Angiostatin Suppresses Malignant Glioma Growth in Vivo

Matthias Kirsch, Jon Strasser, Rafael Alledea, Lorenzo Bello, Jianping Zhang, and Peter McL. Black

Neurosurgical Laboratories and Brain Tumor Center, Brigham and Women’s Hospital, Children’s Hospital, Dana-Farber Cancer Institute, Joint Center for Radiation Therapy, Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Human malignant gliomas are among the most malignant and most intensely vascularized solid tumors. Angiostatin, an internal fragment of plasminogen, was recently discovered as an endogenous inhibitor of tumor-related angiogenesis by selective inhibition of endothelial cell growth. Using xenograft transplants of rat and primary human glioma cells in immunodeficient mice we investigated the effects of systemic administration of angiostatin purified from human plasma on tumor growth. The rat C6 and 9L glioma and the human U87 glioma cell lines implanted either s.c. or intracranially in Swiss nude mice responded to angiostatin in a dose-dependent fashion with growth inhibition to 11% of controls (P < 0.01), without detectable signs of toxicity. The inhibition of treated tumors was accompanied by a marked reduction of vascularity to 38% of controls (P < 0.01) in the presence of an up to 6-fold increased apoptotic index (P < 0.01), consistent with the hypothesis that angiostatin acts tumorstatic by inhibiting tumor-induced endothelial cell proliferation. Expression analysis of growth factors in angiostatin-treated tumors revealed an up to 3-fold decrease in vascular endothelial growth factor-mRNA and an up to 4-fold increase in basic fibroblast growth factor-mRNA, as compared with untreated controls in rat gliomas (P < 0.01). This suggests that inhibition of the tumorigenic phenotype may be mediated in part by a downregulation of vascular endothelial growth factor expression within the tumor.

Our data demonstrate that systemic administration of angiostatin efficiently suppresses malignant glioma growth in vivo. The tumorstatic activity against intracranial tumors independent of the blood brain barrier suggests that targeting the vascular compartment may offer novel therapeutic strategies against malignant gliomas.

INTRODUCTION

Malignant gliomas, the most common of primary brain tumors, are highly aggressive tumors characterized by a recurrence rate of virtually 100%, even in the presence of aggressive treatment with surgery, radiation, and chemotherapy. Despite significant advances in neuroimaging and neurosurgical techniques, the median survival time of patients with glioblastoma multiforme has barely improved over the past 50 years and remains 12–18 months (1). Malignant gliomas are characterized by rapidly dividing cells, invasion into normal brain, and a high degree of vascularity. In fact, vascularity and endothelial cell proliferation constitute morphological features that distinguish malignant gliomas from other low-grade astrocytomas. Thus, histological grading based on vascular proliferation, endothelial cell hyperplasia, microvessel count, and grade have been shown to correlate with the degree of malignancy and prognosis of gliomas (2). Recent experimental evidence indicates that tumor-related angiogenesis contributes significantly to the malignant phenotype, as shown in the rabbit corneal pocket model in which the angiogenic response induced by glioma xenografts correlates with the degree of malignancy (3).

An adequate vascular network is required for tumor development, progression, and metastasis. The concept of angiogenesis-dependent tumor growth was formulated and experimentally demonstrated by Folkman (4). Tumor cells can induce angiogenesis by secretion of growth factors that act on endothelial cells in a paracrine fashion. Inhibition of tumor-related angiogenesis may provide a novel strategy to combat solid tumor growth. To date, several substances known to modulate angiogenesis have been tested for their efficacy against brain tumors with promising results. For example, administration of fumagillin derivatives (5), the combination of heparin and angiostatic steroids (6), and antibiotics (7) have been shown to prolong survival in animals with brain tumor implants. Recently, O’Reilly et al. (8) identified the first endogenous specific inhibitor of tumor-related endothelial cell proliferation, angiostatin. Angiostatin is a cleavage product of plasminogen that can be purified from urine and plasma of tumor-bearing mice and has been effectively used against murine and human xenotransplanted tumors, including breast, prostate, and lung carcinoma (8, 9).

