Antiangiogenic Therapy by Local Intracerebral Microinfusion Improves Treatment Efficiency and Survival in an Orthotopic Human Glioblastoma Model

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ABSTRACT

Targeting active angiogenesis, which is a major hallmark of malignant gliomas, is a potential therapeutic approach. For effective inhibition of tumor-induced neovascularization, antiangiogenic compounds have to be delivered in sufficient quantities over a sustained period of time. The short biological half-life of many antiangiogenic inhibitors and the impaired intratumoral blood flow create logistical difficulties that make it necessary to optimize drug delivery for the treatment of malignant gliomas. In this study, we compared the effects of endostatin delivered by daily systemic administration or local intracerebral microinfusion on established intracranial U87 human glioblastoma xenografts in nude mice. Noninvasive magnetic resonance imaging methods were used to assess treatment effects and additional histopathological analysis of tumor volume, microvessel density, proliferation, and apoptosis rate were performed. Three weeks of local intracerebral microinfusion of endostatin (2 mg/kg/day) led to 74% ($P < 0.05$) reduction of tumor volumes with decreased microvessel densities (33.5%, $P < 0.005$) and a 3-fold increased tumor cell apoptosis ($P < 0.002$). Systemic administration of a 10-fold higher amount of endostatin (20 mg/kg/day) did not result in a reduction of tumor volume nor in an increase of tumor cell apoptosis despite a significant decrease of microvessel densities (26.9%, $P < 0.005$). Magnetic resonance imaging was used to successfully demonstrate treatment effects. The local microinfusion of human endostatin significantly increased survival when administered at 2 mg/kg/day and was prolonged further when the dose was increased to 12 mg/kg/day. Our results indicate that the local intracerebral microinfusion of antiangiogenic compounds is an effective way to overcome the logistical problems of inhibiting glioma-induced angiogenesis.

INTRODUCTION

For the past several decades, the standard therapy for malignant gliomas consisting of surgical removal and postoperative radiation has undergone extensive improvements from a technical viewpoint. However, the clinical outcome of these patients still remains poor, with an average survival after diagnosis of only 12–18 months (1). Tumor-induced angiogenesis is a promising therapeutic target and its inhibition has shown efficacy in a variety of experimental tumor models (2–5). The increased metabolic requirements during the malignant progression of a tumor and the dependency on paracrine factors provided by angiogenic active endothelial cells are the major rationales for the therapeutic concept of inhibiting the tumor-induced neovascularization (6, 7). Because angiogenesis depends on the local net result between positive and negative regulators it has been suggested that the prolonged, daily delivery of antiangiogenic therapy is the most effective way to obtain long-term suppression of tumor angiogenesis (6, 8).

An important advantage of a direct antiangiogenic approach is that it targets primarily the angiogenic active endothelial cells and not the tumor cell compartment. Therefore, the blood–brain barrier does not need to be overcome, and the logical delivery method of antiangiogenic compounds seems to be the systemic delivery, for example, by intravascular administration. Daily systemic administration of antiangiogenic compounds requires large quantities of purified protein and a high patient compliance. Furthermore, it is well known that tumors display a highly impaired and heterogeneous blood flow that decreases toward the center of the tumor, inevitably leading to hypoxia and tumor necrosis (9). Thus, even under optimized conditions, it is questionable whether sufficient concentrations of therapeutic compounds within and throughout the tumor can be achieved (10).

The direct infusion of therapeutic compounds into brain tumors can overcome some of the obstacles of drug delivery. First clinical trials using intratumoral infusions of cytotoxic compounds have recently demonstrated encouraging therapeutic effects with an acceptable local safety and minimized systemic toxicity (11, 12). Although it circumvents the blood–brain bar-

rier, local intracerebral (i.c.) microinfusions were effectively used using various conventional chemotherapeutic drugs in experimental brain tumor models (13, 14). The drug containing fluids distribute by bulk flow (convection-enhanced) homogeneously through the interstitial space, and, depending on the infusion pressure, higher local drug concentrations and more distant areas of the brain can be reached (15). Because 90% of the malignant gliomas recur within 2–3 cm of the resection site (16), local infusions of therapeutic compounds may be able to delay or prevent tumor recurrence.

In this study, we demonstrate that the local i.c. microinfusion of the well-known antiangiogenic compound endostatin is safe and more effective in the treatment of established orthotopic human glioblastoma xenografts than is the daily systemic delivery. These results suggest a potential role for the direct i.c. infusion of antiangiogenic compounds for the clinical management of malignant glioma.

