PF-4/CTF and cyclo-VEGI were administered together (93.5% tumor volume inhibition at 0.5 mg/kg/day; Fig. 4*A*). The combination was more effective than the administration of a single inhibitor alone. No significant difference in activity was observed when the experiments were performed in nude or immunocompetent BALB/c mice (Fig. 4*B*).

The Combinatorial Administration of Human PEX, PF-4/DLR, PF-4/CTF, and Cyclo-VEGI Sustains a Prolonged Inhibition of Glioma Growth *in Vivo*. We next studied the ability of the combinatorial administration of inhibitors to sustain a prolonged inhibition of glioma growth in long-term *in vivo* experiments. In addition, we identified which combination afforded the longest therapeutic response.

The inhibitors were administered at different concentrations (0.25, 0.5, and 1 mg/kg/day), alone or in combination, by s.c. osmotic minipumps, starting 12 days after tumor cells injection. The pump reservoir was replaced four times over a 140-day period. Animals were sacrificed at the occurrence of any signs of distress or neurological deficits.

The combinatorial administration of human PEX, PF-4/DLR, PF-4/CTF, or cyclo-VEGI resulted in a prolonged inhibition of glioma growth in all of the cases (Fig. 5*A*). The effect was dose dependent and stronger than that observed when a single inhibitor was administered alone, independently from the type of inhibitor used (Fig. 5*B–D*). The longest survival was observed when human PEX and PF-4/DLR were used in combination (50% survival of 113, 87, and 68 days at 1, 0.5 and 0.25 mg each, respectively), the shortest when PF-4/CTF and cyclo-VEGI were combined (50% survival of 78, 58, and 49 days at 1, 0.5, and 0.25 mg each, respectively).

The treatment was always very well tolerated without the occurrence of any side effects.

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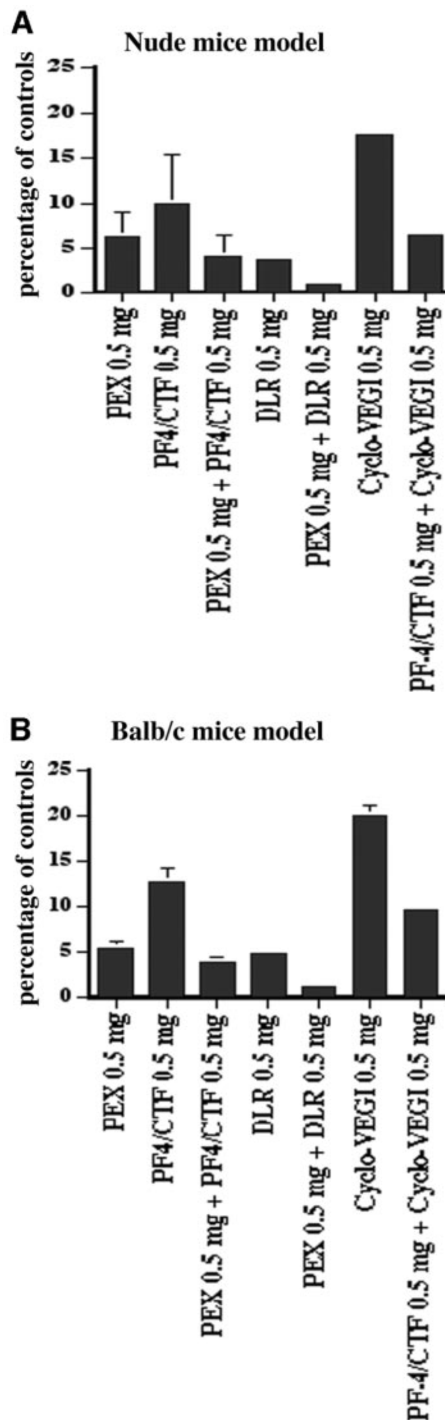
Histological Analysis. Histological and immunohistochemical analysis of tumors from treated and untreated animals showed that the inhibition of glioma growth was associated with a decrease and change in tumor vasculature, an increase in apoptosis, and in some cases a decrease in cell proliferation and a change in the pattern of glioma cell invasion (Fig. 6). The significance of effect was dependent on the type of inhibitors used in the combination.