The aim of the present study was to investigate the effects of systemic administration of human angiostatin on glioma xenografts in vivo.

MATERIALS AND METHODS

Cell Culture. The C6 and 9L rat glioma and the human U87 glioma cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10,000 units/ml penicillin/streptomycin/fungizone (Life Technologies, Inc., Gaithersburg, MD) in a 5% CO2 incubator at 37°C.

Purification of Angiostatin from Human Plasma. Human plasma was generously provided by the Blood Bank of Brigham and Women’s Hospital. Angiostatin was obtained after enzymatic digestion of plasminogen purified from human plasma, as described by O’Reilly et al. (8). Briefly, centrifuged and filtered plasma was diluted 1:2 in PBS and applied to a lysine-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with PBS. The column was re-equilibrated with PBS followed by 0.3 M phosphate buffer containing 3 mM EDTA (pH 7.4). Plasminogen was eluted as single peak (A280) with 200 mM aminocaproic acid (pH 7.4). The eluate was diluted with an equal volume of chloroform, the aqueous phase was removed, and was then dialyzed against 20 mM Tris-HCl (pH 7.6). Angiostatin was obtained by limited proteolysis with 0.8 units of porcine elastase/mg plasminogen (Calbiochem, San Diego, CA) for 5 h at 37°C. The reaction was quenched by applying the digest to a lysine-Sepharose column equilibrated with 50 mM phosphate buffer (pH 7.4). The column was re-equilibrated with 50 mM phosphate buffer (pH 7.4), followed by PBS. Angiostatin was eluted as single peak (A280) with 0.2 aminocaproic acid (pH 7.4) and dialyzed against PBS, followed by water. Angiostatin was then lyophilized and resuspended in PBS before use. One liter of human plasma yielded 60–85 mg of angiostatin.

Capillary Endothelial Cell Proliferation Assay. The activity of purified angiostatin was determined by assessing its ability to inhibit bFGF-induced proliferation of bovine capillary endothelial cells in vitro (8). Bovine capillary endothelial cells were kindly provided by Dr. Judah Folkman (Children’s Hospital).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a fellowship grant by the Deutsche Forschungsgemeinschaft (to M. K.).
2 These authors contributed equally to this work.

Received 5/11/98; accepted 8/17/98.

The abbreviations used are: bFGF, basic fibroblast growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; EGFr, epidermal growth factor; VEGF, vascular endothelial growth factor; TUNEL, terminal deoxyxynucleotidytransferase-mediated nick end labeling.

4654
ANGIOSTATIN SUPPRESSES GLIOMA GROWTH

Hospital, Boston, MA). Briefly, cells were plated as triplets in DMEM supplemented with 10% FCS (Life Technologies, Inc.) onto gelatinized 6-well culture plates and incubated at 10% CO2 at 37°C. After 24 h, the medium was replaced with media supplemented either with 1 ng/ml recombinant human bFGF (Scios Nova, Mountainview, CA) or with 1 ng/ml bFGF in combination with 100 ng/ml angiostatin. After 72 h cells were washed, trypsinized, and counted twice in a modified Neubauer hemocytometer. Purified angiostatin at a concentration of 100 ng/ml medium was sufficient to suppress bFGF-induced bovine endothelial cell proliferation. Angiostatin had no direct antiproliferative effects on C6, 9L, or U87 cells in vitro (data not shown).

Animal Studies. All animal work was carried out in the animal facility at Brigham and Women’s Hospital in accordance with federal, local, and institutional guidelines. Swiss nude mice (Taconic Farms, Inc., Germantown, NY) were housed in a barrier care facility. Animals were anesthetized with methoxyflurane before all procedures and observed until fully recovered. Animals were sacrificed by CO2 asphyxiation.

