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Successful inhibition of intracranial human glioblastoma multiforme xenograft growth via systemic adenoviral delivery of soluble endostatin and soluble vascular endothelial growth factor receptor-2:

Laboratory investigation

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Abstract

Object—Glioblastoma multiforme (GBM) is characterized by neovascularization, raising the question of whether angiogenic blockade may be a useful therapeutic strategy for this disease. It has been suggested, however, that, to be useful, angiogenic blockade must be persistent and at levels sufficient to overcome proangiogenic signals from tumor cells. In this report, the authors tested the hypothesis that sustained high concentrations of 2 different antiangiogenic proteins, delivered using a systemic gene therapy strategy, could inhibit the growth of established intracranial U87 human GBM xenografts in nude mice.

Methods—Mice harboring established U87 intracranial tumors received intravenous injections of adenoviral vectors encoding either the extracellular domain of vascular endothelial growth factor receptor-2-Fc fusion protein (Ad-VEGFR2-Fc) alone, soluble endostatin (Ad-ES) alone, a combination of Ad-VEGFR2-Fc and Ad-ES, or immunoglobulin 1-Fc (Ad-Fc) as a control.

Results—Three weeks after treatment, magnetic resonance imaging-based determination of tumor volume showed that treatment with Ad-VEGFR2-Fc, Ad-ES, or Ad-VEGFR2-Fc in combination with Ad-ES, produced 69, 59, and 74% growth inhibition, respectively. Bioluminescent monitoring of tumor growth revealed growth inhibition in the same treatment groups to be 62, 74, and 72%, respectively. Staining with proliferating cell nuclear antigen and with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

showed reduced tumor cell proliferation and increased apoptosis in all antiangiogenic treatment groups.

Conclusions—These results suggest that systemic delivery and sustained production of endostatin and soluble VEGFR2 can slow intracranial glial tumor growth by both reducing cell proliferation and increasing tumor apoptosis. This work adds further support to the concept of using antiangiogenesis therapy for intracranial GBM.

Keywords

antiangiogenesis; endostatin; gene therapy; glioblastoma multiforme; vascular endothelial growth factor

High-grade astrocytic tumors are the most common primary tumors of the central nervous system. Despite combined multimodal treatments (surgery, radiotherapy, and chemotherapy with oral alkylating agents such as temozolomide), the median survival time for patients with GBM is approximately 12–18 months.^{7,27,45,78}

The identification and development of novel therapeutic strategies therefore remains an important priority in this disease. Because GBM is characterized by endothelial cell proliferation and a high degree of vascularity, it has been suggested that antiangiogenic approaches may be an especially useful adjunct to other therapies.

Use of antiangiogenic therapy for brain tumors is based on the premise that the progressively higher grade of glial tumors requires an extensive network of blood vessels.^{20,25,34,60,61,91} Strategies to abrogate new blood vessel formation have been explored in preclinical models of GBM and have shown some success.^{23,31–33,37,41,62,69,81} In tumor-induced angiogenesis of high-grade gliomas, VEGF and its cognate receptors (VEGFR1 and VEGFR2) play a major role,^{9,17,22,44,53,59,67,75,76,82} and this receptor–ligand interaction has been the target of several antiangiogenic strategies in preclinical brain tumor models.^{8,23,31,33,51,62,81} Treatment of human GBM xenografts with monoclonal antibodies specific to VEGF inhibits neovascularization.^{8,90} The inhibition of endogenous VEGF expression by an antisense VEGF sequence significantly reduced vascularization and tumorigenicity of high-grade glioma cell lines.^{15,69} In addition to inhibitors of the VEGF pathway, other tested angiogenesis inhibitors for brain tumor therapy have included interferon- α ,^{4,18,19} angiostatin,^{18,35,36,50,56,58,66,83,92} and endostatin.^{3,6,28,38,39,52,55–57,64,65,70,77,88}

Because effective antiangiogenic therapy requires both long-term and sustained therapeutic levels at the tumor site^{21,73} we sought to explore whether a systemic gene therapy approach, delivering a sustained dose of antiangiogenic protein via gene transfer, could demonstrate an antitumor effect against GBM grown in the intracranial compartment. In this report we show that systemic delivery of recombinant adenoviral vectors encoding either a soluble VEGFR (Ad-VEGFR2-Fc) or endostatin (Ad-ES) were efficacious in slowing GBM growth in vivo. This work lays a foundation for considering the systemic delivery of these proteins in human studies for patients with GBM.