The most significant changes were observed when human PEX and PF-4/DLR were used together. Tumors from animals treated with human PEX and PF-4/DLR showed a marked decrease in microvessel count (human PEX + PF-4/DLR *versus* human PEX or PF-4/DLR = $P < 0.001$) and change in vessel morphology. Tumor vessels were composed mostly by capillary-like tubes, few large telangectatic vessels, and without glomeruloid structures. The same tumors showed the highest apoptosis index ($P < 0.001$) and the lowest proliferation rate ($P < 0.001$). Tumors from animals that received the combination of PF-4/CTF and cyclo-VEGI showed a decrease in tumor microvessel count (PF-4/CTF + cyclo-VEGI *versus* PF-4/CTF or cyclo-VEGI = $P < 0.001$ or $P < 0.01$) and a change in tumor vessel morphology. As in the tumors from animals treated with human PEX and DLR, tumors from animals treated with PF-4/CTF and cyclo-VEGI showed mostly capillary-like tubes, fewer large vessels, mainly in the tumor center and delimitated by a unilayer of endothelial cells, and no glomeruloid structures. Although the decrease in microvessel count was similar to that observed when other inhibitors were administered in combination, the same tumors showed the lowest increase in apoptosis index and no change in tumor cell proliferation. Tumors from animals treated with human PEX and PF-4/CTF were characterized by a similar decrease in microvessel count in comparison to the other two groups of combinations and a similar change in vessel morphology (human PEX + PF-4/CTF *versus* human PEX or PF-4/CTF = $P < 0.05$ or $P < 0.01$). The changes observed in apoptosis index and proliferation rate did not significantly differ from those observed in tumors from animals treated with human PEX alone.

The most intriguing finding consisted in the modification in the pattern of tumor cell invasion at the tumor-normal brain parenchyma interface (Figs. 6 and 7). Tumors from animals belonging to the control group and those submitted to PF-4/CTF and cyclo-VEGI showed a similar pattern of invasion, consisting

Fig. 4 Efficacy of the combinatorial administration of human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI on glioma growth *in vivo*. *A*, groups of male nude mice received injections of U87 cells intracranially. Twelve days later, mice were s.c. implanted with osmotic minipumps containing 0.5 mg/kg/day of the inhibitors, alone or in combination. Twenty-nine days later, corresponding to the working period of the pumps, mice were sacrificed, the brains removed, and the tumor volume measured. The combinatorial administration of human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI produced an increase in the tumor growth inhibition in comparison when the inhibitors were used alone ($P < 0.05$). The highest inhibition was observed when human PEX and DLR were given in combination (human PEX + PF-4/DLR *versus* human PEX + PF-4/CTF or PF-4/CTF + cyclo-VEGI = $P < 0.001$ or $P < 0.001$). Data are expressed as percentages of untreated controls ± SE (100%, not indicated in the figure) and representative for three animal experiments ($n = 30$ mice in each group). *B*, the efficacy of the combinatorial administration of the inhibitors produced a similar reduction in tumor volumes in BALB/c mice models. BALB/c mice received injections of GL261 cells. Twelve days later, mice received osmotic minipumps implanted s.c. in the right flank, which the reservoirs were filled with 0.5 mg/kg/day human PEX, PF-4/CTF, PF-4/DLR, or cyclo-VEGI, administered alone or in combination. Twenty-nine later, mice were sacrificed, their brains removed, and the tumor volumes measured. Data are expressed as percentages of untreated controls ± SE (100%, not indicated in the figure) and representative for three animal experiments ($n = 30$ mice in each group).

