Conditionally replicating adenoviruses (CRAd) are a promising class of gene therapy agents that can overcome already known glioblastoma (GBM) resistance mechanisms but have limited distribution upon direct intratumoral (i.t.) injection. Collagen bundles in the extracellular matrix (ECM) are an important role in inhibiting virus distribution. In fact, ECM pre-treatment with collagenases improves virus distributions to tumor cells. Matrix metalloproteinases (MMPs) are an endogenous class of collagenases secreted by tumor cells whose function can be altered by different drugs including anti-angiogenic agents, such as bevacizumab. In this study we hypothesized that upregulation of MMP activity during anti-angiogenic therapy can improve CRAd-S-pk7 distribution in GBM. We find that MMP-2 activity in human U251 GBM xenografts increases ($P = 0.03$) and collagen IV content decreases ($P = 0.01$) during vascular endothelial growth factor (VEGF-A) antibody neutralization. After proving that collagen IV inhibits CRAd-S-pk7 distribution in U251 xenografts (Spearman rho = −0.38; **$P = 0.003$), we show that VEGF-blocking antibody treatment followed by CRAd-S-pk7 i.t. injection reduces U251 tumor growth more than each individual agent alone (**$P < 0.0001$). Our data propose a novel approach to improve virus distribution in tumors by relying on the early effects of anti-angiogenic therapy.

Keywords: anti-VEGF; bevacizumab; oncolytic virus; adenovirus; metalloproteinase; glioma; glioblastoma; brain tumor
In this study we investigated the effects of short-term VEGF neutralization via blocking antibody (VEGF-Ab), on the brain tumor microenvironment. Specifically, we show that MMP-2 levels are increased upon treatment with VEGF-Ab in different glioma cell lines, as well as in glioma xenograft models. In turn, these high MMP-2 levels degrade collagen IV bundles present in the ECM of GBM. Moreover, when oncolytic adenovirus injection follows anti-angiogenic therapy, it results in increased i.t. distribution and higher adenoviral titers. Finally, we show that combination therapy with VEGF-blocking antibody and CRAd-S-pk7 inhibits tumor growth more effectively than each individual agent alone.

RESULTS
Anti-angiogenic therapy has no additive effect to oncolytic adenovirus toxicity in vitro

We first evaluated whether in vitro VEGF antibody blocking (VEGF-Ab) can alter expression of adenovirus receptors in glioma cells. CRAd-S-pk7 attachment and entry into the host cell relies on the interaction between the adenoviral fiber and surface receptors such as coxsackie-adenovirus receptor (CAR), integrins (\(\alpha v\beta 3\) and \(\alpha v\beta 5\)) and CD138. We did not detect any significant changes in expression of these receptors on U251 glioma cells after VEGF blockade (Figure 1a). Coxsackie-adenovirus receptor expression varied from 29.2\(\pm\)4.8% in the control group to 29.1\(\pm\)1.4% for the VEGF-Ab-treated cells (\(P = 0.98\)). Similarly, the level of integrins \(\alpha v\beta 3\) (from 48.7\(\pm\)2.6 to 43\(\pm\)7%; \(P = 0.4\)), \(\alpha v\beta 5\) (from 75.2\(\pm\)4 to 75.3\(\pm\)4.8%; \(P = 0.98\)) and CD138 (from 12.4\(\pm\)1.1 to 12.5\(\pm\)0.1%; \(P = 0.86\)) did not change significantly after treatment.

Next we tested whether VEGF-Ab therapy could increase adenovirus replication in vitro. In glioma cells, we quantified adenoviral E1A copy numbers (Figure 1b), through quantitative real-time (qRT)-PCR, and the infectious progeny titers produced (Figure 1c). On day 5 we noticed that in cells treated with VEGF-Ab, adenoviral E1A copies were significantly higher compared with Ig-control-treated cells (9.1\(\times\)10^7 vs 3.6\(\times\)10^7 E1A copies per ng DNA; \(*P = 0.01\)). Also, the infectious progeny produced was fourfold higher in the VEGF-Ab-treated group (4.13\(\times\)10^9 vs 1.02\(\times\)10^9 infectious units per ml (IU ml\(^{-1}\)); \(*\ast P < 0.0001\).

