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

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## 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 and 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 others 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

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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 stereotaxically 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 Hagedron 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-Turax 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

(11). 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, [$^3$H]thymidine (1 μCi/mL) was added to each well, and cells were incubated for 24 hours in 37°C, 10% $CO_2$. 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 and analyzed with β-counter for radioactivity. For the controls, medium from empty microspheres taken after the same incubation times as the respective experimental groups or by adding PBS only. The results are expressed in counts per minute (cpm).

### PF-4/CTF-Binding Studies

To evaluate the biological activity of PF-4/CTF as an inhibitor of FGF-2 to its receptor, a binding assay was done as described previously (27). FGF-2 was labeled with $^{125}$I-Na using Iodo-Gen (Pierce) as a coupling agent according to former protocol (27). Binding experiments of FGF-2 to high- and low-affinity sites were done on BCE cells. Cells were seeded at 2.5 cm × $10^5$/cm, cultured in complete medium onto 3.5 cm$^2$ dishes, and grown for 2 days. The cells were washed twice with ice-cold PBS before binding and incubated with $^{125}$I-FGF-2 in DMEM containing 20 mmol/L HEPES (pH 7.4), 0.15% gelatin for 2 hours at 4°C in the presence or absence of indicated concentration of PF-4/CTF. The PF-4/CTF samples for this study were taken from the 11th day of the release study of PLGA microspheres. At the end of the incubation period, the cells were washed twice with ice-cold PBS. $^{125}$I-FGF-2 was dissociated from its cellular low-affinity binding sites by two 20-second washes with ice-cold 20 mmol/L HEPES (pH 7.4), 2 mol/L NaCl and from its cellular high-affinity binding by two 20-second washes with ice-cold 20 mmol/L NaAc (pH 4.0), 2 mol/L NaCl. Bound $^{125}$I-FGF-2 was quantified using a Kontron MR 250-counter (Saint-Quentin-Yvelines, France). Nonspecific binding was determined by incubating separate dishes with $^{125}$I-FGF-2 and 100-fold excess of unlabeled ligand. Specific binding was determined by subtracting nonspecific binding from total binding.

### Animal Studies

Male BALB/C nude mice (4-6 weeks old, Harlan Laboratories, Rehovot, Israel) were inoculated s.c. with 5 × $10^6$ U87-MG cells. Ten days later, when tumors reached ~200 mm$^3$, animals were randomly divided into four treatment groups of 15 animals each. The first group received a single s.c. injection of PLGA microspheres containing a total of 50 μg PEX. In the second group, each mouse received a single s.c. injection of PLGA microspheres containing 100 μg PF-4/CTF. The third group received a single s.c. injection of empty PLGA microspheres as a control, and the fourth group did not receive any treatment. S.c. tumor growth was measured transcutaneously with a caliper every 2 days for 30 days, and the tumor volume was then calculated (6). All mice were sacrificed 30 days post-injection, tumors were weighed, and volumes were measured. Statistically significant differences in tumor growth among the groups were analyzed by ANOVA. In all experiments, $P < 0.01$ was considered statistically significant. These studies were done according to the Animal Ethics Committee at the Technion-Israel (Haifa, Israel). The weight and behavior of the mice were followed during the *in vivo* studies.

### Immunohistochemistry

Tumors were embedded in OCT (Tissue-Tek, Sakura Fineteck, Inc., Torrance, CA), frozen on dry ice/butane, and stored at −80°C. Frozen sections (8 μm) were cut using a cryostat. Sections of each specimen were stained using H&E (data not shown). Immunohistochemistry was carried out using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Primary antibodies included CD31 (BD Biosciences, San Jose, CA) for microvessel staining and cleaved caspase-3 (Cell Signaling, Beverly, MA) for *in situ* detection of apoptosis. 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 obtained by omitting the primary antibody. Apoptosis was quantified by determining the percentage of positively stained cells (stained cells divided by total nuclei) in 10 randomly chosen fields per tissue section at 400×. Microvessel density was determined by image analyses software using 10 randomly chosen fields per section in at least five sections at 200×.