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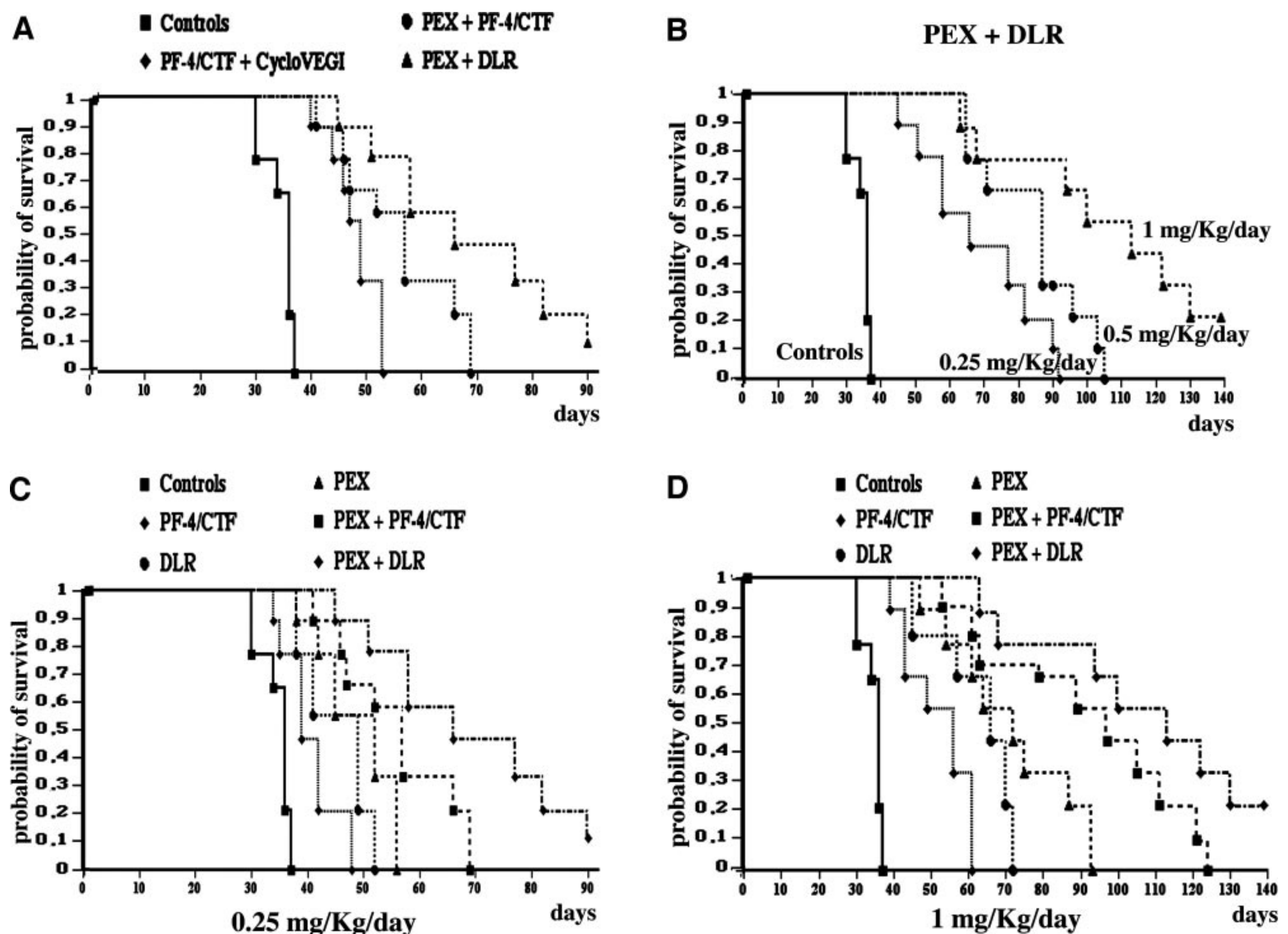

Fig. 5 Long-term efficacy of the combinatorial administration of human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI on glioma growth *in vivo*. *A*, long-term efficacy of the combinatorial administration of human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI in the nude mice glioma model. Twelve days after injection of U87 cells intracranially, nude mice were implanted s.c. with osmotic minipumps filled with 0.25 mg/kg/day of the inhibitors, alone or in combination. The pump reservoirs were changed three times to afford a period of treatment of 112 days. Animals were sacrificed at the onset of signs of distress or neurological deficits. Kaplan-Meier survival curves were designed. The longest survival was observed when human PEX and DLR were combined (50% survival of 68 days). *B*, the significance of the effect depends on the dose of the inhibitor administered. Mice that received injections of U87 cells intracranially were implanted 12 days later with osmotic minipumps, which reservoirs were filled with 0.25, 0.5, and 1 mg/kg/day of human PEX or PF-4/DLR, given in combination. The pumps were replaced four times to afford a period of treatment of 140 days. *C* and *D*, the combinatorial administration of human PEX, PF-4/CTF, or PF-4/DLR was more effective than the administration of a single inhibitor alone ($P < 0.001$). The pump reservoirs were filled with 0.25 mg/kg/day (*C*) or 1 mg/kg/day (*D*) of the inhibitors, given alone or in combination. The pumps were replaced four times to afford a period of treatment of 140 days. All experiments have been repeated three times. Data are representative of 30 mice for each group.

