Continuous Delivery of Endogenous Inhibitors from Poly (Lactic-Co-Glycolic Acid) Polymeric Microspheres Inhibits Glioma Tumor Growth

Ofra Benny,1 Maayan Duvshani-Eshet,1 Theresa Cargioli,1 Lorenzo Bello,2 Andreas Bikfalvi,3 Rona S. Carroll,4 and Marcelle Machluf 1

1Laboratory of Cancer Drug Therapeutics and Mammalian Cell Technology, Faculty of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa, Israel; 2Molecular Mechanisms of Angiogenesis, Universite de Bordeaux I, Talence, France; 3Department of Neurological Sciences, University of Milan, Milan, Italy; and 4Laboratory of Neurosurgical Oncology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Purpose: There is an urgent need for modalities that can localize and prolong the administration of the antitumor agents, particularly antiangiogenic, to achieve long-term tumor inhibition. However, one of the major obstacles is designing a device in which the biological activity of sensitive endogenous inhibitors is retained. We have designed a biodegradable polymeric device, which provides a unique and practical means of localizing and continuously delivering hemopexin (PEX) or platelet factor 4 fragment (PF-4/CTF) at the tumor site while maintaining their biological activity. The potential and efficacy of this system is shown in vitro and in vivo in a human glioma mouse model.

Experimental Design: Polymeric microspheres made of poly(lactic-co-glycolic acid) (PLGA) were loaded with very low amounts of PEX or PF-4/CTF. The release profiles of these factors from PLGA and their biological activity were confirmed in vitro using proliferation assays done on endothelial and tumor cells. Tumor inhibition using this system was studied in nude mice bearing a human s.c. glioma.

Results: PEX and PF-4/CTF released in vitro from PLGA microspheres were biologically active and significantly inhibited the proliferation of human umbilical vein endothelial cells, bovine capillary endothelial cells, and U87-MG cells. A single local s.c. injection of PLGA microspheres loaded with low amounts of PEX or PF-4/CTF resulted in an 88% and 95% reduction in glioma tumor volume 30 days post-treatment. Immunohistochemical analysis of the treated tumors showed a marked decrease in tumor vessel density compared with untreated tumors.

Conclusion: Our findings show that polymeric microspheres are a very promising approach to locally and efficiently deliver endogenous inhibitors to the tumor site leading to a significant inhibition of the tumor.

INTRODUCTION

Malignant gliomas, the most common primary brain tumors, present a major therapeutic challenge. Even with surgery followed by chemotherapy and radiation, the majority of the tumors recur within 1 year (1). The marked induction of angiogenesis and the correlation between the degree of glioma malignancy and angiogenesis (the sprouting of new blood vessels from existing ones) suggests that this process plays an important role in their tumorigenesis. Therefore, gliomas are potentially responsive to antiangiogenic therapies, as these tumors are highly vascularized and overexpress angiogenic factors (1). Currently, there are more than 30 inhibitors of angiogenesis in clinical trials, and the efficacy of several others is being extensively studied on a variety of experimental tumors (2, 3). For these inhibitors to be therapeutically effective, they are usually given on a daily basis using high quantities (grams of purified protein) to achieve significant tumor suppression. Moreover, most of these studies concluded that this form of therapy would need to be carried out for the rest of the patient’s life to avoid tumor recurrence (4–8). The purification of large quantities of angiogenic inhibitors, which are needed to achieve sufficient tumor suppression along with the daily administration, results in huge costs and low patient compliance.

In recent studies, to overcome the daily administration, a continuous systemic and local modality has been used to administer inhibitors resulting in significant therapeutic outcome for longer times, with decreased protein quantities (9–13). However, systemic administration may not assure sufficient concentration of the inhibitors at the tumor bed. Therefore, other delivery modalities need to be developed which can be localized, have a constant release rate, and use lower amounts of the proteins particularly when treating brain tumors.