To assess whether increased adenoviral replication in U251 glioma would result in more toxicity, we treated CRAd-S-pk7-infected cells with VEGF-Ab and performed a viability assay 7 days later. VEGF-Ab treatment of infected cells in vitro did not increase CRAd-S-pk7 toxicity to glioma cells (Figure 1d).

VEGF neutralization increases MMP-2 levels in glioma cell lines

To understand how VEGF neutralization can alter the glioma microenvironment, we quantified the expression of two major MMPs related to glioma invasion: MMP-2 and MMP-9, following anti-angiogenic therapy. In all four glioma cell lines that were tested, there was a dramatic increase in MMP-2 levels after VEGF-Ab (Figure 2a). No difference was noted in MMP-9 expression after treatment.

![Figure 1. In vitro effects of VEGF neutralization on adenovirus replication. (a) Flow cytometry analysis of U251 glioma cell line for expression of surface receptor CAR, \(\alpha v\beta 3\), \(\alpha v\beta 5\) and CD138. The percentages of positive cells for the respective receptors are shown in bar diagrams below the flow cytometry histograms. (b, c) CRAd-S-pk7 replication in U251 glioma treated with VEGF-Ab was quantified via quantitative real-time PCR for E1A (b) or adenovirus progeny titer (c). (d) Toxicity of CRAd-S-pk7 in combination with VEGF-Ab in U251 glioma cells 5 days after infection (NS: not significant; \(*P < 0.05; \ast \ast \ast P < 0.001\).](image-url)
To understand how VEGF-Ab upregulates MMP-2, we quantified its mRNA expression at different time points. We observed that VEGF-Ab upregulates MMP-2 gene transcription in all four cell lines differently (Figure 2b), as compared with IgG control-treated cells. In the case of U87 glioma cell line, we noticed that induction of MMP-2 transcription reached its peak within 24 h after VEGF-Ab therapy. For U251, RNA MMP-2 peak levels were detected on day 3 of VEGF-Ab treatment; whereas in U118 and A172 MMP-2 transcription was affected similarly. There was no significant alteration during the first 3 days and the peak was reached on day 5 of therapy. Additionally, the effect of VEGF blocking on the peak levels of MMP-2 varied among the cell lines. U251 glioma cell line achieved the highest MMP-2 transcription, approximately sevenfold higher than control-treated cells. Compared with control-treated cells, U87 maximal transcription following VEGF-Ab therapy was fourfold higher. In U118 and A172, the mRNA levels reached an upregulation of only twofold.

We also looked at the level of collagen IV expression in the same glioma cell lines after VEGF-Ab therapy. Despite upregulation of its collagenase (MMP-2) levels, there were no detectable changes in collagen IV levels after in vitro therapy (Figure 2c).

High MMP-2 levels reduce collagen IV content in glioma xenografts

To detect the microenvironmental changes induced after short-term (5 days) anti-angiogenic therapy, we relied on the highly tumorigenic U251 and U87 glioma cell lines. Flank tumors were left to grow up to 0.5 cm in diameter before starting treatment with VEGF-Ab or Ig-control. Similar to our in vitro findings, U251 glioma xenografts upregulated more than threefold MMP-2 expression on day 5 after bevacizumab therapy (Figures 3a and b; \( P = 0.0274 \)). The difference was even more evident in smaller tumors, which in the absence of anti-angiogenic therapy expressed barely detectable levels of MMP-2. Consistent with our in vitro observation, MMP-9 expression was not altered during bevacizumab therapy (data not shown).

Moreover, we tested the possibility that CRAd-S-pk7, by itself, can induce changes in MMP-2 levels in presence or absence of anti-angiogenic therapy. Mice bearing U251 xenografts (\( n = 5 \)) were treated as above, with VEGF-Ab or Ig-control for 5 days, and then injected with \( 10^{11} \) IU of CRAd-S-pk7 or phosphate-buffered saline (PBS) i.t. Mice were killed 3 days later. We did not find any difference in MMP-2 expression between mice that received CRAd-S-pk7 or PBS, irrespective of whether they were treated with VEGF-Ab or Ig-control before (Figures 4a and b).

Furthermore, VEGF-Ab therapy not only upregulates MMP-2 but also was associated with significantly reduction of collagen IV content in U251 (Figures 3a and c; \( * P = 0.0133 \)) and U87 xenografts (Figures 4c and d; \( ** P = 0.006 \)).