## RESULTS

### Microspheres Size and Surface Morphology

The external surface morphology of the PLGA microspheres prepared by the double-emulsion technique was analyzed by scanning electron microscopy. The micrographs exhibited a spherical shape with smooth and uniform surface morphology (Fig. 1*A*). Microspheres size was measured using a Coulter counter. The average diameter of lyophilized microspheres loaded with PEX or PF-4/CTF was between 30 and 50 μm (Fig. 1*B*).

### *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 Proliferation by PEX and PF-4/CTF Released from PLGA Microspheres

Endothelial and glioblastoma cell proliferation assays were done to determine whether recombinant PEX and PF-4/CTF

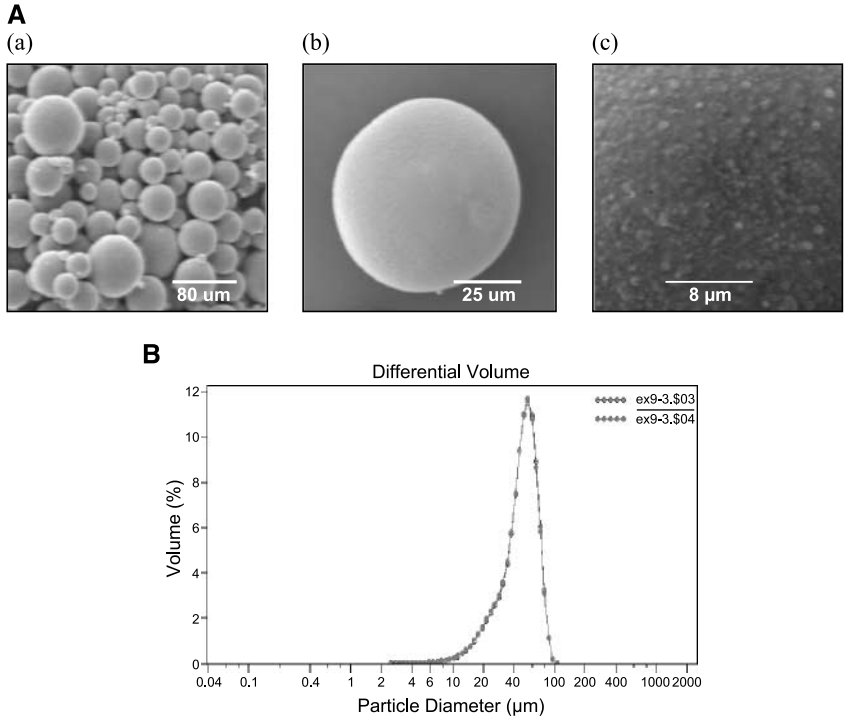


*Fig. 1* PLGA microspheres morphology and size distribution. *A,* morphology of PLGA microspheres studied by scanning electron microscopy: microspheres loaded with protein (*a*), a higher magnification of a single microsphere (*b*), and the surface area of a microsphere (*c*). *B,* microsphere size distribution as obtained by a Coulter counter.

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. 3*A*). PF-4/CTF released at days 1 to 11 inhibited BCE proliferation by 42% to 54% (Fig. 3*B*). A similar pattern was observed for recombinant PEX. PEX released during the first 10 days inhibited HUVEC proliferation by 36% to 62% (Fig. 3*C*). Moreover, PEX released from the PLGA microspheres inhibited the proliferation of U87-MG glioma cell line by 29% to 65% (Fig. 3*D*). 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 $^{125}$I-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