Poly(lactic-co-glycolic acid) (PLGA) microspheres have been used as a controlled delivery system of many proteins, drugs, and other factors, such as cytokines, hormones, enzymes, vaccines, and chemotherapeutic agents (14–19). The protein released from the PLGA microspheres occurs by the degradation of the polymeric matrix. The composition of the PLGA allows the control of the degradation rate and therefore the control of protein release kinetics (20, 21). By varying the co-monomers...
ratio, the time release of the protein can range from weeks to months. Most importantly, PLGA is biocompatible and can be used in humans. Recently, PLGA particles containing radiosensitizer 5-fluorouracil were implanted stereotactically in the brains of human patients (22). This study showed an increase in the efficacy of the radiation treatment. Therefore, the use of PLGA microspheres for therapeutic delivery may provide a prolonged sustained delivery, thus maintaining high levels of the agent at the site of interest, which can be used in humans.

In the present study, for the first time, the potential and efficacy of modified PLGA microspheres to release endogenous antiangiogenic proteins for tumor inhibition is shown in vitro and in vivo in a human glioma mouse model. The modified PLGA particles are loaded with the endogenous inhibitor: hemopexin (PEX) or platelet factor 4 fragment (PF-4/CTF). Systemic administration of human recombinant PEX has been shown to inhibit the growth of human malignant gliomas in vivo and delay the appearance of glioma recurrence in nude mice (10, 23). Human PEX is rapidly cleared from the blood, and prolonged administration of this protein is needed. To achieve inhibition of glioma growth, daily administration (i.p.) is needed (23).

PF-4/CTF also inhibits angiogenesis in vitro and in vivo by inhibiting the association of fibroblast growth factor-2 (FGF-2) to their receptors and thereby restricting their proangiogenic action (24). In addition, systemic PF-4/CTF administration was shown to inhibit the growth of glioma in animal models (10, 25). The present data show the successful loading of PEX and PF-4/CTF in PLGA particles without affecting their biological activity. Furthermore, local administration of these particles significantly inhibited the size and vessel density of human malignant gliomas grown s.c. in nude mice. This significant inhibition was achieved with only a single administration of these particles, which were loaded with very low amounts of inhibitor (50 μg for PEX and 100 μg for PF-4/CTF per mouse).

**MATERIALS AND METHODS**

**Preparation of Protein-Loaded Microspheres**

PEX protein was purified and analyzed as reported previously (23). PF-4/CTF was synthesized as reported previously by Hagedorn et al. (25). PEX- or PF-4/CTF-loaded microspheres were prepared using the double-emulsion solvent extraction method with slight modifications. Briefly, 200 mg PLGA (RG502, Boehringer Ingelheim, Ingelheim, Germany) with 50:50 lactic acid-to-glycolic acid ratio was dissolved in 0.5 mL dichloromethane (Frutarom, Haifa, Israel). A predetermined solution of protein was added to the dissolved polymer, and the solution was homogenized using Ultra-Turrax type DI-18 IKA (Dottingen, Germany) for 1 minute leading to the formation of the first emulsion (W/O). Polyvinyl alcohol of 85 to 89 gr/mol (Aldrich Chemical, Inc., Munich, Germany) saturated with dichloromethane was rapidly added to the first emulsion, and the solution was homogenized again for the second time for 20 seconds. The resulting multiple W/O/W was mixed for 5 minutes, and a volume of 50 mL of 0.1% w/v aqueous polyvinyl alcohol containing 5% (v/v) 2-propanol solution (J.T. Baker, Deventer, Holland) was added to the W/O/W double emulsion. After 30 minutes of extensive stirring, the microspheres were centrifuged and washed twice, and after the final wash, the microspheres were lyophilized (Modulo Edwards, Crawley, United Kingdom) resulting a fine powder of dry PLGA microspheres containing the PEX or PF-4/CTF protein. Empty microspheres were prepared in the same way without PEX or PF-4/CTF.

**Morphologic Studies with Scanning Electron Microscopy**

Scanning electron microscope (Joel, JSM 5400) was used to evaluate the shape and surface morphology of PEX- and PF-4/CTF-loaded PLGA microspheres. After the microspheres were lyophilized, the dried microspheres were mounted on an aluminum stub and sputter coated with a thin layer (100-150 Å) of gold under argon atmosphere. The surface morphology of the microspheres samples was then visualized under a scanning electron microscope.

**Particle Size Distribution**

The particles size distribution was analyzed using a Coulter LS 230 particle size analyzer. Samples were prepared by resuspending 5 to 10 mg microspheres in distilled water. The results were reported as a volume size distribution by a computerized analysis for ideal spheres.