There were several other interesting observations following VEGF-Ab therapy that did not reach statistical significance. First, we observed a trend in reduction of vessel density (Figures 3d and e). Second, CD31, or PECAM, a marker of endothelial cells was found to be non-significantly reduced to 68 ± 9 vessels per mm² after therapy compared with 125 ± 21 vessels per mm² in the control group (\( P = 0.54 \), NS not significant). Similarly, there was a trend in reduction of laminin expression after bevacizumab therapy that did not reach statistical significance (\( P = 0.197 \); NS).

High collagen IV content in intracranial glioma correlates negatively with adenovirus distribution

To understand how collagen contents can affect adenovirus distribution in intracranial (i.c.) glioma, we assessed CRAd-S-pk7 localization in relation to collagen bundles. We stained normal mouse brain and mouse brain bearing i.c. U251 glioma for collagen IV and adenosinar hexon protein. In normal mouse brains (Figure 5a, i–iii) collagen IV was found to be organized around vessels. On the other hand, in i.c. U251 glioma xenografts (Figure 5a, iv–vi) we observed extensive disorganized bundles of collagen similar to flank xenografts. Upon VEGF-Ab therapy, there was a substantial reduction in collagen deposits in i.c. glioma (Figure 5a, vii–ix) that remained disorganized.
To show that collagen bundles can indeed block adenovirus distribution, we investigated the relationship between collagen content and adenovirus hexon distribution in orthotopic glioma xenografts. An analysis of a representative area (Figure 5b) is shown. On the basis of immunofluorescent staining intensity we created a heat map graphical representation for each antigen and analyzed their correlation. We found an inverse correlation between collagen content in U251 glioma and CRAd-S-pk7 distribution (Spearman rho \( \rho \approx -0.38 \); ** \( P = 0.0028 \)).

VEGF neutralization increased CRAd-S-pk7 distribution and replication in glioma xenografts

We quantified the adenoviral hexon distribution in glioma xenografts after CRAd-S-pk7 i.c. injection. We found that short-term anti-angiogenic therapy more than doubled adenovirus distribution (Figures 6a and b; \( \ast P = 0.044 \)) in the orthotopic glioma. Quantification of the VEGF-Ab effect on adenoviral replication within tumors was performed in U251 glioma and CRAd-S-pk7 distribution (Spearman rho \( \rho = -0.38 \); ** \( P = 0.0028 \)).

Combination therapy with VEGF neutralization and CRAd-S-pk7 reduces glioma growth more than each therapy alone

To evaluate whether the higher adenoviral titers and distribution resulted in reduced tumor growth, we treated mice bearing U251 xenografts with VEGF-Ab, CRAd-S-pk7 or combination therapy. Oncolytic adenovirus therapy alone slowed tumor growth at a similar rate to short-term anti-angiogenic therapy. The group that received oncolytic adenovirus after short-term VEGF-Ab therapy had the largest reduction in tumor growth rate (Figure 7; *** \( P < 0.0001 \)). The time needed for the average tumor volume to double was shortest in the PBS-treated animals (2.6 days) and longest in the combination therapy group (11.9 days). Animals that received CRAd-S-pk7 and VEGF-Ab had a tumor doubling time of 7.6 and 8 days, respectively.

DISCUSSION

This study addresses the effect of anti-angiogenic therapy on oncolytic adenovirus replication and distribution both in vitro and in vivo. We found that VEGF neutralization/blocking increases MMP-2 expression in glioma cell lines. In glioma xenografts, VEGF-Ab upregulated MMP-2 and reduced the amount of its substrate, collagen IV. When CRAd-S-pk7 was injected i.t. following VEGF-Ab administration, it resulted in increased viral distribution (Figures 6a and b). Short-term VEGF-Ab therapy followed by adenovirus injection resulted in increased i.t. adenoviral titers (Figure 6d). Moreover, this combination therapy was superior in reducing tumor growth rate than each individual therapy (Figure 7).