Materials and Methods

Experimental Animals

Male Swiss–Webster nude mice 4–6 weeks old were purchased from Charles River Laboratories, and all animal work was conducted at the Animal Research Core Facility at the Harvard Institutes of Medicine in accordance with institutional guidelines.

Construction of Recombinant Adenoviruses

The *E1*- and *E3*-deleted adenoviral vectors were constructed as previously described,⁴² propagated on 293A cells and purified by 2 cycles of CsCl banding.¹⁴ The final products were titrated using the optical absorbance method,^{48,49} and the results were converted to pfu/ml. Viruses produced included: Ad-Fc (batch 62.42, 3.4×10^{10} pfu/ml); Ad-VEGFR2-Fc (batch 275, 1.4×10^{10} pfu/ml; batch 275.11, 1.9×10^{10} pfu/ml; and batch 275.2, 5.2×10^{10} pfu/ml); and Ad-ES (batch 55.6, 7×10^9 pfu/ml).

Cell Cultures

The human GBM cell line U87 was purchased from the American Type Culture Collection and cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin/streptomycin, and 10% inactivated fetal bovine serum (Life Technologies, Inc.). Cells were maintained in tissue culture dishes (10-cm diameter) in 5% CO₂ and 95% air at 37°C in a humidified incubator. The U87-LuciferaseNeo (U87-LucNeo) cell line was kindly provided by Dr. Andrew Kung at Dana Farber Cancer Institute and was created by retroviral transduction with a firefly luciferase/neomycin fusion gene of the parental U87 glioma cells, followed by clonal selection.⁶⁸ The U87-LucNeo cells were grown and maintained in the same conditions as the U87 cells.

Orthotopic Implantation of Tumor Cells and Adenoviral Injections

Orthotopic human GBM xenografts were established in 4–6-week-old Swiss–Webster nude mice. Animals were anesthetized using 100 mg/kg ketamine and 5 mg/kg xylazine and received a stereo-tactically guided injection of 1×10^5 U87-LucNeo cells into the left striatum (2.5 mm lateral and 0.5 mm posterior to the bregma, at a 3.5 mm intraparenchymal depth) using a Kopf stereotactic frame (David Kopf Instruments). Using an automated microinjector (Model 310, Stoelting), the cells were injected in a volume of 2 μ l over a period of 5 minutes. Seven days after tumor cell injection the mice underwent imaging using a Xenogen IVIS system (Xenogen Corporation) to record luciferase-derived bioluminescent signals emitted from the engrafted tumors as previously described.^{12,80} Following BLI, the animals were randomized into the following treatment groups (10 animals/group) and received a single intravenous injection of 1×10^9 pfu/ml of either Ad-VEGFR2-Fc, Ad-ES, the combination of Ad-VEGFR2-Fc and Ad-ES, or Ad-Fc (control group). On Day 35 after tumor implantation, the animals were killed and their brains were harvested for histological examination.

Determination of Transgene Expression Using ELISA

Plasma samples were obtained 3 days after treatment and once a week for 5 weeks using retroorbital puncture with heparinized capillary tubes while the mice were anesthetized. Murine plasma VEGFR2 concentrations were determined by sandwich ELISA using anti-murine VEGFR2 primary antibody (BD Pharmigen) and anti-murine immunoglobulin G2a Fc-horseradish peroxidase secondary antibody (Jackson ImmunoResearch Laboratories) as previously described.⁴² Murine endostatin plasma levels were quantitated using competition ELISA (CytImmune Sciences).

Bioluminescence Imaging

Animals first underwent BLI 1 week after tumor injection, then once a week for 4 additional weeks. Animals were anesthetized with ketamine/xylazine and then received intraperitoneal injections of DLuciferin (Xenogen Corporation) at 300 µg/g body weight concentration. Approximately 5 minutes after D-Luciferin injection, mice were placed in the imaging chamber of the Xenogen IVIS 100 CCD system (Xenogen Corporation) and a grayscale image was acquired. Following this procedure, bioluminescence was acquired for 30–60 minutes while the peak BLI emission was recorded from each animal. Living Image software (Wavemetrics Corporation) was used for data analysis. For each animal an ROI was drawn, encompassing the cranium to calculate maximum photon efflux. Final BLI values are reported as total photon efflux emission (photons/second).