Male mice, 6–8 weeks of age, received s.c. injections of 2 x 10^6 tumor cells suspended in 100 µl of PBS in the proximal dorsum. For further in vivo passages, s.c. tumors were resected under aseptic conditions, minced, and s.c. reimplanted into new animals. For intracranial implantation, 5 x 10^5 cells in a volume of 5 µl of PBS were injected into the right frontal hemisphere using a stereotactic fixation device (Stoelting, Wood Dale, IL).

After a recovery period, mice received i.p. injections of angiostatin or PBS control every 6 or 12 h beginning the day after tumor cell implantation. Intracranial tumor size was determined on resected specimens after cryosectioning. Animals with s.c. implants were sacrificed after 21 days of treatment. Animals with intracranial implants were sacrificed 14 days after implantation or as soon as the mice suffered from tumor burden, or developed neurological symptoms. Resected specimens were embedded in OCT (Tissue-Tek; Miles, Elkhart, IN), quickly frozen in a dry ice butane bath and stored at -80°C.

Histological Studies. Immunohistochemistry was carried out on acetone-fixed 6-µm frozen sections using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, the sections were blocked with horse serum for 30 min, incubated with primary antibody against von-Willebrand Factor (1:250; DAKO Corp., Carpinteria, CA) or Ki-67-nuclear antigen (1:100; DAKO) for 1 h at room temperature and washed with PBS two times for 5 min. Slides were then incubated with biotinylated antirabbit IgG antibody for 30 min and washed. Detection was carried out with avidin-coupled horseradish peroxidase in the presence of chromogen, which resulted in a dark purple staining of positive cells within 5–15 min. Slides where the primary antibody was omitted served as negative controls. Sections were counterstained with 1% methyl green (Sigma Diagnostics, Stobens, MD) and mounted in Gelmount (Biomeda, Foster City, CA).

Vascularity was graded by examination of tumors at low magnification. The area of highest vascularity was identified (neovascular “hot spot”) and subjectively graded on a vascularity scale of 1+ to 4+, as described by Weidner et al. (10) and Leon et al. (2). Gliomas often display complex clusters of microvessels, known as glomeruloid structures. Tumors with glomeruloid structures were given grades of 3 or 4 as these clusters were felt to present an added risk for tumor hemorrhage and increased laterality.

RESULTS

Effects of Angiostatin on s.c. Glioma Xenografts. Untreated s.c. C6, 9L, and U87 tumor xenografts reached an average diameter of 3 cm within 21 days. At this size, the overlying skin began to ulcerate, and the animals exhibited signs of distress and were subsequently sacrificed. To determine the sensitivity of the 9L glioma cell line to angiostatin, a dose-response experiment was performed. Mice received s.c. injections of 9L cells (n = 10) and were then randomized into two groups of five animals each. Tumor growth was monitored in mice with s.c. 9L glioma implants after i.p. injections of 125 ng, 250 ng, or 1 mg of angiostatin twice daily or 1 mg three times daily, over a period of 3 weeks. These tumors were compared with tumors from mock-treated animals who received injections of PBS (n = 5/group). Although 125 ng of angiostatin every 12 h had no effect on tumor growth, 250 ng and 1 mg every 12 h led to a significant growth suppression of 47% and 69%, respectively, compared with untreated controls (Fig. 1). The highest dose of 1 mg every 8 h resulted in a growth suppression of 89% as compared with controls. In the high dose group most of the tumors were difficult to separate from overlying skin which, in fact, added to the weight of the growth inhibited tumors.

To compare the effects of angiostatin on another cell line, mice received injections of C6 or U87 glioma cells and were randomized as before. One group received injections of 1 mg of angiostatin in 100 µl of PBS by i.p. injection every 12 h over a period of 21 days, whereas
the other group received 100 µl of PBS only. Systemic administration of angiostatin led to a significant growth suppression of s.c. C6 and U87 gliomas, which grew only to 26% and 16% of the tumor weight of controls, respectively (Fig. 2). A similar dosing regimen in 9L gliomas allowed growth to only 31% of controls. No signs of toxicity such as weight loss, inactivity, opportunistic infections, or reduced appetite were observed.

