The median survival of glioblastoma multiforme (GBM) is approximately 1 year. Following surgical removal, systemic therapies are limited by the blood–brain barrier. To circumvent this, we developed a method to modify neurons with the genetic sequence for the therapeutic monoclonal antibodies using adeno-associated virus (AAV) gene transfer vectors, directing persistent, local expression in the tumor milieu. The human U87MG GBM cell line or patient-derived early passage GBM cells were administered to the striatum of NOD/SCID immunodeficient mice. AAVrh.10BevMab, an AAVrh.10-based vector coding for bevacizumab (Avastin), an anti-human vascular endothelial growth factor (VEGF) monoclonal antibody, was delivered to the area of the GBM xenograft. Localized expression of bevacizumab was demonstrated by quantitative PCR, ELISA and western blotting. Immunohistochemistry showed that bevacizumab was expressed in neurons. Concurrent administration of AAVrh.10BevMab with the U87MG tumor reduced tumor blood vessel density and tumor volume, and increased survival. Administration of AAVrh.10BevMab 1 week after U87MG xenograft reduced growth and increased survival. Studies with patient-derived early passage GBM primary cells showed a reduction in primary tumor burden with an increased survival. These data support the strategy of AAV-mediated central nervous system gene therapy to treat GBM, overcoming the blood–brain barrier through local, persistent delivery of an anti-angiogenesis monoclonal antibody.

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INTRODUCTION

Glioblastoma multiforme (GBM), the most common central nervous system (CNS) malignancy, is an aggressive human cancer, with a median survival of about 14 months. Current therapy includes a combination of surgery, radiation and chemotherapy, but the invasive growth of the tumor prevents complete removal, and GBM is typically radioresistant. Although a great deal is known about the aberrant biology exhibited by GBM, applying therapies, including anticancer monoclonal antibodies, against these biologic processes is limited by the blood–brain barrier which restricts many systemically administered therapies from reaching the brain parenchyma. On the basis of the knowledge that GBM are highly vascular, and express high levels of the angiogenic mediator vascular endothelial growth factor (VEGF), there have been several clinical studies of systemically administered anti-VEGF monoclonal antibodies, but the results have been disappointing, with little effect on the survival from GBM.

Although several hypotheses have been proposed as to the mechanism of bevacizumab resistance in recent clinical trials, maintenance of therapeutic levels behind the blood–brain barrier is a major concern that has not been fully addressed. This is particularly concerning in areas that fail to display (magnetic resonance imaging, MRI) contrast enhancement and therefore the blood–brain barrier is presumed intact. We hypothesize that the impact of anti-VEGF therapy could be best harnessed if persistent local CNS therapeutic levels of anti-VEGF antibody could be readily achieved.

We propose to bypass the blood–brain barrier to anti-VEGF monoclonal antibody therapy by using adeno-associated virus (AAV) gene transfer vectors to deliver the genetic sequences for monoclonal antibodies directly to neurons, thus enlisting normal CNS cells for long-term delivery of therapeutic monoclonal antibodies in the local milieu. The clinical strategy would be to surgically remove as much of the tumor as possible, and at the same time, administer to the local area an AAV vector encoding the therapeutic antibody. Because neurons do not turn over, the expression of the monoclonal would be persistent, an important feature for treating GBM, where it is not feasible to surgically remove the entire tumor. On the basis of our experience in delivering genes to the CNS for other applications, we chose the AAVrh.10 vector, as it has the property of mediating excellent CNS expression specifically in neurons, and has been shown to be safe in studies of CNS gene transfer.

With this background, the present study evaluates the effectiveness of AAVrh.10BevMab, an AAVrh.10 gene transfer vector coding for bevacizumab (Avastin), to suppress the growth of human GBM in the CNS of immunodeficient mice. The data demonstrate that AAVrh.10BevMab mediates the expression of bevacizumab in CNS neurons, and reduces GBM tumor burden as shown by histology, MRI and increased survival.