MATERIALS AND METHODS

Cell Culture. The human glioblastoma cell line U87 (American Type Culture Collection, Manassas, VA) was cultured in MEMα (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mM L-glutamine, 2 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, and 10% fetal bovine serum (Life Technologies, Inc.). Cells were maintained in T-75 tissue culture flasks in 5% CO_2/95% air at 37°C in a humidified incubator and were routinely passaged at confluency. For the intracranial implantation experiments, U87 cells were dispersed with a 0.05% solution of trypsin/EDTA (Life Technologies, Inc.), were washed with PBS, and were adjusted to a final concentration of 5×10^4 cells/10 μl in PBS.

Animal Studies. All of the animal work was carried out in the animal facility at Brigham and Women's Hospital in accordance with federal, local, and institutional guidelines.

Tumor Models and Treatment Schemes. Orthotopic human glioblastoma xenografts were established in 4–6-week-old male Swiss nude mice (Taconic, Germantown, NY). Mice were anesthetized (100 mg/kg ketamine and 5 mg/kg xylazine) and received a stereotactically guided injection of 5×10^4 human U87 glioma cells into the left forebrain (2 mm lateral and 1 mm anterior to bregma, at a 3-mm depth from the skull surface). Eight days after tumor cell injection, groups of 5–10 mice were randomized. For the systemic treatment groups, mice received a total daily dosage of 20 mg/kg of murine (derived from *Escherichia coli*) or human recombinant endostatin (soluble human protein derived from *Pichia pastoris*; provided by Dr. Judah Folkman, Children's Hospital, Boston, MA and EntreMed, Rockville, MD) as i.p. injections every 12 h. The control animals of the systemic treatment groups were given i.p. injections with PBS only. For the local delivery scheme, Alzet osmotic minipumps, designed to deliver 0.25 μl/h for 4 weeks, were used (Durect Corporation, Cupertino, CA). The pumps were filled with 2 mg/kg/day murine endostatin, or PBS for the control animals, and connected to a brain infusion cannula via a catheter tube according to the manufacturer's instructions (Brain Infusion Kit, Durect). To establish a constant flow before implantation, the filled pumps were incubated overnight in sterile

PBS at 37°C. Previous experiments have demonstrated the stability and biological activity of endostatin contained in a pump over a prolonged time period (17). Eight days after the injection of U87 glioma cells, the pump reservoirs were placed s.c. in the left flank of the anesthetized mice. The brain infusion cannula was tunneled s.c. and fixed on the skull with surgical glue slightly posterior to the initial tumor cell injection site resulting in an intraparenchymal localization of the cannula tip (∼2–2.5 mm deep). Closure of the incisions was performed by suturing. Each experiment was performed in duplicate.

Glioma Surgical Resection Model. To mimic the clinical scenario more closely, we also used the recently developed glioma surgical resection model as described previously (18). Briefly, 20 days after U87 cell injection, established human gliomas were surgically removed using a microsurgical technique. At that time, some degree of tumor cell invasion had occurred resulting in the formation of small tumor satellites distant from the main tumor mass. Microsurgical removal was pursued until clear resection margins were visible. After achieving hemostasis, the incision was closed by suturing. One day after surgery, mice were randomly divided, and systemic treatment with 20 mg/kg/day of murine endostatin started. All of the groups were sacrificed by CO_2 inhalation 21 days after treatment was initiated or when signs of neurological dysfunction were apparent. Brains were removed, embedded in OCT, and stored at −80°C until further processed for histological analysis.

Survival Study. The effects of a local microinfusion of endostatin on the survival of nude mice with established human glioblastoma xenografts were assessed by implantation of osmotic minipumps 8 days after tumor-cell injections, as described above. Pumps were filled either with PBS for the control animals or with human endostatin at 2 mg/kg/day and 12 mg/kg/day in a PBS solution. Animals were sacrificed at the onset of neurological signs.

Magnetic Resonance Imaging (MRI). Five mice from each of the groups with established tumors receiving the systemic (20 mg/kg/day i.p. murine endostatin or PBS) and the local delivery (2 mg/kg/day i.c. murine endostatin or PBS) treatment were randomly chosen at the end of the experiments (day 21 after treatment start). Tumor growth was evaluated on a Bruker 8.5 T DRX vertical bore micro-imaging system (Bruker Instruments, Billerica, MA). Gadopentetate dimeglumine (Gd-DTPA; Berlex Laboratories, Wayne, New Jersey) was administered i.p. (0.8 ml/kg body weight) and the mice were anesthetized with 1% isoflurane (Abbott Laboratory, North Chicago, IL) in oxygen. Brain infusion cannulas were carefully removed before positioning using a rigid bite-bar. Respiratory rates were monitored using a Bruker Physiogard vital sign monitor. Gd-enhanced T1-weighted spin-echo images were obtained for tumor segmentation and three-dimensional rendering to calculate tumor volumes [three-dimensional analysis software 3D Slicer (19)]. T1-weighted post-Gd images were obtained 15 min after contrast injection using TR = 1000 ms, TE = 8.8ms with a slice thickness of 0.75 mm, matrix size of 128×128 and a 2.56×2.56 cm^2 field of view. Immediately after imaging, the animals were sacrificed by CO_2 inhalation, and the brains were removed for histological analysis.