Magnetic Resonance Imaging

Animals underwent MR imaging prior to being killed at Week 4 after tumor injection. Tumor growth was evaluated on a 4.7-T micro-imaging system (Biospec, Bruker BioSpin, Inc.), which consists of 3-axis self-shielded magnetic field gradients, with 30 G/cm maximum gradient amplitude in all 3 channels. Gadopentetate dimeglumine (Magnevist, Schering) was administered intraperitoneally (0.25 mmol Gd/kg body weight), and the mice were anesthetized using 1% isoflurane (IsoFlo, Abbott Laboratories) in O₂ and placed within a radio frequency coil (inner diameter 35 mm). Gadopentetate-enhanced T1- and T2-weighted images were subsequently acquired with a 1-mm slice thickness (gapless) using a conventional spin-echo pulse sequence and a fast spin echo sequence, respectively, on the entire brain. The pulse repetition times and echo times were 1000 and 10 msec for T1-weighted imaging and 2000 and 40 msec for T2-weighted imaging, respectively, with a matrix size of 128 × 128, a 3-cm field of view, and 2 averages, resulting in a total scan time of approximately 4.5 and 2.4 minutes, respectively.

Immunohistochemical Determination of VEGF, PCNA, Factor VIII, and TUNEL

Paraffin-embedded tissues were used for identification of VEGF, PCNA, Factor VIII, and TUNEL. Sections (4–6 µm thick) were mounted on positively charged Superfrost slides (Fischer Scientific Co.) and dried overnight. Sections were deparaffinized in xylene followed by treatment with a graded series of alcohol (100, 95, and 80% ethanol in distilled H₂O by volume) and rehydrated in phosphate-buffered saline (pH 7.5). Sections analyzed for PCNA were microwaved for 5 minutes for antigen retrieval.⁷¹ Sections analyzed for Factor VIII were stained in the Department of Pathology, Brigham and Women's Hospital,

Boston, Massachusetts. All other paraffin-embedded tissues were treated with pepsin (Biomed) for 15 minutes at 37°C and washed with phosphate-buffered saline prior to immunohistochemical staining.⁸⁶

The TUNEL assay was performed using a commercially available apoptosis detection kit with modifications as described previously.^{11,74} Immunofluorescence microscopy was performed using an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products) to select for green fluorescence. Images were captured using a 3 CCD camera (Photometrics), mounted on a Zeiss universal microscope (Carl Zeiss), and analyzed using Optimas Image Analysis software (Bio-scan) installed on a Compaq computer with a Pentium chip, frame grabber, optical disk storage system, together with a Sony Mavigraph UP-D7000 digital color printer. Images were additionally processed using Adobe Photoshop software (Adobe Systems).

Quantification of Microvessel Density, PCNA, TUNEL, VEGF Absorbance, and Data Analysis

For the quantification of Factor VIII staining, 10 random fields (0.159 mm²) at a magnification of 100 were captured for each tumor, and microvessels were quantified according to the method described previously.^{63,85,89} Briefly, the immunostained sections were scanned, and the tumor area with the highest density of distinctly highlighted microvessels (the “hot spot”) was selected. Each stained lumen was regarded as a single countable microvessel, and if no lumen was visible, this cell was also interpreted as a single microvessel. For the quantification of the immunohistochemical reaction intensity, the absorbance of 100 VEGF-positive cells in 10 random fields (0.039 mm²) of tumor tissue at a magnification of 100 was measured using Optimas image analysis software.^{10,40} The VEGF cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis. To quantify PCNA expression, we counted the number of positive cells in 10 random fields (0.159 mm²) at a magnification of 100. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random fields (0.159 mm²) at a magnification of 100.

For bioluminescent data analysis, the ROI encompassing the intracranial space was drawn using Living Image software, and the total photons per second in the ROI was recorded on a spreadsheet. In addition, the time point at which the peak photon emission was observed was also recorded. For MR imaging data analysis, transverse Gd-enhanced T1-weighted sequences were segmented by 3 investigators (O.S., C.H.B., and S.S.B.) in 3 independent settings using National Institutes of Health image software, with a conversion of 1 MR imaging pixel = 0.529 mm³; DTATA software was used for statistical analysis and graphing. Linear regression was performed on log-transformed bioluminescence and MR imaging volume data. The data were analyzed by 2 different methods: 1) using actual peak BLI values and time-to-peak measurements; or alternatively, 2) using a parabolic curve fit (MATLAB software) to the data of each mouse to determine a calculated peak BLI and time-to-peak value.