of undefined interface, with trails of invading tumor cells and distant tumor satellites. On the contrary, tumors from animals that were treated with human PEX and DLR showed a well-defined tumor-parenchyma interface, without trails of invading cells or tumor satellites, and a clear-cut decrease in the peripheral vessel recruitment. In addition, this pattern was particularly prominent in tumors from animals submitted to the long-term treatment, which were small, round, and with well-delineated borders (Fig. 7).

DISCUSSION

It has been shown that the administration of antiangiogenesis and anti-invasive molecules successfully inhibited the growth of human malignant gliomas, as well as that of other solid tumors in various animal models (5–12, 14, 26). This form of therapy is usually free of side effects and may represent a promising therapeutic venue for the treatment of tumors (5–7). For maximum efficacy, molecules should be administered on a long-term basis (5–7). The administration of these inhibitors faces several problems. First, the long-term treatment requires a large amount of proteins (5, 10). Furthermore, selection may facilitate the outgrowth of cell populations, the growth of which may be less dependent on the vasculature. This may be due to selection mechanisms that lead to a diminished susceptibility to apoptosis or facilitate tumor cell dissemination in the surrounding normal tissue through an increase in migration (15–18). Cancer cells can also modify their migration mechanisms during the course of tumor progression or in response to treatment (19).

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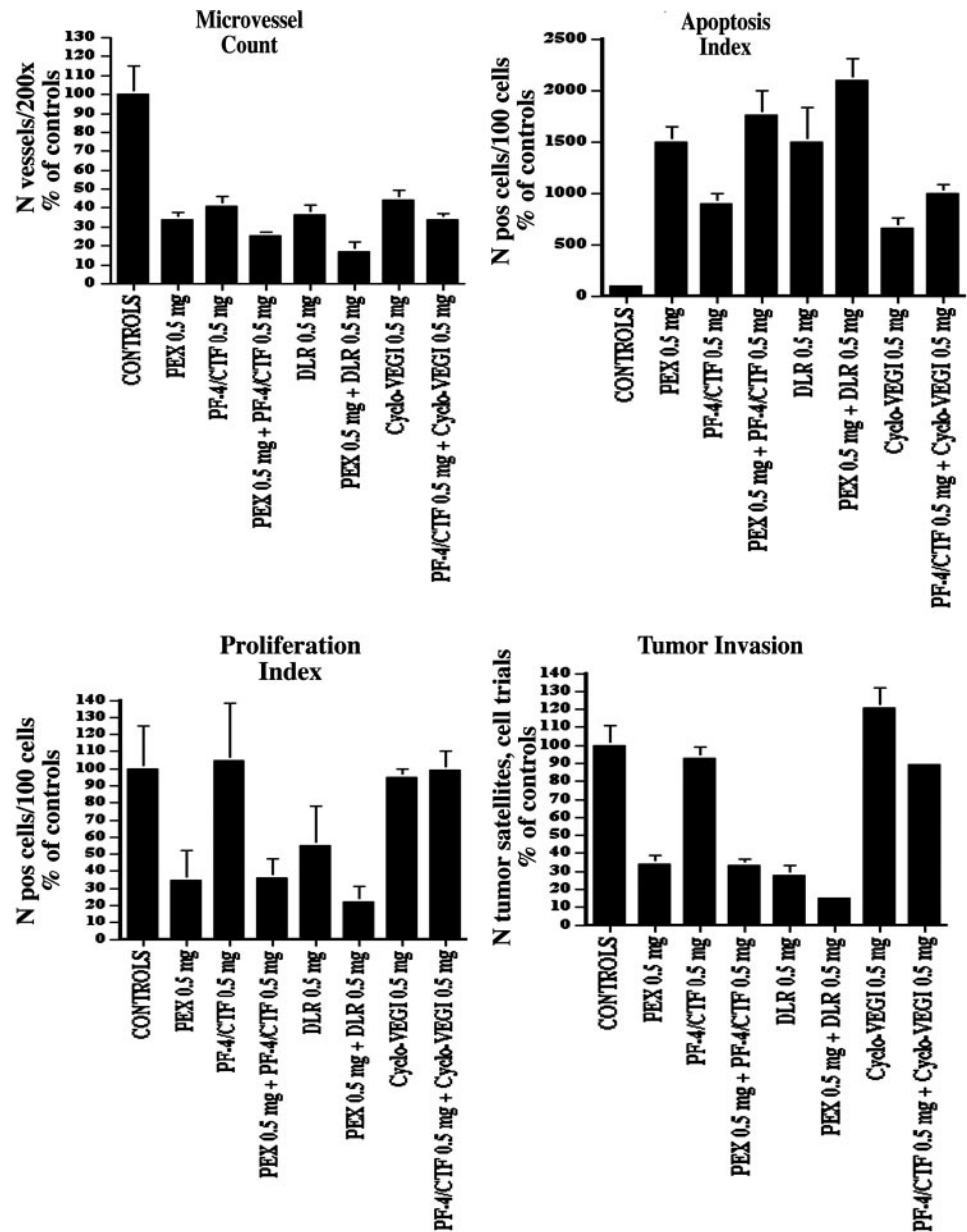