Immunohistochemistry. Frozen brains embedded in OCT were cut in serial 10-μm sections and were stained with

H&E for histological evaluation. Tumor volumes were calculated by the ellipsoid formula. Immunohistochemistry was carried out using standard techniques with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Primary antibodies included anti-CD31 (1:100; BD Biosciences PharMingen) for blood vessels, anti-Ki67 nuclear antigen (1:100, DAKO, Carpinteria, CA) for proliferating cells, and anti-cleaved Caspase 3 (1:100; Cell Signaling Technology, Beverly, MA) for the detection of apoptosis. Sections were counterstained with hematoxylin, and negative control slides were obtained by omitting the primary antibody. The proliferation and apoptosis index were quantified by counting the number of positively stained cells of 100 nuclei in five randomly chosen high-power fields. The microvessel density was assessed as described by Leon *et al.* (20) For the identification of hemosiderin deposits as a sign for micro-hemorrhages, we used the Prussion-Blue reaction with a neutral red counterstain.

Statistics. All of the values were calculated as mean ± SE or were expressed as percentage of control ± SE. Significant differences between tumor volume, microvessel density, prolif-

eration, and apoptosis index were determined using the Mann-Whitney rank-sum test (MW test) or the unpaired *t* test. Kaplan-Meier survival curves were statistically analyzed using Cox regression analysis.

RESULTS

Systemic *versus* Local i.c. Treatment of Human Gliomas with Endostatin. Local i.c. microinfusions were established by implantation of osmotic-minipumps in the near vicinity of the tumor site 8 days after glioma cell injection. The direct intraparenchymal release of a daily total dose of 2 mg/kg of murine endostatin for 21 days resulted in significant tumor growth inhibition (73.6%, $P < 0.05$). A 10-fold higher systemic treatment (2×/day: 10 mg/kg = total daily dosage of 20 mg/kg i.p.) with murine endostatin for 21 days had no significant effect on the inhibition of tumor growth of established orthotopic human glioblastoma xenografts (Fig. 1*A*). In contrast, histological analysis revealed that the intratumoral blood vessel density, as assessed by CD31 immunoreactivity, was significantly re-
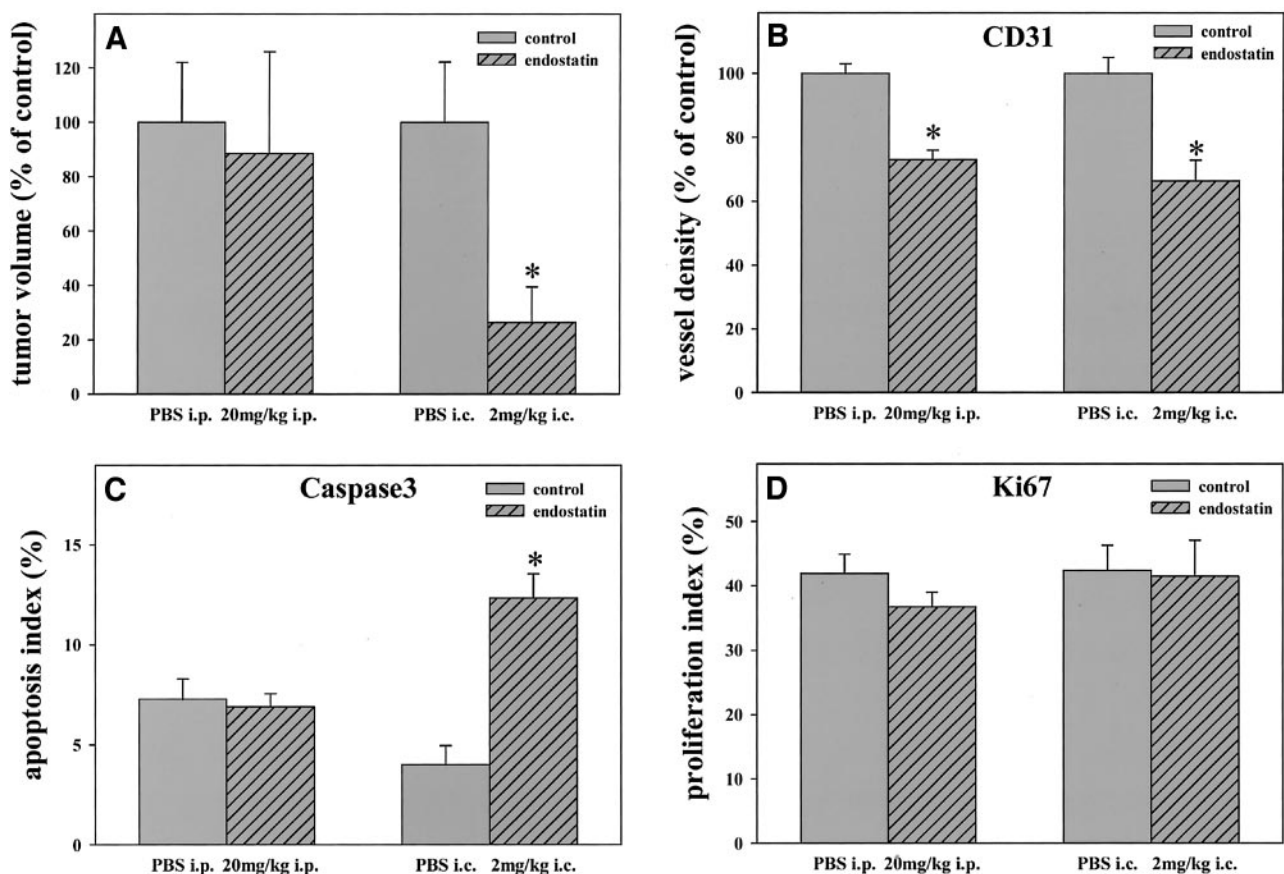