Results

Systemic Treatment of Human GBM by Adenoviral Vectors Encoding Antiangiogenic Proteins

Seven days after intracranial implantation of U87-Luc-Neo GBM tumor cells, 5 mice were killed to confirm the presence of solid tumor lesions. Histological examination (Fig. 1) and BLI confirmed that the lesions were actively growing vascularized GBM. The remaining mice were then randomized into 4 treatment groups of 10 mice each and received tail-vein injections of Ad-Fc (10^9 pfu), Ad-VEGFR2-Fc (10^9 pfu), Ad-ES (10^9 pfu), or a combination of Ad-VEGFR2-Fc (10^9 pfu) and Ad-ES (10^9 pfu).

In the present study, the levels of VEGFR2-Fc and endostatin in the plasma were determined 3 and 35 days (in surviving animals) following viral injection. The average initial peak plasma level of antiangiogenic protein in each treatment group is shown in Fig. 2. Animals treated with Ad-VEGFR2-Fc alone had a mean plasma level of VEGFR2-Fc of 3.86 ± 2.8 mg/ml, which declined to an average of 0.53 mg/ml in surviving animals by Week 5 after treatment. The mean plasma level of endostatin in mice treated with Ad-ES was 22.5 ± 11.49 μ g/ml, which declined to an average of 935 ng/ml in surviving animals by Week 5 after injection. In a series of repeated experiments, the therapeutic effect of Ad-VEGFR2-Fc in inhibiting established intracranial glioma growth was reached at concentrations as low as 1 mg/ml (data not shown).

Tumor Growth Kinetic Calculations Based on BLI and MR Imaging

We examined tumor growth kinetics by obtaining weekly bioluminescence acquisitions from U87-LucNeo-expressing tumors as well as from MR imaging on Week 4 after tumor implantation. Figure 3 demonstrates the increase in bioluminescence from Week 1 to Week 4 for individual mice and the correlation between BLI and MR imaging at Week 4. Using this technique of dual BLI and MR imaging, we measured the therapeutic effect of adenoviral antiangiogenic therapy on intracranial tumor growth. Serial BLI measurements showed that intravenous injection of Ad-VEGFR2-Fc, Ad-ES, or the combination of the 2 significantly inhibited intracranial tumor growth in comparison with the Ad-Fc control group (Fig. 4). Treated animals showed a slower rate of tumor growth as soon as 3 weeks after initiation of therapy, and these differences were maintained until the untreated animals were killed due to terminal neurological symptoms secondary to tumor-associated cerebral herniation. At Week 4, there was a statistically significant difference in total bioluminescence ($p < 0.005$, analysis of variance) between the treatment groups and the control group.

In addition, at Week 4, the solid tumor volume (as detected by MR imaging) in treated animals was significantly smaller than the tumor volume in untreated animals. Average tumor volumes (expressed as mm^3) were calculated from segmented MR images: 35.9 ± 22.9 mm^3 in the Ad-VEGFR2-Fc group, 47.2 ± 23.5 mm^3 in the Ad-ES group, 28.9 ± 11.3 mm^3 in the Ad-VEGFR2-Fc and Ad-ES combined group, and 113.1 ± 50.8 mm^3 in the Ad-Fc control group ($p < 0.005$ for difference in means by analysis of variance; $p < 0.05$ for all treatment groups versus the Ad-Fc control group, pairwise t-tests; $p = 0.48$ for difference of

means between treatment groups). All treatment groups showed statistically smaller tumors than the control group and no statistically significant differences were noted between treatment groups. The ratio of treated versus control tumor volumes (T:C ratio) on MR imaging in the Ad-VEGFR2-Fc, Ad-ES, and Ad-VEGFR2-Fc and Ad-ES combined groups was 0.31, 0.41, and 0.26, respectively.

Comparative analysis of the tumor volume on MR imaging and bioluminescence intensity (a measure of viable tumor cells expressing luciferase) revealed a similar degree of tumor growth inhibition in the Ad-VEGFR2-Fc (BLI = 62%, MR imaging = 69%), Ad-ES (BLI = 74%, MR imaging = 59%), and the combined Ad-VEGFR2-Fc/Ad-ES (BLI = 72%, MR imaging = 74%) treatment groups.