**Effects of Angiostatin on Intracranial Glioma Xenografts.** Mice carrying C6 or 9L glioma xenografts in the right frontal hemisphere were treated with 1 mg of angiostatin in 100 µl of PBS, or PBS alone, over a period of 14 days. Angiostatin-treated mice implanted with C6 gliomas grew to 1.4 ± 0.3 mm in diameter (n = 4), as compared with 4.0 ± 0.9 mm in untreated controls (n = 5). A more pronounced suppression of growth was observed in angiostatin-treated mice with intracranial 9L xenografts. Except for microscopic tumor foci of up to 0.4 mm and avascular growth along the implantation tract (as shown in Fig. 3e), as well as occasional avascular subpial spread, no tumor was visible after 14 days compared with 3.6 ± 1.4 mm in untreated controls.

**Histopathology, Microvessel Morphology, Proliferative and Apoptotic Indices.** Histological sections of s.c. tumors were analyzed for microvessel morphology and neovascularization by staining for von-Willebrand-factor, which labels specifically endothelial cells. Sections of tumors from PBS-treated mice were characterized by high microvessel density with polymorphic capillaries of varying calibers. In contrast, the histological aspect of angiostatin-treated tumors was characterized by remarkably decreased vascularity. Glomeruloid capillary structures were observed only in U87 gliomas. The vessels that were more uniform, less branched, and of relatively strong caliber with fewer capillaries (Table 1; Fig. 3, a and b).

Apart from vessel paucity, angiostatin-treated tumors revealed fewer mitotic figures and hardly any necrotic areas, therefore, resembling tumors of lower pathological grade.

Proliferative indices measured after staining for Ki-67 nuclear antigen revealed no differences in angiostatin-treated tumors when compared with untreated controls (Table 1). However, apoptotic indices quantified in situ by labeling fragmented DNA with terminal transferase using the TUNEL-method were increased in tumors treated with angiostatin (Table 1; Fig. 3, c and d). As for untreated tumors, a high degree of apoptosis was seen at the growing periphery of angiostatin-treated tumors. In addition, apoptotic foci were often concentrated in perivascular areas of treated tumors, suggesting that endothelial cell growth was being controlled by apoptosis rather than inhibition of proliferation. This pattern was observed more frequently in 9L tumors than in U87 or C6 tumors.

**Expression of Growth Factors.** Angiogenesis is influenced by a variety of stimulatory and inhibitory growth factors. Therefore, mRNA expression of the growth factors VEGF, acidic and bFGF, EGF, TGF-α, PDGF-A, and PDGF-B was analyzed by Northern blot analysis and normalized to the mRNA expression of the housekeeping gene ß-actin. Angiostatin treatment was associated with a 4-fold up-regulation of bFGF-mRNA and 3-fold down-regulation of VEGF-mRNA expression as compared with untreated control tumors for both C6 and 9L. Only 9L tumors showed a significant up-regulation of TGF-α. U87 tumors did not show significant changes of bFGF or VEGF mRNA expression. No significant differences in the mRNA expression pattern of PDGF-A, PDGF-B, EGF, acidic FGF for both C6 and 9L, or of TGF-α in C6 gliomas was observed (Table 2).

**DISCUSSION**

Our data demonstrate that systemic application of angiostatin exerts potent tumor suppressing activities against glioma xenografts in nude mice. Furthermore, tumoristatic effects of angiostatin occurred without any evidence of toxicity. The endothelial cell inhibitory activity of angiostatin seems not to require an intact immune system, as primary tumors were grown in immunodeficient mice lacking T-lymphocytes. The dose-dependent effects observed with varying doses of angiostatin are consistent with a biological half-life of human angiostatin in mice of <8 h (8). Furthermore, angiostatin acts from an endovascular site as an endothelial cell inhibitor independent of the blood–brain-barrier.