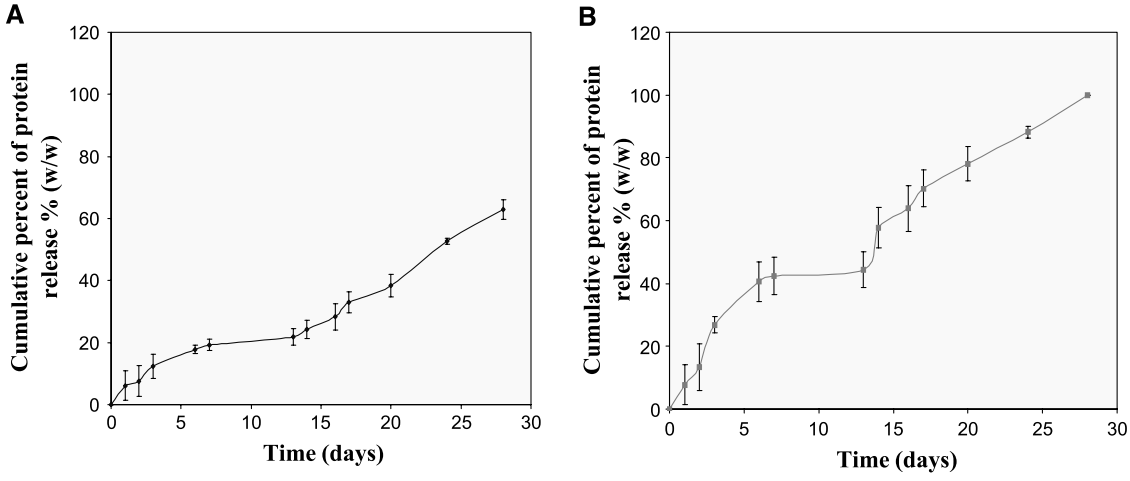


*Fig. 2* Protein release profiles from PLGA loaded with PEX (*A*) and PF-4/CTF (*B*). For each release profile, 20 mg of polymeric microspheres containing 25 µg PEX or 125 µg PF-4/CTF were incubated (separately) in PBS at 37°C and 100 rpm shaking. *Points,* mean of four different release experiments, expressed as a percentage of released protein from total protein loaded in microspheres.

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 $^{125}$I-FGF-2 binding to low-affinity receptors on endothelial cells (Fig. 4*A*). 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. 4*B*). 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% $^{125}$I-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 $5 \times 10^6$ U87-MG cells. The tumors reached a size of $\sim 200 \pm 95$ $mm^3$ 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. 5*A*). 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. 5*C*). 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. 6*A*, *e* and *f*) than tumors treated with empty microspheres as seen by CD31 immunohistochemistry (Fig. 6*A*, *c* and *d*). Sections of tumors harvested from untreated mice (Fig. 6*A*, *a* and *b*) or mice treated with empty PLGA microspheres (Fig. 6*A*, *c* and *d*) were characterized by high microvessel density with a well-developed capillary network. Quantification of microvessel density revealed $52 \pm 9$ capillaries per field in untreated mice (Fig. 6*B*). Microvessel count in mice treated with PLGA-PEX or PF-4/CTF reveled a significant decrease ($P < 0.01$) with a count of $27 \pm 5.7$ and $18.2 \pm 5.2$, 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 reinjected to the brain without the need of an invasive second surgical procedure. Menei 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 $\alpha_v\beta_3$ 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