**Protein Loading Efficiency**

For all the experiments, the PEX and PF-4/CTF amounts per unit weight of microspheres were determined as follows: Fractions of 10 mg microspheres loaded with the desired protein were digested overnight with 0.1 N NaOH containing 5% SDS. NaOH increases PLGA hydrolysis rate, and SDS ensures the complete solubility of the protein. MicroBCA protein assay kit (Pierce, Rockford, IL) was used to determine total protein loading in the microspheres and the amount of protein released from the microspheres at different time points. The loading efficiency was obtained as follows: the total PEX or PF-4/CTF amounts measured in the microspheres at the end of preparation procedure divided by the initial protein amount used for the preparation of the microspheres.

**In vitro Release of PEX and PF-4/CTF from PLGA Microspheres**

For the in vitro release studies, microspheres were incubated in PBS (pH 7.3) and maintained in a shaking incubator at 37°C. After different times of incubation, microspheres were recovered from the release medium by centrifugation, and the medium was replaced with fresh medium. This procedure was repeated every 2 days for at least 16 days. The concentrations of PEX and PF-4/CTF in the release medium were evaluated by Western blot analysis (data not shown) and quantitatively determined using microBCA protein assay. The percentage of the released proteins from total protein loading was calculated for every sample from the different incubation periods and presented as a cumulative curve.

**Cell Proliferation Assays**

Proliferation assays were done on human umbilical vein endothelial cells (HUVEC), which were isolated from umbilical veins as described previously (ref. 26; 20,000 cells per well), bovine capillary endothelial (BCE) cells (Judah Folkman Laboratory, Harvard Medical School, Boston, MA; 20,000 cells per well), and U87-MG cells (American Type Culture Collection, Manassas, VA; 15,000 cells per well) using a thymidine assay.
HUVEC, BCE, and U87-MG cells were seeded on 24-well plates coated with gelatin and incubated overnight with medium 199 (Biological Industry, Beit Haemek, Israel, for HUVEC) or DMEM (Life Technologies, Grand Island, NY for BCE, U87-MG) supplemented with 10% FCS (Life Technologies), glutamine, and 1% (v/v) penicillin/streptomycin. Endothelial cells were also supplemented with 2 ng/mL basic fibroblast growth factor. The medium was replaced with medium containing 5% serum after 24 hours; 250 μL samples were taken from the releasing medium of different incubation periods of the microspheres containing the PEX, PF-4/CTF (total microspheres loading of 25 and 125 μg, respectively), or empty microspheres (data not shown) and added to the cells. After 48 hours, [3H]thymidine (1 μCi/mL) was added to each well, and cells were incubated for 24 hours in 37°C, 10% CO2. The cells were washed twice with PBS and lysed with 250 μL of 0.2 N NaOH for 20 minutes. The samples were suspended in liquid scintillation medium and quantified using a MR 250-counter (Saint-Quentin-Yvelines, France). Nonspecific binding was determined by incubating separate dishes with 125I-FGF-2 and 100-fold excess of unlabeled ligand. Specific binding was determined by omitting the primary antibody. Detection was carried out using a 3,3′-diaminobenzidine chromogen, which results in a positive brown staining. Sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted with glass coverslips. Negative control slides were done to determine whether recombinant PEX and PF-4/CTF exhibited a spherical shape with smooth and uniform surface morphology. The micrographs of the PLGA microspheres were analyzed by scanning electron microscopy. The micrographs exhibited a spherical shape with smooth and uniform surface morphology (Fig. 1A). Immunostaining was carried out using a Coulter counter. The average diameter of lyophilized microspheres loaded with PEX or PF-4/CTF was between 30 and 50 μm (Fig. 1B).

In vitro Release of PEX and PF-4/CTF from PLGA

In this study, the release profile of PEX and PF-4/CTF from the PLGA microspheres was determined by cumulative percentage of protein release (Fig. 2). The release profiles of PF-4/CTF and PEX were characterized by a controlled release of the proteins over a 30-day period. However, overall, the release rate of PF-4/CTF was higher than with PEX. This difference may be due to the difference in the size of the two proteins (2.7 versus 29 kDa).