In vitro VEGF neutralization can disrupt glioma cell metabolism and alter phenotype.\(^{15}\) Cell surface changes can result in a more or less hospitable environment for oncolytic adenovirus replication. Therefore, we first tested whether VEGF neutralization would alter the expression of surface receptors used by adenovirus for attachment and entry into glioma cells. We did not detect any changes in the expression levels of commonly used receptors for adenovirus transduction. Bevacizumab induces expression of hypoxia-induced factor (HIF-1\( \alpha \))-regulated genes, and survivin is downstream HIF-1\( \alpha \).\(^{17,18}\) Of note, adenoviruses lacking survivin promoter did not replicate.

**Figure 3.** Anti-angiogenic treatment alters the ECM architecture of human glioma xenograft in nude mice. (a) Immunohistochemistry staining for MMP-2, Collagen IV, CD31 and Laminin. (b-e) Quantification of staining intensity was done through a computer-based scoring for each of the corresponding IHC slides (\( n = 5 \) animals for each group) and mean values ± s.e. of measurement (s.e.m.) are presented in bar diagrams. Bars = 50 \( \mu \)m; \( \ast P < 0.05 \); NS, not significant.
more in presence of bevacizumab in vitro." Nevertheless, higher adenovirus replication did not result in an increase of oncolytic toxicity toward glioma cells. We expected higher adenovirus replication to result in more toxicity, but the same factors that may have increased replication (survivin upregulation) have been shown to have anti-apoptotic properties.20

In presence of anti-angiogenic therapy, GBMs have demonstrated a propensity to transform their phenotype into a more invasive one.7 This phenotype is characterized by alterations in the microenvironment that results in a lower interstitial pressure. Collagen is one of the main components of the ECM that has a major role in the generation of interstitial pressure and blocks adenovirus distribution upon direct injection. To better understand how VEGF neutralization can alter collagen composition of gliomas, we assessed the expression of collagenases MMP-2 and MMP-9. Overexpression of MMP-9 in neuroblastoma cells resulted in reduced levels of collagen IV and increased oncolytic virus distribution.9 Similarly, oncolytic virus distribution and efficacy was improved in sarcomas expressing MMP-1 and MMP-8.22

In our study we found that short-term anti-angiogenic therapy induced upregulation of MMP-2 expression in U251 glioma cells both in vitro and in vivo (Figures 2a and 3a). Regulation of MMP-2 expression remains complex and understudied.23 MMP-2 transcription is upregulated during HIF-1α stabilization, which does not increase MMP-9 levels; similar to what we saw in our study.24,25 Moreover, multiple downstream targets of HIF-1α are upregulated during anti-angiogenic therapy.18 On the basis of these data, we evaluated HIF-1α expression in U251 glioma cells and found it to be upregulated as early as 8 h post therapy (Supplementary Figure S1). Furthermore, MMP-2 levels and its activity are regulated post-transcriptionally through proteases, such as tissue inhibitor of metalloproteinases (TIMP2), which are themselves altered during anti-angiogenic therapy.18,24 Moreover different glioma subtypes (classical, mesenchymal, proneural and neural) can respond to anti-angiogenic therapy and regulate expression of MMPs differently.27 Our study supports previous and recent findings that suggest and active role for VEGF-A on glioma cells through an autocrine loop.28,29

The importance of MMP-2 in GBM invasion is well established, but its role in tumor growth and ultimately survival remains to be defined. Complete knock down or reduction of MMP-2 to barely distinguishable levels has a significant impact on tumor growth.30 On the other hand, once a threshold of expression is reached, alterations in its levels do not have a significant role in tumor growth progression.31 To further corroborate the role MMP-2 in GBM progression, we run a gene-based Kaplan–Meier survival analysis at The Cancer Genome Atlas project and found that upregulation or downregulation of MMP-2 by a factor of 2 does not have any significant effect on patient survival (Supplementary Figure S2).

During VEGF-A blockade we noted no changes in MMP-9 expression levels, not only in U251 but also in three other cell lines. But higher dose and longer treatment can increase MMP-9