Immunohistochemical Analysis and Microvessel Densities

In vivo cell proliferation and apoptosis were evaluated using anti-PCNA antibodies and the TUNEL method, respectively (Fig. 5). Regardless of treatment, the number of PCNA-positive cells decreased in all treatment groups as compared with the control group, and the mean number of TUNEL-positive cells was inversely correlated with PCNA positivity (Table 1). In tumors in the control group, the mean number of TUNEL-positive cells was 3 ± 2 /hpf, whereas among the treatment groups the highest count was 206 ± 18 apoptotic cells/hpf, found in tumors from mice treated with Ad-ES. Immunohistochemical analysis revealed that production of VEGF by the GBM cells was significantly reduced after treatment with Ad-VEGFR2-Fc, Ad-ES, or Ad-VEGFR2-Fc combined with Ad-ES, as compared with the Ad-Fc control; however, there was no qualitative difference observed between the different treatment groups based on immunostaining (Fig. 5).

Microvessel density, assessed using Factor VIII staining, was determined according to the method of Weidner.⁸⁵ Qualitatively, the blood vessels in brain tumors from mice treated with Ad-Fc (control) were larger and more variable in size than in brain tumors from mice treated with Ad-VEGFR2-Fc, Ad-ES, and Ad-VEGFR2-Fc combined with Ad-ES. Brain tumors in the combination treatment group of Ad-VEGFR2-Fc/Ad-ES demonstrated the smallest microvessel density in a qualitative review of the immunohistochemical results (Fig. 5).

Discussion

In this study, 2 different antiangiogenic proteins—soluble endostatin and soluble VEGFR2 extracellular domain—were systemically delivered to nude mice by adenoviral gene therapy, resulting in inhibition of growth of intracranial GBM xenografts. At Week 4, serial BLI and MR imaging of tumor volume measurements in treated and untreated animals showed that a single intravenous injection of Ad-VEGFR2-Fc, Ad-ES, or the combination of the 2, significantly reduced BLI intensity in the tumor and decreased tumor growth in the treated animals by 64–75% as compared with the control animals injected with soluble Fc.

To better understand mechanisms of action of these antiangiogenic proteins, immunohistochemical analysis of the proangiogenic cytokine, VEGF, and analysis of microvessel density was also undertaken. A hallmark of GBM (Grade IV astrocytoma) is

vascular endothelial cell proliferation and neovascularization. The neovascularization in the region of a GBM is abnormal, however, and the absolute density of new microvessels may not achieve that of the normal brain. In late stages of growth, a necrotic core often develops within the GBM, suggesting that neovascularization is not able to maintain a state of complete vascularization of the solid tumor. One study showed that the microvessel density in patients with GBM is 78% of normal brain tissue, postulated as being related to a lower O₂ consumption rate of tumor cells.⁵ It has also been suggested that lower microvessel density in tumors may relate to increased intercapillary distance as multiple layers of tumor cells surround a blood vessel.²⁶ In other tumor systems, it has been shown that the relative expression of angiogenic factors changes over time, with a resulting imperfect correlation between angiogenic factor expression and micro-vessel density.³⁰ In the present study, immunohistochemical analysis (Fig. 5) revealed that all tumors from treated animals continued to express VEGF at similar levels, but there was a qualitative reduction in both microvessel density and microvessel diameter in tumors from animals treated with Ad-VEGFR2-Fc, Ad-ES, and with combination Ad-VEGFR2-Fc and Ad-ES therapy, consistent with changes in blood vessel angioarchitecture according to therapy type.

Interestingly, the Ad-ES tumors were the most apoptotic as demonstrated by TUNEL assay, raising the question of whether endostatin in the concentrations achieved in this study may exhibit a direct apoptotic effect as well as indirect effects on tumor growth through antiangiogenesis. Li and colleagues⁴⁶ found that adenovirus-associated virus-based delivery of endostatin had direct antitumor proapoptotic effects consistent with our findings, although they did not report serum levels of endostatin. In this report, using an adenoviral delivery system, a relatively high serum level of endostatin was obtained (in the 10s of micrograms/ml range) compared with most studies that have shown efficacy of endostatin in the 150–200 ng/ml range.^{16,47,84} Notably, the initial peak levels of gene expression at Week 1 obtained in these nude mice (3.86 mg/ml for VEGFR2-Fc and 22.5 µg/ml of soluble endostatin) that yielded an antitumor effect were comparable to those achieved in a prior study (2–8 mg/ml for VEGFR2-Fc and > 15 µg/ml for endostatin) that we performed involving other nonneural tumor types growing in the subcutaneous space.⁴² The finding in this study of growth inhibition of U87 tumors by Ad-ES differs somewhat from findings in other tumor types by Kuo and associates⁴⁰ who observed only a small amount of growth inhibition in Lewis-lung-carcinoma (~ 27%) and minimal (< 12%) inhibition of P_xPC3 pancreatic tumor cells. Tjin et al.⁸⁴ produced serum endostatin concentrations of 1–4 µg/ml in a canine Fc/ES fusion protein after intramuscular adenovirus-associated virus delivery and did not observe an antitumor effect, yet found excellent growth inhibition at lower concentrations. These investigators suggested that a U-shaped efficacy curve may best explain the antitumor effects of endostatin in that model system.