Fig. 1 The local intracerebral (i.c.) microinfusion of murine endostatin (*2mg/kg/day i.c.*) via osmotic pumps is more effective than systemic i.p. administration of 20 mg/kg/day on established U87 human glioblastoma xenografts in nude mice. *A,* direct i.c. delivery of 10-fold less murine endostatin (2 mg/kg/day) inhibited the tumor volume by 74% (∗, $P < 0.05$) as assessed by histological volume measurements. *B,* histological analysis after 21 days of treatment indicated a significant reduction of vessel density (CD31) for the systemic treatment by 26.9% (∗, $P < 0.005$) and for the local delivery by 33.5% (∗, $P < 0.005$). *C,* apoptotic cells were identified by immunoreactivity for cleaved Caspase 3. Only the local microinfusion of endostatin induced a highly significant 3-fold increase of the apoptotic index (∗, $P < 0.002$). *D,* the proliferative index based on Ki67 immunoreactivity remained unchanged in both treatment schemes.

duced in both treatment schemes. The local microinfusion of endostatin reduced the blood vessel density by 33.5% ($P <$ 0.005), and the systemic treatment reduced the blood vessel density by 26.9% ($P < 0.005$) as compared with the respective control groups (Fig. 1*B*). However, only the local microinfusion of 2 mg/kg/day endostatin induced a 3-fold increased apoptosis index ($P < 0.002$; Fig. 1*C*). No difference in the apoptosis index of the systemic treatment group was observed, and the proliferative activity of the tumors in all of the treatment groups remained unchanged (Fig. 1*D*). Histological screenings on the brain sections did not reveal any signs of intratumoral hemosiderin deposits, indicating that the antiangiogenic treatment did not induce any i.c. bleeding. Only rarely were minor hemosiderin deposits identified at a distance from the tumor site with no predisposition for control or treated animals or the cannula site of the pump. All of the pump reservoirs displayed a decreased volume at the end of the experiments, indicating that endostatin was delivered as expected.

In the glioma resection model, the cytoreduction by the surgical removal of the main tumor mass followed by the systemic treatment scheme with 20 mg/kg/day of endostatin i.p. did not result in a significant reduction of tumor recurrence when compared with the respective control group (control 82.18 ± 38 mm^3 *versus* treated 45.66 ± 13 mm^3). In both groups, the invading cells that remained in the distant parenchyma after surgery inevitably regrew to form new tumor masses. Histological analysis revealed a significant inhibition of the intratumoral blood vessel density by 34% ($P < 0.05$) and an increase of the apoptotic index (control 8.5 ± 2% *versus* treated 16.14 ± 4.62%; $P < 0.05$). The proliferative activity in tumors of both groups remained unchanged. None of the animals from any of the groups showed any signs of side effects due to the treatment.