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Figure 1. AAVrh.10BevMab-directed expression of bevacizumab in the mouse CNS. (a) Diagram of the mouse brain illustrates regions for expression analysis and site of vector administration. R = right; L = left. (b) Relative quantification of AAVrh.10-directed bevacizumab mRNA expression per microgram of total RNA in each brain region and in peripheral organs (n = 3). The limit of detection is denoted by the dashed line. Statistical difference by two-tailed t-test to R1 and R2, P < 0.01 is marked with asterisk (*). There was no significant difference between R1 and R2 (P > 0.4). (c) Time-dependent quantification of AAVrh.10-directed bevacizumab protein expression in each brain region compared with blood (n = 4). The limit of detection denoted by the dashed line. R = right; L = left. Statistical difference by two-tailed t-test to (R1,R2) P < 0.05 marked with asterisk (*) and P < 0.01 (**). (d) AAVrh.10BevMab-mediated expression of bevacizumab in neurons of the mouse striatum. Shown is immunofluorescent assessment of coronal section of the CNS 4 weeks after administration of AAVrh.10BevMab. Detection of AAVrh.10BevMab-directed expression of bevacizumab was assessed with anti-human IgG antibody (anti-IgG, green), glia cells were assessed with anti-glia fibrillary acidic protein (anti-GFAP, purple) and neurons were assessed with neuronal nuclear antigen (NeuN, red). Bar = 50 μm.
$10^5$ U87MG cells were administered in a volume of 2 μl, followed by $10^{11}$ genome copies AAVrh.10BevMab in a volume of 2 μl. For the 0709 low passage primary cells, $10^5$ cells were administered in a total volume of 3 μl together with $10^{11}$ genome copies AAVrh.10BevMab or control (AAVrh.10 vector coding for an irrelevant antibody or PBS) in a volume of 3 μl (total volume 6 μl). Mice were sacrificed at a time point indicated or upon signs of neurological impairment, cachexia or significant loss of weight (decrease in 1/3 adult body weight). All animal studies were conducted under protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee.

Quantification of bevacizumab

Samples of brain and organ tissue were collected after perfusion with cold PBS (pH 7.4). Coronal sections of mouse brain divided the two hemispheres into four segments (equidistance anterior to posterior). The levels of bevacizumab mRNA were assessed by RNA reverse transcription and TaqMan quantitative PCR (Applied Biosystems, Grand Island, NY, USA). Levels of bevacizumab antibody were determined by ELISA and western blotting, and cell localization of bevacizumab expression assessed by immunohistochemistry (see Supplementary Materials and Methods for details).

MRI quantification

MRI was performed on a 7.0 Tesla 70/30 Bruker Small Animal MRI scanner (Bruker Biospin, Billerica, MA, USA) equipped with an additional small animal imaging gradient set (45 G cm$^{-1}$). A T1-weighted 2D FLASH sequence was used to visualize contrast enhancement with a repetition time of 357 ms and an echo time of 3.8 ms. T2-weighted Turbo RARE sequences were also acquired with repetition and echo times of 2300 ms and 48 ms, respectively, to detect edema. A 20 mm field of view and 256 × 256 matrix produced an image resolution of 78 μm × 78 μm × 500 μm with 20 matching axial slices.

Tumor burden was assessed for the U87GM line T1(gadopentetate dimeglumine-enhanced) and T2-weighted sequences using IDL 8.1 custom coded algorithms (Exels Visual; Boulder, CO, USA). For the U87MG tumor burden, tumor volume (ml) was assessed using T1 gadopentetate dimeglumine-enhanced sequences of the demarcated tumor. For the 0709 primary tumor, tumor burden was assessed using T2-weighted sequences. Given the diffuse nature of the 0709 tumor, tumor burden was calculated on the whole brain volume (ml), by outlining the perimeter of mouse brain.