In contrast to the lack of efficacy of endostatin in some tumor models, Abdollahi and colleagues¹ also observed endostatin-mediated inhibition of subcutaneous U87 tumors (subcutaneous delivery of protein), both as a single therapy and synergistically with VEGF-signaling blockage using SU5416, a VEGFR2 tyrosine kinase inhibitor. The combined therapy with SU5416 and endostatin enhanced the antiangiogenic effects in human endothelial cells in vitro and enhanced subcutaneous tumor growth delay of human xenografts (prostate adenocarcinoma PC3, non-small-cell lung cancer A549, and GBM

U87). Although we did observe an inhibition of intracranial U87 with either agent (Ad-ES or Ad-VEGFR2), we did not observe a synergistic effect of coupling endostatin with VEGF blockade as a combination therapy as has been shown in some reports.²⁴ As of August 2005, recombinant yeast-derived endostatin is no longer used in clinical studies in the US,⁸⁷ although a modified endostatin has shown efficacy in a Phase-III study in China.⁷⁹ Given that VEGF blockade (Avastin) has already demonstrated efficacy in some human tumors, a gene-based blockade of the VEGF pathway is a more feasible target for the systemic gene therapy approach as described in this study.

In terms of translation of these efforts to the clinic, several limitations and unexplored issues are worth noting. First, the tumor model used (U87) tends to grow as a solid mass when engrafted (Fig. 1), whereas high-grade gliomas in general are characterized as an invasive disease. In the invasive disease scenario, a small cluster of invading glioma cells at the leading edge or distant from a solid tumor core may be able to effectively coopt the existing blood supply and be less amenable to treatment using an antiangiogenic approach. Recent data suggest that more invasive growth patterns can be achieved using human tumor cells derived from the CD133+ population,⁷² and it will be useful to assess the efficacy of these cells in such models. Second, the xenograft model in T-cell-deficient nude mice allowed us to have long-term transgene expression over the course of the study without repeated dosing. This model does not, however, fully capture all of the problems faced when trying to achieve long-term transgene expression in the immunocompetent host, in which cellular immunity can destroy initially infected cells and humoral antibody responses markedly inhibit the effect of repeat dosing.⁵⁴ In previous work from our group, systemic intravenous delivery of first-generation adenoviral vectors in immunocompetent mice produced only transient (~ 30 days) expression with presumed immune-mediated loss of gene expression.⁴² The solution to this problem is multifactorial and potentially involves: 1) adoption of local intracerebral delivery of the vector (in the intracerebral peritumoral milieu); 2) the use of third generation helper-dependent adenoviral vectors that allow for longer persistence of gene expression; and 3) the use of immune modulation (such as cyclophosphamide) to reduce innate antiadenoviral responses.^{2,13} Immuno-modulation has been used for other immunogenic vectors such as herpes simplex viral vectors²⁹ and may be required for adenoviral-based vectors as well.⁴³

In addition, this study of “biological” therapy involving delivery of antiangiogenic proteins will need to be compared directly to the emerging array of small molecular inhibitors of angiogenesis. And finally, antiangiogenic therapy is generally not curative; second generation studies that test antiangiogenic therapy in combination with radiation and chemotherapy will be particularly valuable.

Conclusions

Although our results suggest that a systemic antiangiogenic therapy can be efficacious for inhibiting intracranial GBM growth, additional studies will be important to clarify the exact agents and delivery strategy needed to best achieve the antiangiogenic effect in the clinic. Further investigations are currently underway to address these additional questions. We

hope these studies add further support for the future clinical use of antiangiogenic therapies for patients with GBM.

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Oszkar Szentirmai, M.D., and Cheryl H. Baker, Ph.D., contributed equally to this work. Our esteemed colleague, Judah Folkman, M.D., died in January 2008.