Assessment of Treatment Effects by MRI. The evaluation of new therapeutic approaches can benefit from noninvasive MRI methods to quantify *in vivo* antitumor activity in experimental animal models. Randomly chosen subsets of animals ($n = 5$ each) from the original groups having received 21 days of systemic or local i.c. treatment of endostatin, or vehicle only (PBS) were imaged using an 8.5T MRI scanner, and
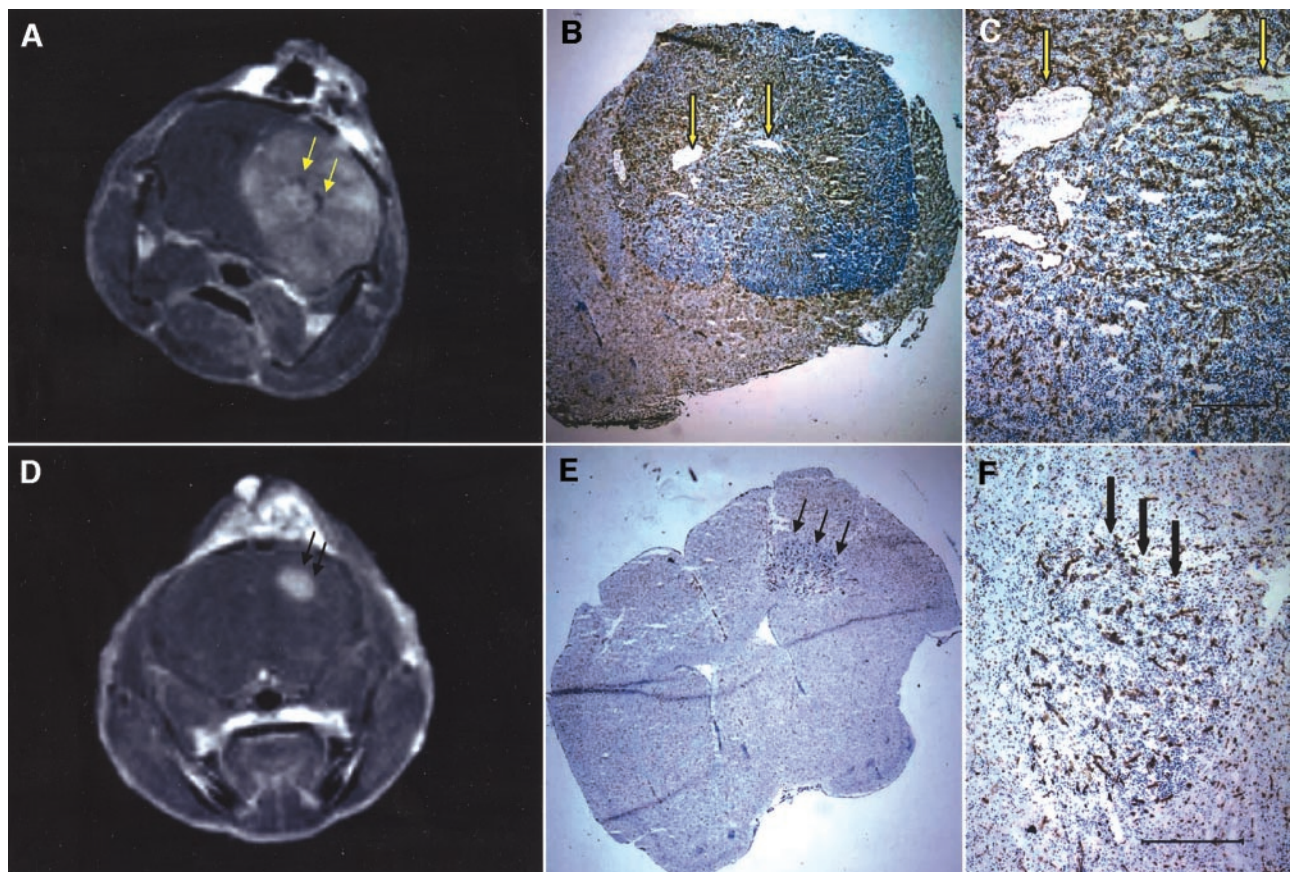