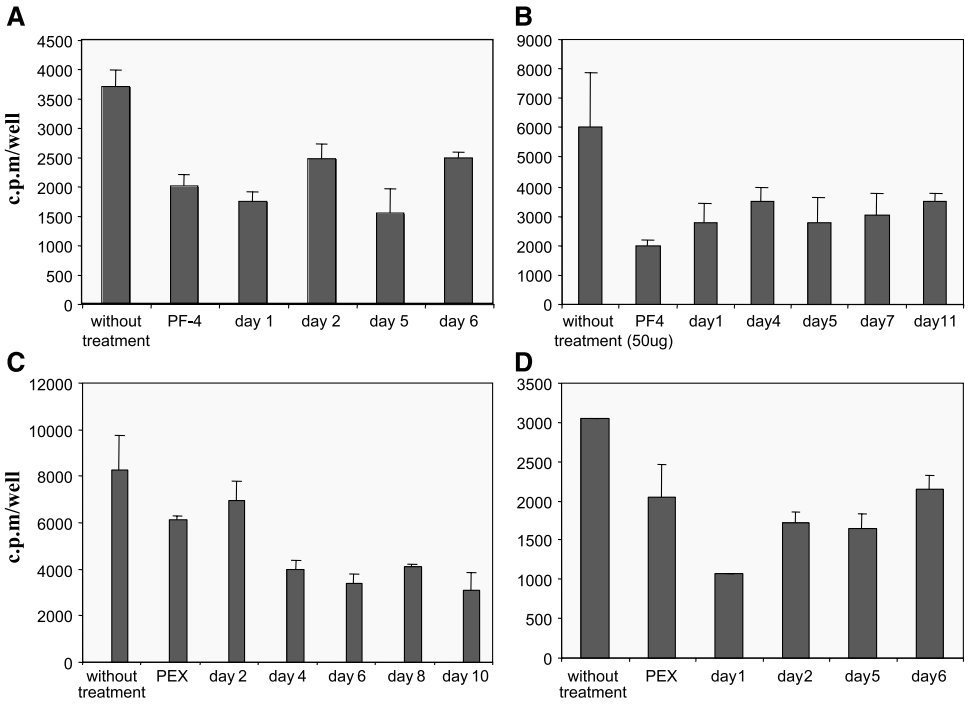


*Fig. 3* Effect of PEX or PF-4/CTF released from the PLGA microspheres on the proliferation of endothelial and tumor cells *in vitro*. Inhibition of HUVEC proliferation by PF-4/CTF released from PLGA during the first 6 days of release (*A*). Inhibition of BCE proliferation during 11 days of release (*B*). In all studies, 50 μg/mL pure PF-4/CTF were added to the cells as positive control. Inhibition of HUVEC proliferation by PEX released from PLGA during 11 days of release (*C*). Inhibition of U87-MG proliferation by PEX released during the first 10 days (*D*). As a positive control, 10 μg/mL PEX were added to the cells. Columns, mean of four different release experiments; bars, SE. $P < 0.05$.

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

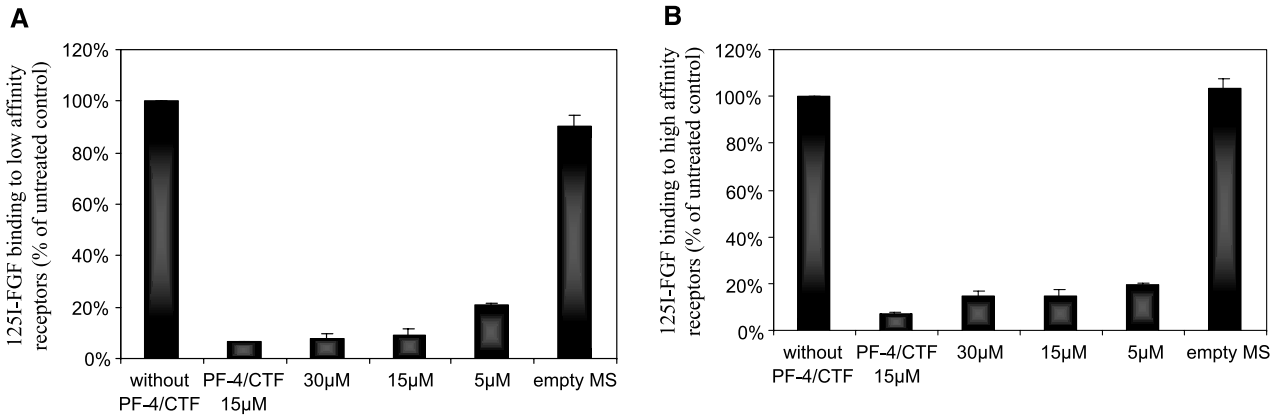


*Fig. 4* Inhibition of $^{125}$I-FGF-2 binding to low-affinity (*A*) and high-affinity (*B*) receptors on endothelial cells treated with PF-4/CTF released from microspheres at the 11th day of incubation in PBS. BCE cells were incubated with $^{125}$I-FGF-2 in the presence or absence of cold ligand (200-fold excess) or competitor, and binding was determined as described in MATERIALS AND METHODS. 100% as 6,033 cpm/well (high-affinity binding). 100% as 122,101 cpm/well (low-affinity binding). Columns, mean of two experiments in duplicates expressed as a percentage in comparison with untreated control; bars, SD. $P < 0.01$.