Inhibition of Endothelial and Glioblastoma Cells

Endothelial and glioblastoma cell proliferation assays were done to determine whether recombinant PEX and PF-4/CTF...
released from the PLGA microspheres retained their biological activity (Fig. 3). Recombinant PF-4/CTF released at days 1 to 6 from the microspheres inhibited HUVEC proliferation by 33% to 47% (Fig. 3A). PF-4/CTF released at days 1 to 11 inhibited BCE proliferation by 42% to 54% (Fig. 3B). A similar pattern was observed for recombinant PEX. PEX released during the first 10 days inhibited HUVEC proliferation by 36% to 62% (Fig. 3C). Moreover, PEX released from the PLGA microspheres inhibited the proliferation of U87-MG glioma cell line by 29% to 65% (Fig. 3D). Samples taken from empty microspheres incubated in PBS as described for the loaded microspheres did not affect endothelial cell proliferation (data not shown).

PF-4/CTF-Binding Assay

It is well known that PF-4/CTF exerts its angiogenic inhibitory effect in vitro by interrupting the binding of FGF-2 to its receptors (24). Therefore, receptor-binding assays were used to assure that PF-4/CTF released from the PLGA microspheres retained its functionality. BCE cells were incubated with 10 ng/mL 125I-FGF-2 before adding the releasing medium from the PLGA microspheres. PF-4/CTF released from the microspheres on day 11 of the release was added with increasing concentrations to the cultured BCE, and binding assays to FGF-2 high- and low-affinity sites on BCE were done (Fig. 4). BCE cells without the addition of PF-4/CTF were used as a negative control.
control, whereas BCE supplemented with 15 μmol/L PF-4/CTF were used as positive control. The results obtained using a γ-counter analyzer were given in cpm/10^6 cells and presented as percentage of the positive control. As seen, PF-4/CTF released from the PLGA microspheres retained its biological activity by inhibiting the 125I-FGF-2 binding to low-affinity receptors on endothelial cells (Fig. 4A). PF-4/CTF concentrations measured in the samples (by protein assay, BCA), and the inhibitory effect was found to be dose dependent; the binding inhibition was 79.9%, 90.7%, and 92.1% for 5, 15, and 30 μmol/L PF-4/CTF, respectively. An inhibition level of 93.5% was achieved when adding 15 μmol/L pure PF-4/CTF protein (with no microspheres), which served as a positive control. No inhibitory effect was detected when using release samples from empty microspheres, excluding a possible effect of the PLGA polymer on the binding assay. Similar results were obtained in the FGF-2 high-affinity receptors assay done on BCE (Fig. 4B). The binding inhibitions achieved were 80%, 84.7%, and 85.1% for 5, 15, and 30 μmol/L PF-4/CTF, respectively. A 92.3% FGF-2-binding inhibition occurred when adding 15 μmol/L pure PF-4/CTF protein, which served as positive control. To exclude the PLGA effect on FGF-2 binding to its receptors, samples were taken after an incubation of empty PLGA microspheres in PBS. The low- and high-affinity binding results show 90.5% and 103.7% 125I-FGF-2 binding for empty microspheres samples (relative to negative control). Differences were considered significant when P < 0.01 (ANOVA test).

In vivo Studies: Effects of PEX and PF-4/CTF PLGA Microspheres on Subcutaneous Human Glioma Xenografts

Mice were inoculated with a right flank s.c. injection of 5 × 10^6 U87-MG cells. The tumors reached a size of ∼200 ± 95 mm^3 10 days post-inoculation, at which time the microspheres were injected adjacent to the tumor. The growth of U87-MG glioma xenografts was significantly inhibited by a single dose of microspheres containing PEX or PF-4/CTF when compared with control groups (P < 0.01, empty microspheres or no microspheres; Fig. 5A). The growth of the glioma tumors was suppressed by 88% and 95% with PEX- or PF-4/CTF-loaded microspheres, respectively, 30 days post-microsphere injection. Animals were sacrificed 30 days after PLGA microspheres injection; tumors were harvested, weighed, and measured (Fig. 5C). Tumors taken from the groups treated with microspheres containing PEX or PF-4/CTF showed 85% or 83% reduction in weight, respectively, compared with controls.