Fig. 6 Microvessels counts, apoptotic index, proliferation index, and tumor invasion in tumors from animals treated with 0.5 mg/kg/day human PEX, PF-4/CTF, PF-4/DLR, and cyclo-VEGI, alone or in combination. Data are expressed as a percentage of controls ± SD.

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Thus, an efficient anti-invasive therapy needs to target more than one mechanism at the same time.

The solution to overcome these problems is to associate molecules that both inhibit angiogenesis and invasion by using different mechanisms. This will both limit potential escape mechanisms and may, in addition, decrease the amount of protein needed for long-term treatment.

We therefore investigated the efficacy of combinatorial administration by associating molecules with pure antiangiogenic activity and molecules that have both antiangiogenic and anti-invasive properties. Different doses for these various associations were used in this study. We hypothesized that the highest and longest therapeutic effect would have been reached when inhibitors displaying the largest activities were combined together, specifically when human PEX and PF-4/DLR would have been used in combination. Consequently, the lowest and more limited action would have been expected when two antiangiogenic drugs, namely PF-4/CTF and cyclo-VEGI, were given in combination.

Association of pure antiangiogenesis molecules (PF-4/CTF, cyclo-VEGI) increased the inhibition of capillary tube formation *in vitro* to some extent. This correlated with a slight increase in survival when the combination was used in comparison to the single agent alone. Furthermore, the analysis of tumors from animals treated with PF-4/CTF and cyclo-VEGI showed a significant decrease and changes in tumor vasculature and an increase in apoptosis without modifications of cell proliferation. These findings are in agreement with an antiangiogenic effect of the molecule (13, 20, 21). When PF-4/CTF, a