Tumor-induced neovascularization results from a homeostatic balance between angiogenic stimulators and inhibitors produced by the tumor cells. Although the degree of proliferation remained at similar levels in tumor tissue irrespective of treatment, angiostatin led to a marked reduction in vascularity that was accompanied by an increased apoptotic index. A reduction in the number of vessels within the tumor in the presence of a steady proliferative rate can be expected to deprive the tumor of nutrients and impede the removal of metabolites.
ANGIOSTATIN SUPPRESSES GLIOMA GROWTH

Fig. 3. Effects of angiostatin in 9L xenografts. Microvessel morphology of sections from PBS (a) and angiostatin-treated (b; 1 mg of angiostatin twice daily) s.c. 9L xenografts after von-Willebrand factor immunostaining. Apoptotic cells stained by TUNEL in PBS (c) and angiostatin-treated (d; 1 mg of angiostatin twice daily) s.c. 9L xenografts. e, H&E staining of intracerebral 9L xenograft 7 days after implantation and angiostatin treatment. Tumor cells are scattered along the implantation needle tract without formation of solid tumor and without vascularization. f, s.c. 9L gliomas 21 days after treatment with 1 mg of angiostatin (top) or with PBS (bottom) twice daily.

but also inhibit synthesis and secretion of endothelial-derived growth factors necessary for tumor growth-associated capillary proliferation. Thus, angiostatin may force the tumor cell population into a dormant state by targeting the vascular compartment of the tumor, thereby shifting the equilibrium between those factors promoting apoptosis and those promoting proliferation, which results in an accelerated rate of apoptosis. Interestingly, we observed in angiostatin-treated tumors apoptotic cells in the perivascular areas in addition to the expected pattern of apoptosis at the growing periphery of a tumor. Apoptotic cells may be of both neoplastic and of endothelial origin, and the clustering of apoptotic cells could be the net result of two processes. First, in intratumoral areas with perivascular apoptosis, endothelial

<table>
<thead>
<tr>
<th>Table 1 Microvessel count, vascularity, apoptotic and proliferative indices in subcutaneous glioma xenografts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Microvessel count</td>
</tr>
<tr>
<td>Vascularity</td>
</tr>
<tr>
<td>Apoptotic index</td>
</tr>
<tr>
<td>Proliferative index</td>
</tr>
</tbody>
</table>

* * P < 0.01.
*** P < 0.001.
* P < 0.05.
n.s., not significant.
cells may have started to migrate and form new endothelial tubes from pre-existing capillaries/arterioles but regress and undergo apoptosis during angiostatin treatment. Secondly, because endothelial cell proliferation is inhibited, capillaries might thrombose secondary to insufficient endothelial maintenance and, therefore, lead to apoptosis of surrounding cells.