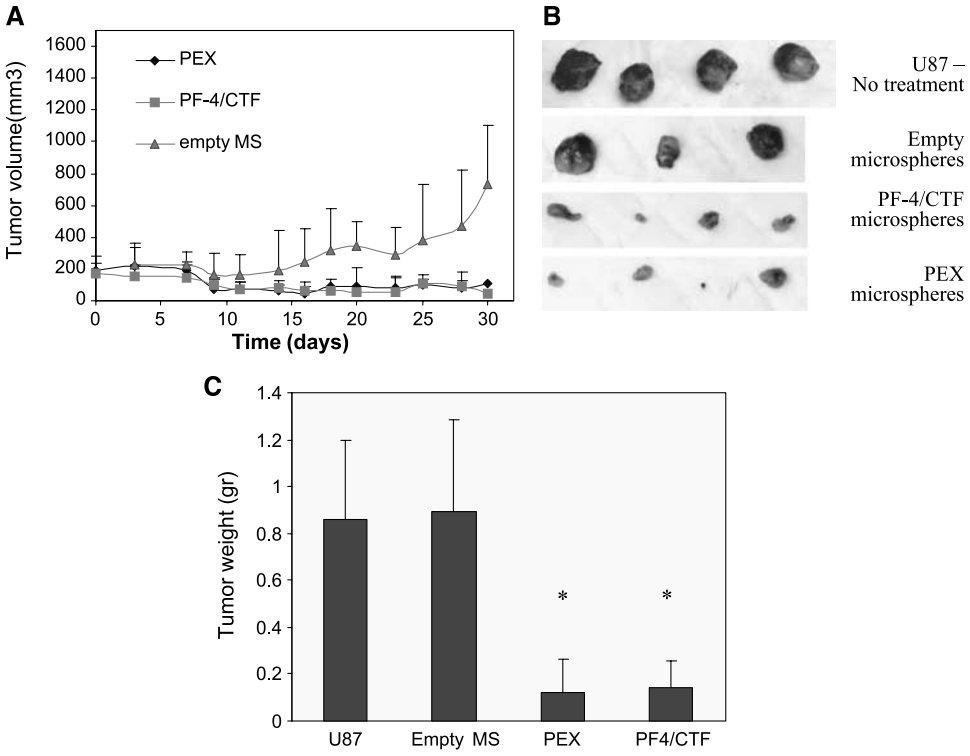


*Fig. 5* Effect of PEX or PF-4/CTF released from PLGA microspheres on tumor growth *in vivo*. *A,* inhibition of s.c. U87-MG human glioma cells xenograft. Mice bearing U87-MG tumors were treated with PLGA microspheres loaded with PEX or PF-4/CTF or with empty microspheres. *B,* tumors harvested from mice 30 days post-treatment and from non-treated mice. *C,* at day 30 post-treatment, U87-MG tumors were harvested and their weights were measured. Bars, SE for 15 animals were used for each group. *, $P < 0.01$.