Fig. 2 Examples of Gd-enhanced T1-weighted MR images with human glioblastoma xenografts and corresponding histological sections. *A,* the T1-weighted image of a large control tumor in the left hemisphere having received a local microinfusion of PBS for 21 days displayed a highly heterogeneous contrast enhancement with some spared areas in the center (*arrows*). *B* and *C,* giving the impression that these correspond to the large blood vessels seen in the center of the tumor (*arrows*) on the corresponding blood vessel stained (CD31) section. The blood vessel stain demonstrates the highly heterogeneous vascularization of the control tumor. *D,* in contrast, the T1-weighted image of a tumor treated with a local microinfusion of endostatin (2 mg/kg/day) for 21 days shows a small contrast-enhancing mass in the left hemisphere (*arrows*). *E* and *F,* the corresponding blood vessel stain reveals a reduced and more regular formed vascularization of the treated glioblastoma xenograft. *Scale bar,* 500 μm.
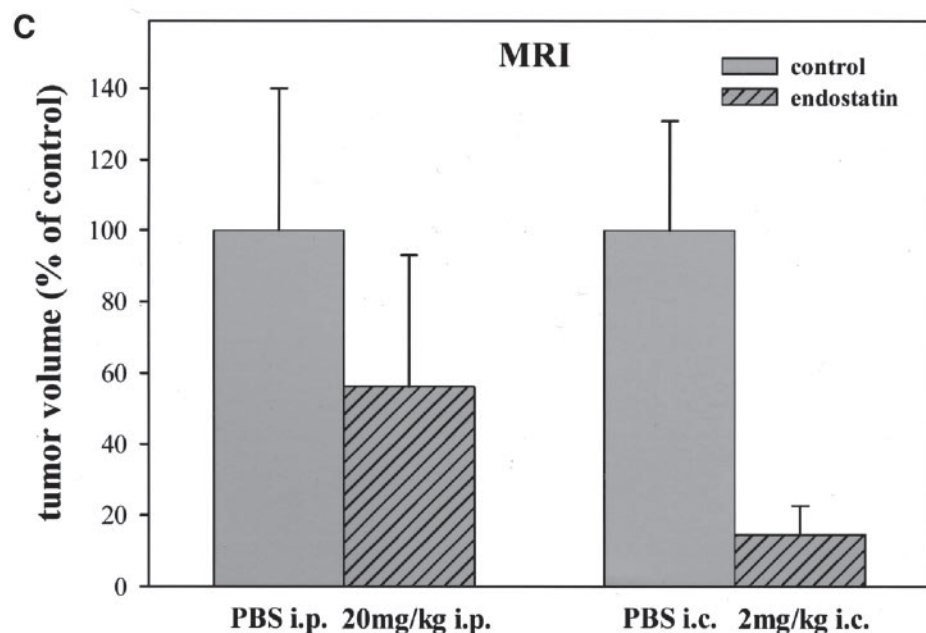
Gd-enhanced T1-weighted images were acquired. Orthotopic human glioblastoma xenografts were seen as very heterogeneous high-signal-intensity areas in the left hemisphere correlating with the corresponding histological sections (Fig. 2, *A–C* and 2, *D–F*). Three-dimensional reconstructions of Gd-enhanced T1-weighted images demonstrated the differences in tumor size between the control and treated mice having received local i.c. microinfusions (Fig. 3, *A* and *B*). The local microinfusion of endostatin resulted in an 85.3% reduction of tumor volume based on the quantification of the MR images compared with the control group. Systemic treatment showed no significant difference in tumor volume (Fig. 3*C*), as has also been demonstrated by histological analysis.