Statistics

Data are expressed as means ± s.e., and comparisons between groups were conducted by a two-tailed unpaired t-test, comparisons between treatment groups at multiple time points were conducted by two-way analysis of variance (MedCalc Software, Ostend, Belgium). The survival
data were generated using Kaplan–Meier survival plot and groups were compared using the Mantel-Cox test (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

The area of AAVrh.10BevMab CNS administration was compared to other regions of the CNS to assess bevacizumab mRNA and protein levels in and around the targeted region and absence in the non-targeted left hemisphere and posterior regions of the CNS (Figure 1a). Regional sections of the mouse brain and peripheral organs assayed by reverse transcription quantitative PCR showed AAVrh.10BevMab-directed expression of bevacizumab mRNA in the targeted region of the right hemisphere to be significantly greater (more than 8-fold) than the highest level observed in the left hemisphere (L2) and significantly greater (more than 10-fold) than the highest levels of a peripheral organ, the liver, with much lower levels in other organs (n = 3; P < 0.01; Figure 1b). Detection of expressed bevacizumab protein levels by ELISA and analysis of total protein showed localized expression of the bevacizumab monoclonal antibody in the area of the administration (n = 4; Figure 1c). The targeted area of the right striatum showed the highest expression among the sections at 2 weeks, with increased levels at 6 weeks, the last time point measured. There was minimal expression in posterior regions and opposite (left) hemisphere of the mouse brain. Importantly, significantly lower or below limit of detection levels of bevacizumab mRNA were measured in non-targeted peripheral organs (liver, lung, heart and kidney) and blood (P < 0.01; Figure 1b). Likewise, only minimal bevacizumab protein was found in the blood (50-fold less than the injected region of the brain; P < 0.02; Figure 1b, c).

Immunohistochemical assessment of the CNS showed AAVrh.10BevMab-directed expression of bevacizumab in the area of administration. Coronal sections taken at 1 mm anterior of the AAVrh.10BevMabPBS AAVrh.10BevMab AAVrh.10control Tumor volume (mm³)

| Tumor growth | PBS AAVrh.10BevMab |
|--------------|-------------------|
| Tumor Growth | p<0.02            |

| Tumor Growth | AAVrh.10control AAVrh.10BevMab |
|--------------|-------------------|
| Tumor Growth | p<0.04            |

Figure 3. MRI assessment of tumor volume of mice with U87MG glioblastoma treated with AAVrh.10BevMab or control. U87MG and AAVrh.10BevMab was administered simultaneously (a, b) or 6 days after xenograft (c, d). In (a) and (c), arrows indicate site of tumor on representative coronal image of striatum. Each coronal MRI image corresponds to site of xenograft implantation in four distinct mice. (a) MRI, PBS-treated control mice (n = 4) and AAVrh.10BevMab-treated mice (n = 4). The scans were carried out at 18 days after U87MG implantation. (b) Quantification of tumor volumes (from multiple coronal MRI images of whole brain), PBS-treated control (n = 4) vs AAVrh.10BevMab-treated mice (n = 4) at 18 days. (c) MRI, PBS-treated control mice (n = 4) and AAVrh.10BevMab post-xenograft treated mice (n = 4). The scans were carried out at 20 days after U87MG implantation. (d) Quantification of tumor volumes in PBS vs post-xenograft treated mice (n = 4 per group) at 20 days.
Concurrent treatment with AAVrh.10BevMab increased the survival time in mice that received the AAVrh.10BevMab vector in the previous group. The results were similar to the group with PBS instead of the viral vector.