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 $^{125}$I-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 nice 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

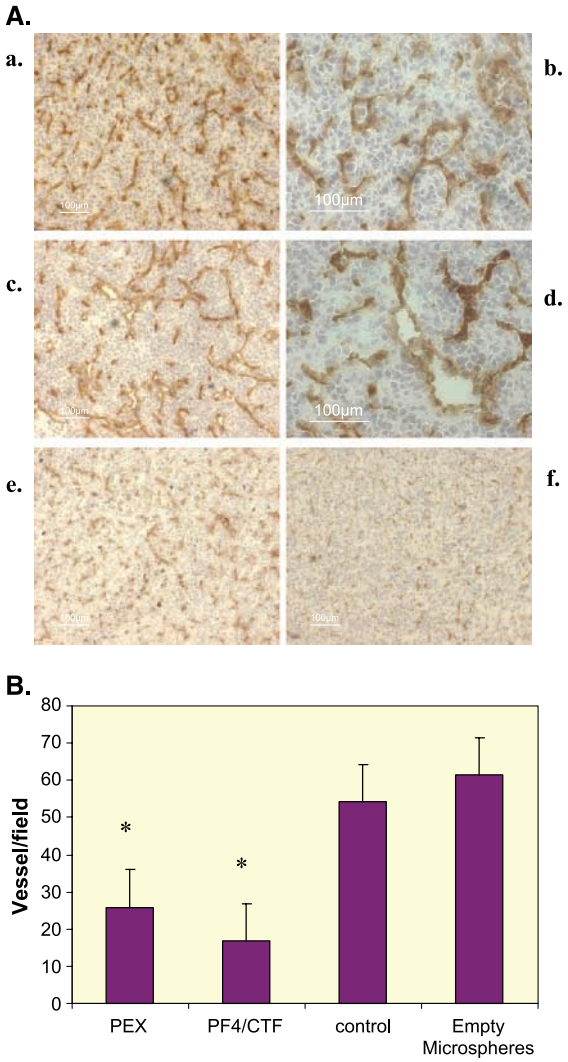


*Fig. 6* Immunohistochemistry of tumors treated with PLGA loaded with PEX or PF-4/CTF. *A,* CD31 staining of nontreated tumors, original magnification, ×200 and ×400 (*a* and *b*, respectively), and tumors treated with empty microspheres, original magnification, ×200 and ×400 (*c* and *d*, respectively). CD31 staining at original magnification, ×200 for PLGA-PEX-treated tumors (*e*) and PLGA-PF-4/CTF-treated tumors (*f*). *B,* microvessel density was determined by counting 10 high-power fields from the CD31-stained sections in *A*. Bars, SE. $P < 0.01$.

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.