Effects of the Local Microinfusion of Endostatin on the Survival of Human Intracranial Glioma-Bearing Nude Mice. To assess whether the local i.c. microinfusion of endostatin would result in an increased survival time of nude mice with established orthotopic human glioblastoma xenografts, minipumps were implanted in the near vicinity of the tumor 8 days after tumor cell injection. The pump reservoirs were filled with either PBS for the control group or human endostatin at a total daily dosage of 2 mg/kg or 12 mg/kg. Similar to the findings using murine endostatin, the systemic treatment with human endostatin at 20 mg/kg/day was not effective in the treatment of established glioma xenografts (data not shown). We found no significant changes between the control and treated animals in blood vessel density, proliferation, or apoptosis index. In contrast, the i.c. microinfusion of 2 mg/kg/day human endostatin by osmotic minipumps significantly increased the survival time compared with the control group ($P < 0.006$; Fig. 4). Increasing the total daily dosage to 12 mg/kg endostatin prolonged the survival time ($P < 0.003$; Fig. 4). The median survival time was increased by eight days compared with the controls. At day 36, the pump reservoirs were empty, and the
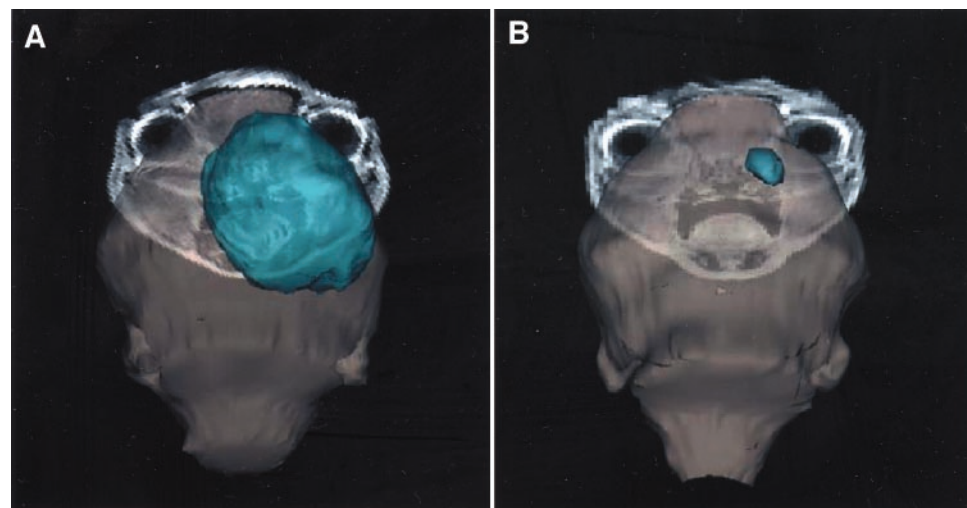
Fig. 3 Treatment effects as assessed by magnetic resonance imaging. Two representative three-dimensional reconstructions of Gd-enhanced T1-weighted images, obtained from animals 21 days after treatment start with the local intracerebral (i.c.) microinfusion of PBS (*A*) or endostatin (*B*) at 2 mg/kg/day. The local i.c. microinfusion of 2 mg/kg/day endostatin clearly induced a reduction of tumor volume. On the basis of the quantification of the Gd-enhanced T1 MR images in the randomly selected subset of animals (*n* = 5/group) systemic endostatin treatment with 20 mg/kg/day endostatin did not result in a significant reduction of tumor volume (*C, left panel*). In contrast, local i.c. delivery of a 10-fold lower amount of endostatin resulted in an 85.3% reduction of tumor volume (*C, right panel*).
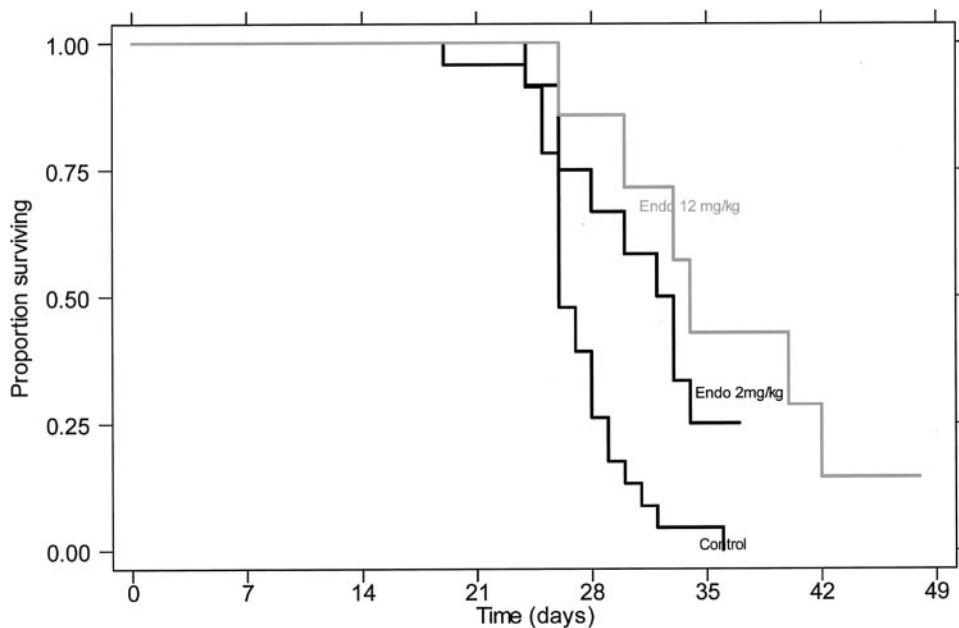
remaining mice of the 2 mg/kg/day endostatin group were sacrificed. Reservoirs from animals of the 12-mg/kg/day-endostatin group were not changed. As expected from previous experiments, withdrawal of antiangiogenic therapy leads to progression of tumor growth (21), and the experiment was terminated at day 48 after tumor cell injection.