In the first set of experiments, NOD/SCID mice received U87MG cells and the AAVrh.10BevMab vector concurrently. The control groups received tumor cells and PBS instead of the viral vector. Animals underwent serial MRI imaging and were assessed for tumor volume and sacrificed upon evidence of significant cachexia or neurological impairment. Upon sacrifice of the mice, the brain tissue was assessed for vascular density as evidence for neo-angiogenesis, as well as tumor histology. All animals had evidence of surviving tumor cells with distinct borders (Figure 2a). Immunohistochemistry for the endothelial marker CD31 demonstrated a substantial decrease in blood vessel density in the tumor area of AAVrh.10BevMab-treated mice, compared with PBS controls (n = 3, P < 0.05, Figure 2b and c). The vessels seen in the AAVrh.10BevMab-treated animals display a more normal morphology as compared with the large sinusoidal vessels seen in the untreated tumors.

Serial MRI using gadopentetate dimeglumine enhancement demonstrated a progressive increase in tumor volume in the untreated mice, whereas the treated group exhibited reduced tumor growth by greater than fivefold at 18 days post injection (Figure 3a and b). To more closely approximate the clinical setting, a second group of mice received tumor cell implants followed 6 days later by administration of the AAVrh.10BevMab vector. The animals were monitored and assessed by MRI as with the previous group. The results were similar to the group with concurrent administration. MRI showed a statistically significant 2.4-fold decrease in tumor volume at 20 days (n = 4, AAVrh.10BevMab post xenograft treated vs AAVrh.10control, P < 0.04; Figure 3c and d).

Survival data demonstrated a statistically significant increase in survival time in mice that received the AAVrh.10BevMab vector in both experimental strategies (concurrent and post-xenograft). Concurrent treatment with AAVrh.10BevMab increased the median survival time of mice with GBM xenografts by 42% (n = 9, AAVrh.10BevMab-treated vs AAVrh.10control, P < 0.003; Figure 4a), whereas treatment post tumor establishment led to a larger 64% increase in survival (n = 6, AAVrh.10BevMab post-xenograft treated vs AAVrh.10control, P < 0.004; Figure 4b). A similar experimental paradigm was repeated in NOD/SCID mice implanted with GBM cells obtained from a freshly dissociated patient tumor and passaged briefly in serum-free media. MRI analysis over time showed a reduced tumor burden in the treatment group (Figure 5a). MRI volumetric assessment showed a reduction in primary tumor burden by 3.3-fold at 12 weeks in the treated group (n = 3, treated vs control-treated, P < 0.002 Figure 5a). Survival was also extended in the AAVrh.10BevMab group through week 15 (n = 21, treated vs control-treated, P < 0.006; Figure 5b).

Tissue analysis demonstrated large infiltrating tumors in the control group whereas the AAVrh.10BevMab-treated mice showed clusters of tumor growing in areas well beyond the site of AAV administration. Immunohistochemistry demonstrated the absence of bevacizumab antibody secretion in areas of tumor growth, with few single cells infiltrating around the area of vector administration (n = 3; Figure 5c). Interestingly, tumor cells growing in the AAVrh.10BevMab-treated animals demonstrated an increase in the expression of phosphorylated c-Met, which has been proposed recently to represent an evasive mechanism in response to anti-angiogenic inhibition (n = 3; Figure 5d).

**DISCUSSION**

The blood–brain barrier represents a major challenge in the development of an effective therapy directed toward glioblastoma, as it restricts systemically administered large molecules from reaching the targeted area in the brain. Although the blood–brain barrier can be permeable in areas of high neo-angiogenesis and leaky vasculature, these ‘contrast-enhancing’ regions are frequently the target of debulking surgery.

However, tumor cells are known to persist in an infiltrative manner in the surrounding regions, usually in areas where the blood–brain barrier is intact (non-contrast-enhancing regions). These cells are frequently responsible for tumor recurrence and are more difficult to target with conventional therapies.