Immunohistochemistry

Tumors treated with PLGA microspheres containing PEX or PF-4/CTF had significantly less vascularization (Fig. 6A, e and f) than tumors treated with empty microspheres as seen by CD31 immunohistochemistry (Fig. 6A, c and d). Sections of tumors harvested from untreated mice (Fig. 6A, a and b) or mice treated with empty PLGA microspheres (Fig. 6A, c and d) were characterized by high microvessel density with a well-developed capillary network. Quantification of microvessel density revealed 52 ± 9 capillaries per field in untreated mice (Fig. 6B). Microvessel count in mice treated with PLGA-PEX or PF-4/CTF revealed a significant decrease (P < 0.01) with a count of 27 ± 5.7 and 18.2 ± 5.2, respectively. PEX and PF-4/CTF released from the PLGA microspheres did not result in a change the apoptotic index (data not shown).

DISCUSSION

Malignant gliomas are aggressive, highly vascularized tumors, which recur within 2 cm of the original tumor core and are rarely metastatic. Studies have shown that endogenous inhibitors, such as endostatin, PF-4, and PEX, inhibit the angiogenic process in glioma tumors leading to tumor growth inhibition (10, 12, 23–25, 28). These studies and others also emphasize the urgent need for modalities that can localize and prolong the administration of the antitumor agents to achieve long-term tumor inhibition. Implantable biodegradable polymeric devices provide a unique practical means of localizing the anticancer agents at the tumor site. The strategy of using a controlled delivery device reduces the amount of protein needed, relative to systemic administration, to achieve similar tumor inhibition. In addition, an efficient delivery of labile proteins with short half-lives may be advantageous when using such systems. To date, the efficacy of different control delivery devices for brain tumor therapy in animal models and human gliomas have been tested. These systems were mainly used to deliver chemotherapeutic agents, which are known for their stability and toxicity (29–33). These devices are macroscopic, nonbiodegradable, or biodegradable wafers (a disc shape), which because of their size (several centimeters) cannot be injected stereotactically and must be placed near the tumor site after craniotomy. Microspheres, on the other hand, may be easily injected to any site in the brain due to their size and spherical shape. Therefore, even once the microspheres release their total content, new freshly loaded particles can be re-injected to the brain without the need of an invasive second surgical procedure. Menet et al. (22) have recently published a study in which PLGA microspheres containing radiosensitizer (5-fluorouracil) were injected in patients after surgical resection of malignant glioma. The authors concluded that the implanted microspheres were well tolerated by the patient, with no episodes of edema or hematologic complication. In addition, the efficacy of the radiation was increase in the presence of the 5-fluorouracil. Another advantage of PLGA microspheres is their preparation procedure, which does not involve any chemical reaction between the polymers, and the proteins thus can serve as a universal system in which endogenous proteins as well as synthetic factors can be entrapped. Most importantly, microspheres made from PLGA with different co-polymer ratio may be formulated to release their content for different times ranging from weeks to months. Various microspheres containing different agents that work independent mechanisms of inhibition may be combined and simultaneously injected to better achieve tumor inhibition.

In the present studies, we show for the first time the use of PLGA microspheres to deliver two endogenous inhibitors PEX and a fragment of PF-4 (PF-4/CTF) for human glioma therapy. These two agents differ in size, stability, and mechanism of action. PEX (29 kDa) regulates the interaction between integrin α5β1 and matrix metalloproteinase-2, which is one of the critical steps controlling endothelial cell invasion (34–36). In vivo studies have shown that PEX is a powerful molecule, which
inhibits glioblastoma growth (s.c. and i.c.), and the appearance of glioma recurrence. PEX also inhibits tumor cell proliferation, migration, and invasion, making it unique from other antiangiogenic agents because its activity is not restricted to endothelial cells. In previous studies, we showed that mice treated with PEX ranging from 1 to 5 mg/kg/d did not show any toxicity in terms of weight loss, inactivity, opportunistic infections, or reduced appetite. Moreover, organs removed from mice treated with PEX of a higher concentration than was used in this study did not show any gross pathologic changes (23).

On the other hand, PF-4/CTF (2.7 kDa) inhibits angiogenesis in vitro and in vivo by associating directly with angiogenic factors (basic fibroblast growth factor and vascular endothelial growth factor) and altering their conformation, thus preventing them from stimulating endothelial cell proliferation (24, 25). PF-4/CTF was also shown to be safe in terms of toxicity in mice bearing glioma (10, 37). The PLGA preparation procedure involves the use of organic solvents due to the nature of polymers and may affect the biological activity of the loaded protein, particularly PEX, which is an unstable protein. Therefore, we did in vitro release studies of both agents from the PLGA microspheres and confirmed their biological activity. We showed that both PEX and PF-4/CTF released from the PLGA microspheres were able to inhibit endothelial cell proliferation.
proliferation. PEX released from PLGA was also able to inhibit U87-MG cell proliferation, which support earlier evidence, indicating that this potent endogenous factor could also inhibit tumor cells in addition to its angiogenic ability to inhibit the proliferation of endothelial cells.