## REFERENCES

1. Black PM. Brain tumors. Part 2. N Engl J Med 1991;324:1555–64.
2. Satchi-Fainaro R, Pudar M, Davis JW, et al. Targeting angiogenesis with conjugate of HPMA coploymer and TNP-470. Nat Biotechnol 2004;10:255–61.
3. National Cancer Institute [Web site]. U.S. NIH [cited 2004 Sep 28]. Available from: http:/cancertrials.nci.nih.gov.
4. Cao Y. Endogenous angiogenesis inhibitors and their therapeutic implications. Int J Biochem Cell Biol 2001;33:357–69.
5. Jain RK, Schlenger K, Hockel M, Yuan F. Quantitative angiogenesis assays: progress and problems. Nat Med 1997;3:1203–8.
6. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
7. Folkman J. Tumor angiogenesis. In: Mendelson J, Howley PM, Israel MA, Liotta LA, editors. The molecular basis of cancer. Philadelphia: W.B. Saunder Co.; 1995. p. 206–33.
8. Folkman J, Browder T, Palmblad J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 2001;86:23–33.
9. Kisher O, Becker CM, Prox D, et al. Continuous administration of endostatin by intraperitoneally implanted osmostic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. Cancer Res 2001;61:7669–74.
10. Bello L, Carroll RS, Giussani C, Carrabba G, Pluderi M, Lucini V, et al. Suppression of malignant glioma recurrence in a newly developed animal model by fragments of endogenous inhibitors. J Clin Cancer Res 2002;8:3539–48.
11. Joki T, Machluf M, Atala A, et al. Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol 2001;19:35–9.
12. Schmidt NO, Ziu M, Carrabba G, et al. Antiangiogenic therapy by local intracerebral microinfusion improves treatment efficiency and survival in an orthotopic human glioblastoma model. Clin Cancer Res 2004;10:1255–62.
13. Kim JS, Chang JH, Yu HK, et al. Inhibition of angiogenesis and angiogenesis-dependent tumor growth by the cryptic kringle fragments of human apolipoprotein(a). J Biol Chem 2003;278:29000–8.
14. Alonso MJ, Gupta RK, Siber GR, Langer R. Biodegradable microspheres as controlled release tetanus toxoid delivery system. Vaccine 1994;12:299–306.
15. Seagal J, Edry E, Keren Z, et al. A fail-safe mechanism for negative selection of isotype-switched B cell precursors is regulated by the Fas/FasL pathway. J Exp Med 2003;198:1609–19.
16. Mullerad J, Cohen S, Benharroch D, Apte RN. Local delivery of IL-1α polymeric microspheres for the immunotherapy of an experimental fibrosarcoma. Cancer Invest 2003;21:720–8.
17. Mullerad J, Cohen S, Voronov E, Apte RN. Macrophage activation for the production of immunostimulatory cytokines by delivering interleukin 1 via biodegradable microspheres. Cytokine 2000;12:1683–90.
18. Sanchez A, Gupta RK, Alonso MJ, Siber GR, Langer R. Pulsed controlled-released system for potential use in vaccine delivery. J Pharm Sci 1996;85:547–52.
19. Roullin VG, Lemaire L, Venier-Julienne MC, Faisant N, Franconi F, Benoit JP. Release kinetics of 5-fluorouracil-loaded microspheres on an experimental rat glioma. Anticancer Res 2003;23:21–5.
20. Arshady R. Preparation of biodegradable microspheres and microcapsules:polyactideandrelatedpolymers.JControlRelease1991;17:1–121.
21. Edlund U, Albertsson A-C. Degradable polymer microspheres for controlled drug delivery. Adv Polym Sci 2002;157:67–112.

22. Menei P, Jadaud E, Faisant N, et al. Stereotaxic implantation of 5-fluorouracil-releasing microsphees in malignant glioma. Cancer 2004;100:405–10.

23. 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.

24. 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.

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

26. Neufeld G, Gospodarowicz D. Identification of the fibroblast growth factor receptor in human vascular endothelial cells. J Cell Physiol 1988; 136:537–42.

27. Moscatelli D. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J Cell Physiol 1987;131:123–30.

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

29. Brem H, Piantadosi S, Burger PC, et al. Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-Brain Tumor Treatment Group. Lancet 1995;345: 1008–12.

30. Valtonen S, Timonen U, Toivanen P, et al. Interstitial chemotherapy with carmustine-loaded polymers for high-grade gliomas: a randomized double-blind study. Neurosurgery 1997;41:44–8.

31. Westphal M, Hilt DC, Bortey E, et al. A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. Neuro-oncol 2003; 5:79–88.

32. Menei P, Daniel V, Montero-Menei C, Brouillard M, Pouplard-Barthelaix A, Benoit JP. Biodegradation and brain tissue reaction to poly(D,L-lactide-co-glycolide) microspheres. Biomaterials 1993;14: 470–8.

33. Fournier E, Passirani C, Vonarbourg A, et al. Therapeutic efficacy study of novel 5-FU-loaded PMM 2.1.2-based microspheres on C6 glioma. Int J Pharm 2003;268:31–5.

34. Gohlke U, Gomis-Ruth F, Crabbe T, Murphy G, Docherty AJ, Bode W. The C-terminal (haemopexin-like) domain structure of human gelatinase A (MMP2): structural implications for its function. FEBS Lett 1996;378:126–30.

35. Brooks PC, Stromblad S, Sanders LC, et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with $\alpha_v\beta_3$ integrin. Cell 1996;85:683–93.

36. Brooks P, Silletti S, von Schalscha T, Friedlander M, Cheresh D. Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell 1998;92: 391–400.

37. 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.