Figure 4. Adenovirus infection does not increase MMP-2 expression. U251 xenografts sections (a) were stained for MMP-2 with HRP and counterstained with hematoxylin; then scanned for intensity of HRP staining, presented as percent of areas with similar intensity (b). Bars represent mean intensity values of all xenografts. NS, not significant; ***P<0.001. (c, d) Sections from U87 xenografts were stained for collagen IV content and scanned for HRP positivity. (c) Representative images from each group; the insert is the ScanSoft rendered intensity image of the same slide. (d) Bar graph representation of the staining intensities for each group (n=4). **P = 0.008.
and other metalloproteinases in U87. Therefore, we have to distinguish between the short- and long-term effects of VEGF-blocking therapy. We focused in the normalization window period (days 5–8) induced by VEGF neutralization. During the early days of bevacizumab therapy, there is a reduction in vessel permeability and interstitial pressure that results from ECM alterations. We found that tumors in animals treated with VEGF-Ab had lower content of collagen IV, the substrate of MMP-2. But this early effect of VEGF neutralization seems to rebound during long-term therapy, resulting in tumors that express higher levels of collagen.32 GBMs inherently express high levels of collagen that block adenovirus expression upon injection. Therefore, any therapy that reduces collagen content, even for a short therapeutic window, can be of benefit to oncolytic virus delivery. VEGF blockade has been shown to increase adenovirus distribution only when given a few days before the i.t. injection.19 When given simultaneously no additive effect was noted.33 It was these observations that argued in favor of VEGF-Ab treatment to precede CRAd-S-pk7 injection.

VEGF neutralization increased adenovirus distribution in intracranial glioma. This was associated with a reduction in the collagen IV content in glioma xenografts (Figures 3b and 5a).

Figure 5. Collagen inhibits adenovirus distribution in intracranial glioma xenografts. (a) Collagen IV immunostaining of normal mouse brains (i), mock-treated glioma (iv) and VEGF-Ab treated IC glioma (vii). Representative areas are presented enlarged in (ii), (v) and (viii), respectively; and counterstained with DAPI (iii, vi and ix). (b) Representative area within glioma that depicts the distribution patterns of adenovirus in proximity to collagen bundles. This area was divided in 60 small quadrants and for each of them the percentage of area covered by collagen or adenoviral hexon staining was determined. The threshold was set at 5% and a heat map was constructed for each antigen based on the legend shown in the figure. The quantified distribution patterns were statistically analyzed for possible correlation. Bars = 150 μm.
Moreover, collagen IV impediment to oncolytic virus distribution has been demonstrated previously in neuroblastoma and was also found true in our U251 GBM model (Figure 5b). We discovered an inverse correlation in the distribution of collagen IV and CRAd-S-pk7 in glioma xenografts. Most importantly, with combination therapy (VEGF-Ab followed by CRAd-S-pk7), we found adenovirus replication to be increased (Figures 6c and d) in glioma xenografts and reduced tumor growth compared with each agent alone (Figure 7).

Bevacizumab therapy is currently approved for use in GBM, advanced colon and kidney carcinomas. At the same time, oncolytic virotherapy is an expanding novel field that is targeting the same cancers, but falling short of expectations due to limited i.t. distribution. Therefore, based on our findings, we propose that when combining these agents the bevacizumab therapy should precede i.t. virus injection. For GBM patients with unresectable tumors that currently receive bevacizumab at their relapse this would mean that oncolytic virotherapy, when introduced in the clinic, should follow bevacizumab during the ‘normalization window’ for best results. Better virus injection techniques, such as convection-enhanced delivery, can even increase the benefits we observed with simple injection.

In conclusion, our study shows that anti-angiogenic therapy given before adenovirus injection can increase its distribution and replication. Our findings emanate from previous studies on solid tumors, characterizing changes in the microenvironment induced during anti-angiogenic therapy, and therefore are widely applicable outside glioma model. Moreover, these results should also apply to other gene therapy vectors as well.

**MATERIALS AND METHODS**

**Cell lines and adenoviral vectors**

The human glioma cell lines U251, U87, A172 and U118 were purchased from the American Type Culture Collection (Manassas, VA, USA). U87, A172 and U118 cells were grown in minimal essential medium with 10% fetal bovine serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. Human glioma cell line U251 human embryonic kidney cell line (HEK-293) and grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 mg/ml penicillin and 100 mg/ml streptomycin (Cellgro, Mediatech Inc., Manassas, VA, USA). All cells were grown in a humidified atmosphere, with 5% CO2 and 37°C conditions. Cells were subcultured using 1 ml per 106 cells of a 0.25% trypsin/2.21 mmol l−1 EDTA solution. Trypsin activity was quenched using the appropriate media for each cell type; then washed at 300 relative centrifugal forces and plated at the indicated densities.