The presence of biologically activity PF-4/CTF in the released medium of PLGA microspheres was further supported by the binding assay done on endothelial cells. PF-4/CTF samples taken from the PLGA release medium strongly inhibited 125I-FGF-2 binding to the high- and low-affinity receptors on endothelial cells. To exclude the possibility that polymer composites affect FGF-2 binding to its receptors, samples were also taken after incubation with empty PLGA in PBS. The in vitro studies clearly show that the PLGA formulation procedure does not affect the biological activity of PEX or PF-4/CTF.

It is well documented in the literature that systemic treatment of tumors, including gliomas, with antiangiogenic factors requires daily administration of high doses to achieve tumor inhibition. A daily systemic administration of 2.5 mg/kg endostatin for 16 days was needed to achieve 53% inhibition of Lewis lung carcinoma (6). For PEX, 5 mg/kg/d for 30 days were needed to achieve 99% inhibition of glioma growth in a nude mouse model (23). However, when using local administration of antiangiogenic agents, the amount of the agent used to achieve tumor inhibition is reduced significantly. Alzet mini osmotic pumps implanted i.p. and used to administer endostatin continually to s.c. Lewis lung carcinoma led to 81% inhibition in tumor size compared with a single bolus administration of 100 mg/kg/d for 23 days, which led to 90% in tumor inhibition. However, these minipumps had to be refilled every 7 days to achieve this level of inhibition. A similar modality was used to locally administer endostatin to i.c. glioma xenografts (12). Three weeks of local i.c. microinfusion of endostatin (2 mg/kg/day) led to 74% reduction of glioma tumor volume. In this study, systemic administration of a 10-fold higher amount of endostatin did not result in tumor volume inhibition. Minipumps have also been used to locally infuse PEX and PF-4/CTF in an i.c. glioma model for the inhibition of i.c. glioma tumors (37). The pump reservoir was filled with 0.5 mg PF-4/CTF or human PEX, which correspond to 0.5 mg/kg/d. In these studies, animal tumor volume was inhibited by 81% using PEX and 72% with PF-4/CTF.

In the present study, we show that the use of PLGA microspheres further reduces the amount of PEX and PF-4/CTF needed to achieve significant glioma inhibition in nude mice when compared with systemic and minipump administration. Only a single s.c. injection of PLGA microspheres loaded with micrograms of PEX adjacent to the tumor inhibited glioma tumor volume by 88% 30 days post-administration. Similar studies done with PLGA microspheres loaded with low amounts of PF-4/CTF led to a 95% inhibition 30 days post-treatment. Tumors harvested from animals 30 days post-injection of PLGA microspheres loaded with PEX or PF-4/CTF showed a significant reduction of 85% or 83%, respectively, in tumor weight when compared with control animals. Immunohistochemistry done on tumor section from all groups revealed a significant decrease in microvessel density in animals treated with PLGA loaded with PEX or PF-4/CTF when compared with
controls. These data correlated with previously published data of glioma treated with PF-4/CTF or PEX (37). By injecting the microspheres adjacent to the tumor, we show that the proteins released from the microspheres are able to diffuse and arrive to the tumor bed. Thus, they do not necessarily need to be injected inside the tumor, which is not always practical or accessible possible when treating brain tumors.

In summary, the presented data show the advantage of PLGA microspheres as a continuous delivery system of angiogenic inhibitors for the treatment of glioma. The PLGA microspheres allow the continuous release of biological active PEX and PF-4/CTF for >30 days. The system may overcome problems associated with high doses and daily administration of angiogenic inhibitors. The use of microspheres also offers a way to combine different inhibitors as well as chemotherapy in one administration. We are in the process of studying the effect of a combined treatment of PEX and PF-4/CTF in a long-term s.c. model and injecting these microspheres for the treatment in an orthotopic glioma model.

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