Abbreviations used in this paper

Ad-ES	adenoviral endostatin
BLI	bioluminescence imaging
ELISA	enzyme-linked immunosorbent assay
GBM	glioblastoma multiforme
MR	magnetic resonance
PCNA	proliferating cell nuclear antigen
pfu	plaque-forming unit
ROI	region of interest
TUNEL	terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor

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FIG. 1.

Photomicrographs demonstrating the vascularized tumor at initiation of treatment. Human intracranial U87 GBM tumors established in nude mice were harvested and sectioned for histological examination 1 week after injection. A: A solid 3D tumor mass is demonstrated after H & E staining. B: The subsequent paraffin-embedded section was stained with hematoxylin and Factor VIII, demonstrating numerous blood vessels within the 1-week-old tumor xenograft. Original magnification $\times 4$ (A) and $\times 20$ (B).

FIG. 2.

Bar graph showing serum levels of endostatin and soluble VEGFR2 after systemic adenoviral-based delivery. Peripheral blood was collected from all animals 3 days following adenoviral administration and plasma levels of secreted VEGFR2-Fc and endostatin proteins were quantitated using ELISA assays. On the y axis the log of concentration of the various proteins analyzed is shown in $\mu\text{g/ml}$. On the x axis the different treatment groups and the type of ELISA that is represented (in parentheses) are shown. ES = endostatin.

FIG. 3.

Images showing the assessment of antitumor effect using BLI and MR imaging.

Representative cases from each treatment group show MR imaging sequences at Week 4 and serial bioluminescent images at Weeks 1 and 2. The ROI calculation of total photons/second was performed using Xenogen Living Image software and is represented by the *colored circle outlines*. Magnetic resonance imaging volumes were calculated following manual segmentation by measurement of gadolinium-enhancing areas of the tumor in serial axial sections.

FIG. 4.

Line graph showing the effects of the various treatments on intracranial tumor growth kinetics detected by serial BLI. The log of concentration (BLI) expressed in photons/second is shown on the y axis. The length of this study is shown on the x axis (4 weeks following the initiation of therapy). All treatments inhibited the aggressive growth of solid GBM tumors, with no significant differences observed between the treatment groups. This effect is first observed at Week 3, and the therapeutic window expands further by Week 4, indicating a sustained tumor static effect.

FIG. 5.

Photomicrographs demonstrating the results of immunohistochemical analysis of H & E, VEGF, PCNA, Factor VIII, and TUNEL staining in the 4 treatment groups. At the termination of the study at Week 4 the brains were harvested and processed for histological examination. The tissue sections were immunostained for VEGF, PCNA (the PCNA stain was used as a marker for cellular proliferation), Factor VIII (to show the presence of endothelial cells and to permit the calculation of microvessel density), and TUNEL (to show the degree of apoptosis, stained with fluorescein isothiocyanate conjugate). The highly vascularized GBM tumor is shown with Factor VIII staining. A decrease in VEGF and PCNA expression was observed in tumors from mice in all treatment groups as compared with controls. Treatment with Ad-ES significantly increased the number of TUNEL-positive tumor cells. Original magnification $\times 40$ (*top row*), $\times 20$ (all remaining *rows*).

TABLE 1

Summary of results of treatment of orthotopically implanted human GBM U87 cells by adenoviral antiangiogenic therapy

Treatment Group [*]	PCNA-Positive Cells [†]	TUNEL-Positive Cells [‡]
Ad-Fc (control)	600 ± 69	3 ± 2
Ad-VEGFR2-Fc	299 ± 54	42 ± 5
Ad-ES	398 ± 32	206 ± 18
Ad-VEGFR2-Fc + Ad-ES	289 ± 28	110 ± 22

^{*} U87 GBM cells (1×10^5) were injected into the left striatum of nude mice. Seven days later, mice were treated with a single intravenous injection of 1×10^9 pfu/ml of Ad-Fc (control), Ad-VEGFR2-Fc, Ad-ES, or Ad-VEGFR2-Fc in combination with Ad-ES. All mice were killed at Week 4.

[†] Mean ± standard deviation PCNA-positive cells/field determined by measuring 10 random fields (0.159 mm^2) at a magnification level of 100.

[‡] Mean ± standard deviation TUNEL-positive cells/field determined by measuring 10 random fields (0.159 mm^2) at a magnification level of 100.