By direct administration of the AAVrh.10BevMab vector to the CNS of immunodeficient mice with human GBM, we have...
bypassed the blood–brain barrier to deliver to neurons the genetic code for the monoclonal antibody bevacizumab. The data demonstrate persistent expression of bevacizumab resulting in reduced tumor blood vessel density in the area of tumor, a significant reduction in tumor growth and a significant increase in survival in both concurrent and post-xenograft models. AAV-mediated gene transfer of therapeutic monoclonal antibodies directly to the CNS overcomes a common hurdle to anti-tumor therapy, the blood–brain barrier, and through local and persistent expression, provides an effective barrier to neo-angiogenesis.
Glioblastoma multiforme
GBM accounts for the majority of all primary brain tumors.\textsuperscript{1-3} Despite advances in the molecular and physiological characterization of GBM, the median overall survival of GBM patients remains little more than 1 year. The standard therapy to increase survival in GBM patients consists of maximal surgery followed by radiation and various chemotherapy agents. Nonetheless, these tumors are remarkably resistant to both radiation\textsuperscript{4-6} and chemotherapy and typically recur within months of treatment.\textsuperscript{28,29}

GBM is distinguished by prominent vascular proliferation, and rapid and invasive growth, and was thus thought to be a prime target for anti-angiogenesis inhibitors. The introduction of bevacizumab to the clinic was met with initial excitement, as normalization of the vessels by this agent leads to significant palliation of symptoms related to leaky vessels and cerebral edema. However, recent data indicate that the response of GBM to bevacizumab in humans is relatively short-lived, with emerging evidence of tumor refractoriness and no significant impact on overall survival.\textsuperscript{30}

The mechanisms of this failure remain unclear, but several possibilities have been proposed.\textsuperscript{31} Those include the amplification of pro-angiogenic genes in resistant tumors, thus requiring higher doses of anti-angiogenic treatment, or an escape to a different mode of angiogenesis such as vasculogenesi.\textsuperscript{32} Alternative mechanisms of angiogenesis, including the Notch pathway, or a transition to a more invasive phenotype have also been suggested.\textsuperscript{33,34} However, studies show that disruption of the blood–brain barrier can enhance permeability to drugs like bevacizumab; therefore, a key element pertaining to the failure of bevacizumab in the clinic could be the degree of penetration across the blood–brain barrier, especially in areas of relatively normal vasculature.\textsuperscript{15} Although studies of angiogenic growth and proliferation pathways of primary tumors has uncovered GBM-sensitive targets, the systemic delivery of immunotherapies to block these targets in GBM clinical trials has not increased survival.\textsuperscript{7,8,11,12,34,35} The disadvantage of current GBM therapy with monoclonal antibodies is the necessity for repeat administration delivered by the intravenous route and at high dose, resulting in systemic distribution of the therapeutic monoclonal antibody.\textsuperscript{35,36} Although the targeted area is within the CNS, systemic delivery results in drug distribution to healthy non-targeted organs and there is limited diffusion of the monoclonal into the tumor.\textsuperscript{37-39} AAV-directed gene therapy to the CNS overcomes these challenges, potentially eliminating side effects while maintaining a long-term and effective dose directed at the site of the tumor.\textsuperscript{16}

Anti-VEGF therapy for glioblastoma
GBM is characterized by extensive vasculature and thus is susceptible to angiogenesis inhibitors. Like many other tumors, most GBM produce VEGF, a major inducer of angiogenesis.\textsuperscript{3} The classical form of VEGF (VEGF-A), functions through three receptors and a transition to a more invasive phenotype have also been suggested.\textsuperscript{33,34} However, studies show that disruption of the blood–brain barrier can enhance permeability to drugs like bevacizumab; therefore, a key element pertaining to the failure of bevacizumab in the clinic could be the degree of penetration across the blood–brain barrier, especially in areas of relatively normal vasculature.\textsuperscript{15} Although studies of angiogenic growth and proliferation pathways of primary tumors has uncovered GBM-sensitive targets, the systemic delivery of immunotherapies to block these targets in GBM clinical trials has not increased survival.\textsuperscript{7,8,11,12,34,35} The disadvantage of current GBM therapy with monoclonal antibodies is the necessity for repeat administration delivered by the intravenous route and at high dose, resulting in systemic distribution of the therapeutic monoclonal antibody.\textsuperscript{35,36} Although the targeted area is within the CNS, systemic delivery results in drug distribution to healthy non-targeted organs and there is limited diffusion of the monoclonal into the tumor.\textsuperscript{37-39} AAV-directed gene therapy to the CNS overcomes these challenges, potentially eliminating side effects while maintaining a long-term and effective dose directed at the site of the tumor.\textsuperscript{16}