**Gene Therapy (2013) 318 – 327**
The conditionally replicative adenoviral vector CRAd-S-pk7 has been described previously. Briefly, the competent vector derives from wild-type adenovirus 5 with human survivin promoter incorporated to drive expression of E1 region. Fiber modification was achieved by insertion of 7 poly-Lysine repeats (pk7) in the C-terminal of knob domain.

Antibodies and reagents

The antibodies anti-human αvβ3 and anti-human αvβ5 is were purchased from Chemicon/Millipore (Billerica, MA, USA); the Ig controls and anti-human CD138 from Ebioscience (San Diego, CA, USA); coxsackie adenovirus receptor, collagen IV, adenovirus-biotin and rabbit anti-human HF-1x from Abcam (Cambridge, MA, USA); Laminin from ABR/Thermo-Fisher Scientific (Newington, NH, USA); MMP-9 from Cell Signaling (Danvers, MA, USA) and MMP-2 from Santa Cruz (Santa Cruz, CA, USA). Secondary antibodies or conjugates used were FITC-conjugated anti-mouse IgG from Millipore, Streptavidin-AlexaFluor 555 and Anti-rabbit AlexaFluor 647 from Invitrogen (Carlsbad, CA, USA).

The VEGF trapping antibody (VEGF-Ab) (clone B20-4.1.1), a cross species reacting monoclonal antibody to VEGF-A, was provided by Genentech (San Francisco, CA, USA). The concentration of VEGF-Ab for in vitro studies (50 μg/ml) was based on the clinically used regimen (10 mg/kg−1) and the extracellular fluid volume where antibodies disperse, to couple better with physiologically relevant concentrations. For in vivo experiments mice received two intraperitoneal (i.p.) injections, 3 days apart, of VEGF-Ab (5 mg/kg−1 diluted in PBS). As an antibody control we used the same amount of a whole mouse IgG from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

Flow Cytometry for cell surface receptors

Cells were plated in 6-well plates at 100 000 cells per well and treated with VEGF-Ab or IgG-control for 72 h. Collection was done by scraping the cells, washing and then staining the live cells with the indicated antibodies. Data were acquired and analyzed in BD FACSCanto with CellQuest (Becton Dickinson, San Jose, CA, USA) and FlowJo (TreeStar, Ashland, OR) software. Experiments were performed twice independently, in triplicates.

Western blotting

For western blotting, samples were loaded with an equal amount of total proteins, as measured by Bradford assay read at 630 nm (Bio-Rad, Hercules, CA, USA). To detect levels of MMP-2 and MMP-9, 250 000 cells were grown in T25cm2 flasks and treated with VEGF-Ab for 5 days. On the last day cell secretion was blocked with GolgiPlug (Ebioscience) for 6 h before collection of cells. Protein lysates were prepared by using a fresh protease inhibitor and RIPA lysis buffer. Fifty μg of proteins were separated in 4–20% ready-to-use gradient gel (Bio-Rad), transferred to the polyvinylidene difluoride membrane following hybridization with either mouse anti-human MMP-2 (Santa Cruz), MMP-9 (Cell Signaling) or anti-human Actin (Sigma, MO, USA) antibodies. Western blotting assay were done with SuperSignal West Pico chemiluminescent substrate (Bio-Rad). Images were collected in aBio-Rad image station.

Glioma viability assay

To test the toxicity of oncolytic adenovirus in combination with VEGF-Ab, cells were plated in a 96-well, 5000 per well, in triplicates. Twenty-four hours later cells were infected with CRAd-S-pk7 in 10-fold dilutions from 10 IU per cell to 0.11 IU per cell and then treated with VEGF-Ab 50 μg per ml or control Ig for 3 days before trypan blue exclusion assay. Cells were collected using trypsin-EDTA 0.25% and counted in a cytometer after resuspended in trypan blue. Cells that excluded trypan blue were considered viable. Graphs express percentage of live cell in experimental treatment were then presented as fold of their control-treated cells.