Angiogenesis is a highly complex process involving multifaceted interactions between specific growth factors, proteolytic systems, and cell-matrix components. The molecular mechanism by which angiostatin inhibits endothelial cell proliferation and secondary increases tumor cell apoptosis is unknown. One explanation may be that angiostatin exerts an inhibitory effect on tumor-derived factors that operate in a paracrine and autocrine fashion to maintain vascular growth and to ensure continuous supply of nutrients and oxygen to the growing tumor mass. These factors are secreted either by tumor or endothelial cells, mobilized from extracellular matrix-components, or released from activated macrophages. VEGF is the only growth factor known to date to exhibit both potent endothelial mitogenic and vascular permeability-inducing activity (13). It has been implicated in physiological angiogenic processes during embryonic development and wound healing (14, 15). High levels of VEGF expression have been shown in a number of highly vascularized brain tumors such as gliomas and hemangioblastomas, but only rarely in less vascularized tumors (16–18). Expression of VEGF and its cognate receptors has been demonstrated along capillaries adjacent to the necrotic foci characteristic of malignant gliomas (13). This has recently been attributed to the presence of a hypoxia-responsive element within the regulatory sequence of the gene (17). Further evidence for a major role of VEGF in glioma neovascularization stems from experiments in which targeting of VEGF with specific antibodies or its receptor with a dominant negative mutant led to a inhibition of the rat C6 glioma growth in nude mice (19, 20). The finding of a decreased VEGF expression in angiostatin-treated C6 and 9L tumors suggests that down-regulation of VEGF may represent one mechanism by which reversal of the angiogenic phenotype is achieved. Angiostatin might mediate its effect by more than one mechanism that cannot be explained by current data alone, as suggested by the unchanged bFGF or VEGF mRNA expression pattern of U87 in contrast to C6 and 9L tumors. Expression of other growth factors such as EGF, PDGF-A, and PDGF-B that have been shown to be overexpressed in gliomas and to act as stimulatory modulators of angiogenesis (21–25), but was not altered in tumors after angiostatin treatment. Interestingly, expression of bFGF and TGF-α, which have been implicated as angiogenic mitogens for glioma cell proliferation (26), was found to be increased in angiostatin-treated C6 and 9L glioma xenografts. This may suggest the presence of counter-regulatory responses to angiostatin-inhibited angiogenesis that might occur independent of VEGF-regulation. These findings of differential growth factor expression may be secondary to the inability of the tumor to recruit neovascularization and are consistent with recent observations that the expression of VEGF, but not of bFGF or TGF-α, correlates with vascular density of gliomas (27). Alternatively, angiostatin treatment could have led to down-regulation of receptors for bFGF and TGF-α, resulting in loss of sensitivity of these cells for the ligands and in a reduced bFGF and TGF-α-mediated induction of VEGF. Because U87 tumors did not show similar changes in growth factor expression, further investigations into the mechanism of angiogenesis inhibition by angiostatin on a molecular level will be necessary to examine whether the outlined hypotheses or secondary effects of inhibition of angiogenesis explain the observed effects on growth factor expression.

The histopathological appearance of angiostatin-treated tumors resembles lower grade tumors than the PBS-treated tumors, as determined by the frequency of cellularity, vascularity, and necrosis. It is most likely attributable to secondary effects of an effectively inhibited angiogenic process rather than a direct differentiating effect of angiostatin.

The effective tumor suppression achieved by angiostatin treatment provides further support for a role of the endothelial compartment in the control of glioma growth. Although vascularization constitutes an integral part of any tumor, it is of particular relevance in glioma biology as it highly correlates with the degree of malignancy and clinical outcome (2). Furthermore, treatment of glioma is complicated owing to a high degree of heterogeneity on multiple levels such as their morphological appearance, genetic alterations, and sensitivity toward radiation or chemotherapy. This may explain why gliomas remain highly resistant to conventional therapeutic measures despite numerous advances in cancer treatment. The difficulty to target this heterogeneous compartment of tumor cells mandates the continuous search for novel therapeutic concepts. The absolute requirement of glioma growth for neovascularization and the fact that endothelial cells constitute a homogenous cell population suggests that the endothelial cell compartment may prove to be the key target for successful treatment of this highly aggressive tumor. Recently, Boehm et al. (29) showed that angiogenic treatment with endostatin can eventually lead to complete tumor suppression without apparent toxicity. Indeed, the potency and lack of toxicity demonstrated here supports the notion of the vascular compartment of malignant gliomas as a novel therapeutic target.

ACKNOWLEDGMENTS

We are grateful to Drs. Rona S. Carroll, Takashi Maruyama, and Jaime Imitola for helpful discussions. We are indebted to Drs. Michael O'Reilly and Judah Folkman for their advice. We thank the Blood Bank of Brigham and Women's Hospital for generously providing human plasma.

REFERENCES

ANGIOSTATIN SUPPRESSES GLIOMA GROWTH


Angiostatin Suppresses Malignant Glioma Growth in Vivo
Matthias Kirsch, Jon Strasser, Rafael Allende, et al.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/20/4654

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.