DISCUSSION

Endostatin, a M_r 20,000 cleavage product of collagen XVIII, is a potent inhibitor of tumor-angiogenesis (4, 5, 17) by negatively affecting a variety of endothelial cell functions like proliferation, migration, survival, and proteinase activity (22). Achieving and maintaining therapeutic effective levels of endostatin is hindered by the short half-life of the protein (17). We have previously demonstrated that the continuous release of endostatin from locally implanted alginate encapsulated cells can inhibit the growth of s.c. human glioma xenografts by 72% inhibition (5). However, angiogenesis is thought to be regulated differently depending on the tissue environment of the tumor (23). Therefore, we conducted the present study in an i.c., orthotopic model and started the treatment schemes after the injected glioma cells had grown to a well-established vascularized tumor mass.

We demonstrated that local i.c. administration of endostatin at a total daily dose of 2 mg/kg via osmotic minipumps inhibited the growth of established intracranial gliomas by 73.6% and was associated with a decrease in blood vessel density and a significant increase in apoptosis. In contrast, the systemic administration of a 10-fold higher daily dosage delivered at 10 mg/kg every 12 h in animals bearing established gliomas or after surgical removal of the main tumor mass did not cause a significant therapeutic effect, although the tumors were significantly less vascularized when compared with the respective control groups. This indicates that systemically administered endostatin at a dose of 20 mg/kg/day actively inhibited the tumor-induced angiogenesis, but was not sufficient to suppress the tumor vascularization below the metabolic demands of the tumor and, therefore, eliciting a reduction of glioma growth. Noteworthy, in this context, the efficiency of an antiangiogenic therapy is not directly reflected by changes of the intratumoral blood vessel density and is, rather, an indicator of antiangiogenic activity of a compound (24). Although the same dosage has been effective in a variety of other cancer types (4, 17), our results are in line with Sorensen *et al.* (25) who also demonstrated only a modest effect of systemically administered endostatin at 20 mg/kg/day when tested in an intracranial rat gliosarcoma model.

One possibility for the failure of systemically delivered endostatin may be the impaired blood flow in a growing tumor mass reducing the availability of systemically administered endostatin in some angiogenic active areas. Especially newly formed tumor vessels are very often not functional and display a diminished and fluctuating blood flow or do not participate in the microcirculation at all (10, 26). We were recently able to demonstrate by perfusion MRI that the orthotopic U87 glioblastoma xenograft model used in this study follows the classical view of a heterogeneous and decreasing blood flow toward the center of a tumor (27).

The higher therapeutic efficacy of local i.c. delivery can be due to numerous factors: (*a*) local delivery directly to the tumor site circumvents the presence of the blood–brain barrier, which can impede entry of the drug into the brain; (*b*) it reduces the likelihood of degradation of the protein by endogenous proteases before it reaches the target 3. Higher concentrations and a more homogeneous distribution are achieved at the tumor site. Conflicting results exist about how endostatin affects endothe-

lial cell physiology, and the structure of a potential primary endostatin receptor is still not known (22). It cannot be excluded that the therapeutic effects of locally applied endostatin are enhanced because of the additional exposure of the angiogenic active endothelial cells from the blood vessel abluminal side, therefore, directly disturbing essential cell-matrix interactions (22, 28). Furthermore, recent studies raise a controversy that endostatin may have direct effects on tumor cells (28, 29). However, these effects have been cell line-dependent and need to be further evaluated.

This study confirms previous work in the treatment of experimental brain tumors that local delivery can decrease the quantity of drug needed (30). In our study, endostatin induced a reduction of tumor angiogenesis but did not result in increased glioma invasiveness, as has been seen by others when inhibiting vascular endothelial growth factor-mediated angiogenesis in experimental gliomas (5, 31). In addition, no signs of intracranial bleeding due to the presence of the pumps or antiangiogenic treatment were observed, which lends further support for direct clinical application. Furthermore, we successfully implemented the use of MRI for assessing the treatment effects of endostatin in our animal model. Using this noninvasive technique allows us to monitor dynamic changes of tumor growth in response to treatment over a period of time. Delivery and scheduling of angiogenesis inhibitors in combination with other therapeutic approaches are of particular importance for the successful clinical integration of antiangiogenic therapies (8). In this respect, MRI in experimental animal models will provide an important tool for the development of new treatment schemes.

In summary, our data demonstrate that the local microinfusion of endostatin is able to inhibit tumor growth and increase survival of animals with orthotopic malignant gliomas. Local continuous i.c. delivery is more effective than daily systemic delivery. I.c. infusions for malignant gliomas are in clinical trials, appear safe and feasible, and could immediately be used for the delivery of endostatin or other angiogenesis inhibitors in patients with malignant tumors.

ACKNOWLEDGMENTS

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