Quantification of viral titers and E1A copy determinations

To determine the infectious units per ml viral stock (IU ml−1), viral progeny in vitro and in vivo, we used the Adeno Rapid-X Titer Kit (Clontech, Mountain View, CA, USA). For in vitro study non-infected HEK-293 cells were used and in vivo the plates, 2 × 106 cells per well; incubated 24 h later with serum-free media containing CRAd-S-pk7, for 1 h, at the indicated concentrations. Infected cells were collected 3 and 5 days later, and freeze-thawed three times to release adenovirus. After spinning down the cells the supernatant was used to infect confluent layers of HEK-293 at different dilutions and 48 h after viral titers (IU ml−1) were determined by counting infected HEK-293 cells. In a similar way, for in vivo experiments, the tissue was collected and homogenized at the time points indicated; the same amount of tissue from each group was taken and freeze-thawed three times.

As adenovirus replication/packaging is not very effective, we also determined E1A copy number in vitro and in vivo. DNA was isolated by using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). The expression levels of adenoviral DNA were detected by using qRT-PCR with primer sequences recognizing E1A area, which have been described before. Samples were run in triplicates and standardized to ng of DNA.

Evaluation of MMP-2 gene expression by qRT-PCR

Relative expression of mRNA transcripts by glioma cell lines was evaluated for the expression of MMP-2. Glioma cells, 106 cells per well in triplicate, were treated with VEGF-Ab or IgG control and cells were collected at different time points. Total cellular RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer’s protocol and in each instance 1 μg of purified mRNA was reverse transcribed to complementary DNA using the iScript cDNA conversion kit (Bio-Rad). Quantitative PCR was conducted using the SYBR Green quantitative PCR kit (Invitrogen) for all experiments. Optimization of annealing temperatures for each transcript was first conducted. Each transcript of interest was amplified in triplicate at its proper annealing temperature and products were analyzed using the Opticon 2 software (Bio-Rad). Relative expression was evaluated using the ΔCt method (ΔCt = Ct gene of interest – Ct GAPDH). Before plotting them in the graph, the MMP-2 expression for each cell line was normalized to their IgG-control-treated condition, to which was given an arbitrary value of 1. The relative expression levels of MMP-2 for all four cell lines upon VEGF-Ab treatment were then presented as fold of their control-treated cells.

Animal experiments

Animals were cared for according to a study-specific animal protocol approved by The University of Chicago Institutional Animal Care and Use Committee.

For immunohistochemistry and adenoviral replication in vivo, 7- to 8-week-old male nude mice (Harlan Laboratories, Madison, WI, USA) were injected i.c. or subcutaneously (s.c.). In brief, mice were anaesthetized with an i.p. injection of ketamine hydrochloride (25 mg kg−1) /xylazine (2.5 mg kg−1) cocktail. For i.c. injection, a midline incision was made, and a 1-mm burr hole centered 2-mm posterior to the coronal suture and 2-mm lateral to the sagittal suture was made. Animals were placed in a stereotactic frame and 25 000 U251 cells, in a 2.5 μl volume, were injected with a Hamilton needle 3 mm deep into the brain. Twenty-one days after tumor implantation, mice received two i.p. injections of 5 mg kg−1 VEGF-Ab 3 days apart or IgG control. Animals underwent MRI, on day 26 after U251 implantation mice were injected i.c. with 108 IU of CRAd-S-pk7. Mice were killed 3 days later and viral distribution was quantified.

For flank tumors, 105 U251 or U87 glioma cells in 100 μl were injected in the dorsal chamber (s.c.) bilaterally with a 20 Gage needle. Three weeks later mice underwent the same therapeutic schedule, two i.p. injections of VEGF-Ab 5 mg kg−1 or IgG control 3 days apart (n = 4 animals per group) and on day 26 mice were killed for IHC or received a dose of 108 IU CRAd-S-pk7 i.t. injection. The latter were killed after 3 and 7 days for the determination of viral titers and E1A expression in xenografts. For determination of the possible role of adenovirus injection in MMP-2 expression, animals were killed 3 days after receiving 108 IU of CRAd-S-pk7.

For assessment of flank tumor volume progression, we injected 105 U251 cells s.c. bilaterally; 3 weeks later, we separated the animals in four groups of five animals per group with similar mean volume. Mice received either VEGF-Ab (two i.p. injections of 5 mg kg−1 3 days apart) or IgG control, followed 5 days later by direct i.t. injection of 108 IU CRAd-S-pk7. The day of the adenovirus injection was considered day 0. Tumor dimensions were measured twice a week, with caliper, and volume was determined by the formula: a × b × c/2. Growth progression was determined for each tumor by comparing the volume at the indicated time vs day 0.