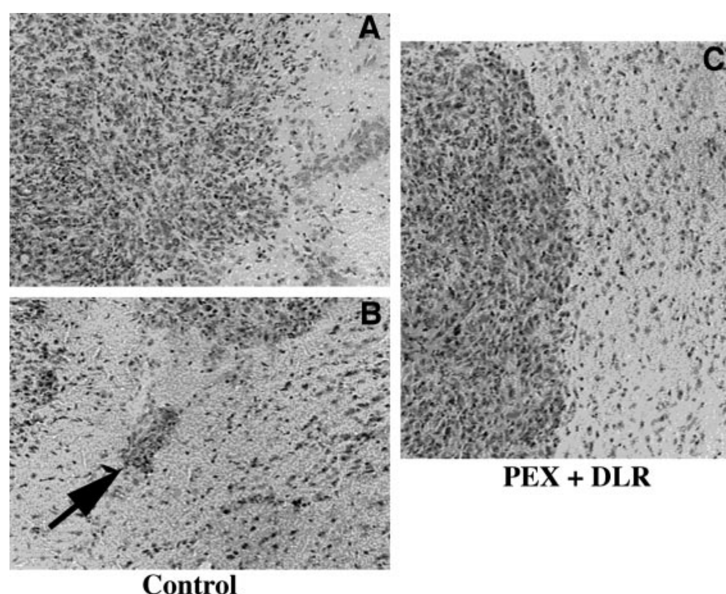

Fig. 7 Histology of tumor and brain parenchyma interface in tumors from animals treated with long-term combination of human PEX and PF-4/DLR and from control group animals. Tumors from control group animals (*A* and *B*) were characterized by the presence of many trails of tumor cells (*A*) along with tumor satellites (*B*, *arrow*) invading the normal brain parenchyma. Tumors from animals treated with human PEX and PF-4/DLR given in combination (0.5 mg/kg/day; *C*) showed a clear-cut interface with a very well-delineated border. Section were stained with H&E and showed at ×100 magnification.

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pure antiangiogenic molecule, was associated with human PEX, which inhibits angiogenesis and invasion, the migration and proliferation *in vitro* of endothelial cells but not of tumor cells were increased to some extent. This was also reflected by some benefits on long-term tumor growth *in vivo*, with an increase in survival of the animals submitted to this type of treatment. When two molecules were associated that both inhibited angiogenesis and invasion (human PEX and PF-4/DLR), a substantial increase in efficacy both *in vitro* and *in vivo* was observed. Tumors treated with human PEX and PF-4/DLR showed a similar inhibition of angiogenesis but a higher degree of cell apoptosis and a marked decrease in cell migration and proliferation. In addition, tumors from animals treated with human PEX and PF-4/DLR were characterized by a well-defined tumor-brain parenchyma interface, indicating a significant inhibition of glioma cell invasion (9). The increase in survival benefit was due to the additional effect of both molecules on invasion. This indicates that when antiangiogenesis and anti-invasive strategies are combined, they provide a stringent control of glioma growth *in vivo* and limit the capacity of the tumor to escape inhibition.

Another significant advantage of giving the inhibitors in combination came from the fact that the combinatorial administration was more effective than the administration of a single inhibitor alone. This allowed to significantly reduce the amount of protein needed to reach a therapeutic efficacy, although, the significance of the effect was still dependent on the dose at which the inhibitor was administered. In fact, the longest inhibition was reached when the inhibitors were given together at 1 mg/kg/day. On the other hand, the efficacy reached by the combinatorial administration was stronger than that observed when each inhibitor was administered alone at the same or even higher concentrations (9, 14). This additionally supports the ability of the combinatorial administration to maintain a prolonged tumor growth inhibition with a lower amount of protein.

How can we explain the beneficial effect of the association of human PEX and PF-4/DLR? Human PEX and DLR act on angiogenesis and invasion by two different mechanisms. It has been shown that human PEX binds to $\alpha_v\beta_3$ integrin and impairs the activation of pro-matrix metalloproteinase-2 and activation cascade of MT1-matrix metalloproteinase (27). In opposite, PF-4/DLR inhibits the interaction of growth factors with cell surface receptors (11, 20). PF-4/DLR inhibits not only the binding of VEGF to its receptors but also the binding of FGF to its receptors (11, 20). FGF is a pleiotrophic growth factor that not only interacts with endothelial cells but also binds to tumor cells, including glioma cells. PF-4/DLR has a much stronger competitive activity on FGF receptors than PF-4/CTF, which is needed to efficiently impair binding on tumor cells (11, 20). Thus, targeting these two distinct pathways should lead to a more stringent and/or complementary modulation of secondary players implicated in angiogenesis and invasion. It is well known that FGFs are critical for the regulation of plasminogen activators and inhibitors (28). Plasminogen activators are not only important for angiogenesis but also for glioma invasion and migration (26). Thus, inhibiting FGF activity by PF-4/DLR may impair plasminogen activator activity needed for angiogenesis and invasion. This is complementary to the inhibitor effect of human PEX, which targets integrins and the matrix metalloproteinases system.

Taken together, our data indicate that the combinatorial administration of compounds that simultaneously inhibit angiogenesis and tumor cell invasion results in a significant increase in the therapeutic efficacy. The achievement of a prolonged response requires the simultaneous delivery of compounds that target both tumor and endothelial cells and act by separate mechanisms. In addition, a significant therapeutic response can be reached with a considerably lower amount of protein.