Bevacizumab is widely used to treat metastatic colon and non-small cell lung cancer, and ocular vascular proliferative disorders.\textsuperscript{34-36} Systemic administration of bevacizumab has been tried as a therapeutic for GBM with limited success.\textsuperscript{9,34,48} Unlike the application of bevacizumab for the treatment of macular degeneration where bevacizumab is injected directly into the eye,\textsuperscript{44} the trials for GBM with bevacizumab to date use systemic delivery and thus are limited by the blood–brain barrier in achieving therapeutic levels in the CNS.\textsuperscript{10,49} The data in the present study demonstrate a novel mode of delivery showing efficacy with direct CNS delivery of the AAVrh.10BevMab vector to the local area surrounding the tumor xenograft. In the human GBM tumors tested, AAVrh.10BevMab inhibited angiogenesis, reduced tumor growth and increased survival in mice.

In the clinical scenario, AAVrh.10-mediated therapy would be applied at the time of GBM surgical removal. As complete removal is not always feasible, the therapy vector would be administered in the milieu of the excised tumor, and within 1 week, local neurons would begin expression of a therapeutic dosage of the monoclonal antibody inhibiting GBM recurrence. The stability of neurons would ensure persistent expression of the monoclonal antibody, thereby preventing angiogenesis and proliferation of the remaining tumor cells. In the current study, primary tumor cells survived and proliferated in regions beyond the area of bevacizumab expression, suggesting that the escape mechanism may be related to region of AAVrh.10BevMab administration or dosage rather than resistance. These data suggest that the high persistent levels of AAVrh.10-mediated bevacizumab expressed within tumors are effective to inhibit tumor growth. Furthermore, tumor growth in the treated animals initiated in regions of the brain relatively removed from the area of AAVrh.10BevMab administration. In human translation, AAV vector dose and antibody expression could be modulated to deliver optimal levels of the anti-VEGF monoclonal antibody in critical regions to sufficiently block VEGF-sensitive GBM.

Interestingly, the upregulation of phosphorylated c-Met in AAVrh.10BevMab-treated tumors observed in this study indicates a tumor refractory response due to either low dose bevacizumab within regions of the parenchyma, or alternatively, the selection of subclones of tumor cells with elevated c-Met. Because activation of the c-Met pathway is associated with an increased capacity for invasion and migration, these results suggest the selection of the c-Met subclones in treated tumors. This study illustrates a proof of principle for the strategy of AAV delivery of a monoclonal antibody beyond the blood–brain barrier. Future studies that make use of combination treatments, targeting additional tyrosine kinases, such as c-Met, may be necessary for a more effective and significant tumor control. In effect, AAV vectors could be readily modified to encode for distinct monoclonals blocking these and other oncogenic signaling pathways, such as the SDF1/CXCR4 and Notch pathways.\textsuperscript{32,50} A combinatorial approach using AAV-monoclonal delivery would favorably alter (and bolster) the therapeutic tumor microenvironment. Our experience with AAV-directed delivery of genes to the CNS has demonstrated that the AAVrh.10 vector mediates an excellent CNS expression profile with specificity for neurons, and has been shown to be safe in studies of CNS gene transfer.\textsuperscript{16,19} Efficacy in this study opens the door to future research with AAV-mediated delivery of other anti-tumor antibodies in the treatment of glioblastoma and other malignancies within the CNS.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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