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REFERENCES

1. Hahahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.

2. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1997;64:327–36.

3. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature (Lond.) 2000;407:249–57.

4. Bjerkvig R, Lund-Johansen M, Edvarsen K. Tumor cell invasion and angiogenesis in the central nervous system. Curr Opin Oncol 1997;9:223–9.

5. Cao Y. Endogenous angiogenesis inhibitors and their therapeutical implications. Int J Biochem. Cell Biol 2001;33:357–69.

6. Bikfalvi A, Bicknell R. Recent advances in angiogenesis, antiangiogenesis, and vascular targeting. Trends Pharmacol Sci 2002;23:576–82.

7. Hagedorn M, Bikfalvi A. Target molecules for anti angiogenic therapy: from basic research to clinical trials. Crit Rev Oncol Hematol 2000;34:89–110.

8. Bello L, Lucini V, Carrabba G, et al. Simultaneous inhibition of glioma angiogenesis, cell proliferation, and invasion by a naturally occurring fragment of human metalloproteinase-2. Cancer Res 2001;61: 8730–6.

9. Bello L, Giussani C, Carrabba G, et al. Suppression of malignant glioma recurrence in a newly developed animal model by endogenous inhibitors Clin Cancer Res 2002;8:3539–638.

10. Giussani C, Carrabba G, Pluderi M, et al. Local intracerebral delivery of endogenous inhibitors by osmotic minipumps effectively suppresses glioma growth in vivo. Cancer Res 2003;63:2499–505.

11. Hagedorn M, Zilberberg L, Wilting J, et al. Domain swapping in a COOH terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors. Cancer Res 2002;62:6884–90.

12. Kunkel P, Ulbricht U, Bohlen P. Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor 2. Cancer Res 2001; 61:6624–8.

13. Kirsch M, Strasser J, Allende R, Bello L, Zhang J, Black PM. Angiostatin suppresses malignant glioma growth in vivo. Cancer Res 1998;58:4654–9.

14. Bello L, Carrabba G, Giussani C, et al. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo. Cancer Res 2001;61:7501–6.

15. Yu JL, Rak JW, Carmeliet P, Nagy A, Kerbel RS, Coomber B. Heterogeneous vascular dependence of tumor cell populations. Am J Path 2001;158:1325–34.

16. Yu JL, Coomber BL, Kerbel RSA. paradigm for therapy induced microenvironmental changes in solid tumors leading to drug resistance. Differentiation 2002;70:599–609.

17. Pennacchetti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met proto-oncogene. Cancer Cell 2003;3:347–61.

18. Steeg PS. Angiogenesis inhibitors: motivators of metastasis? Nat Med 2003;9:822–3.

19. Friedl P, Wolf A. Tumor cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 2003;3:362–74.

20. Hagedorn M, Zilberberg L, Lozano RM, et al. A short peptide domain of platelet factor 4 blocks angiogenic key events induced by FGF-2. FASEB J 2001;15:550–2.

21. Jouan V, Canron X, Alemany M, et al. Inhibition of in vitro angiogenesis by platelet factor 4-derived peptides and mechanism of action. Blood 1999;94:984–93.

22. Zilberberg L, Shinkaruk S, Lequin O, et al. Structure and inhibitory effect on angiogenesis and tumor development of a new vascular endothelial growth inhibitor. J Biol Chem 2003;278:35564–73.

23. Deroanne CF, Hajitou D, Calberg CM, Nusgens BV, Lapiere CM. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. Cancer Res 1997;47:5590–7.

24. Joki T, Heese O, Nikas D, et al. Expression of cyclooxygenase 2 (COX2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS398. Cancer Res 2000;60:4926–31.

25. Leon SP, Folkerth R, Black PM. Microvessel density is a prognostic indicator for patients with astroglial brain tumors. Cancer (Phila.) 1996; 77:362–72.

26. Rao JS. Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 2003;3:489–501.

27. Brooks PC, Silletti S, von Schalcha TL, Friedlander M, Cheresch DA. Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell 1998;92:391–400.

28. Javerzat S, Auguste P, Bikfalvi A. The role of fibroblast growth factors in vascular development. Trends Mol Med 2002;8:483–9.

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