Immunohistochemistry

Flank s.c. tumor tissues were cut in half and dropped in a 10% formalin solution or frozen in OCT in a dry ice–methylbutane bath. The brain tissues were snap frozen in a mixture of 2-N-methyl-Bromide and Methylbutane and then cut coronally at the injection site in two pieces and embedded in OCT in a dry ice–methylbutane bath. Sections of 5 μm for SC tumors,
or 10 μm for brain tumor, spanning over 2 mm of tissue, were cut and stained with the described antibodies. For quantitative evaluations of IHC staining with HRP-Hematoxylin two different automated image-scanning system were used: Automated Cellular Imaging System (Clariant, Aliso Viejo, CA, USA) gives output in brown dots per area (Figure 3) and ScanScope XT (Aperio, Vista, CA, USA), which uses color deconvolution to depict different intensities of staining and gives output in percent of areas that stain at four different levels: no staining, weak, moderate and strong staining (Figure 4).

Immunofluorescent staining for adenosine hexon and collagen was done based on Abcam protocol. Shortly, brain sections were let to dry at room temperature for 10 min before fixation and permeabilization with 0.5/0.5 mixture of acetone-methanol. After washing with PBS and blocking with 10% BSA, sections were stained for with the described antibodies as suggested in their information sheet. Sections were incubated overnight with the primary antibody and 1 h with the secondary antibody. After the final wash sections were covered with Prolong Gold antifade reagent with DAPI (Invitrogen). For capturing fluorescent IHC images we used an inverted Zeiss microscope.

Statistical analysis

The statistical significance difference between means of two groups was evaluated based on unpaired Student's t-test. One way analysis of variance was used to test for difference between two or more independent groups. The level of significance was set as P < 0.05. Calculations were done using SigmaPlot version 8.02 (SPSS Inc., Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Feifei Liu for statistically analyzing the data and Derek A Wainwright for optimizing the immunofluorescence staining protocol. A cross species reacting monoclonal antibody to VEGF-A (clone B20-4.1.1) was provided by Genentech (U01NS069997), and the American Cancer Society (RSG-07-276-01-MGO).

REFERENCES

1 Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Ilyasova D et al. Brain tumor epidemiology: consensus from the brain tumor epidemiology consortium. Cancer 2008; 113(7 suppl): 1953–1968.
2 Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus temozolomide alone as a standard treatment for patients with newly diagnosed glioblastoma (EORTC 22033): a randomised phase III study. Lancet 2009; 375: 1067–1072.
3 Ulasov IV, Zhu ZB, Tyler MA, Han Y, Rivera AA, Bork SG, Carter JP et al. Increased antitumor activity of bevacizumab in combination with hypoxia inducible factor-1 inhibition. Mol Cancer Ther 2009; 8: 1867–1877.
4 Libertini S, Iacuzzo I, Pernuolo G, Scala S, Ierano C, Franco R et al. Bevacizumab induces vascular invasion in human anaplastic thyroid carcinoma xenografts and enhances the effects of E1A-defective adenovirus ΔE12-97. Clin Cancer Res 2008; 14: 6505–6514.
5 Reichert S, Rodel C, Mirsch J, Harter PN, Mittelbronn M et al. Survivin inhibition and DNA double-strand break repair: A molecular mechanism to overcome radioresistance in glioblastoma. Radiat Oncol 2011; 6: 51–58.
6 de Groot JF, Fuller G, Kumar AJ, Piao Y, Eterovic K, Ji Y et al. Tumor invasion after treatment of glioblastoma with bevacizumab: radiographic and pathologic correlation in humans and mice. Neuro Oncol 2010; 12: 233–242.
7 Mok W, Boucher Y, Jain RK. Matrix metalloproteinases-1 and -6 improve the distribution and efficacy of an oncolytic virus. Cancer Res 2007; 67: 10664–10668.
8 Chemov N, Soumni NE, Remacle AG, Strongin AY. Epigenetic control of the invasion-promoting MT1-MMP/MMP-2/TIMP-2 axis in cancer cells. J Biol Chem 2009; 284: 12727–12734.
9 Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3: 721–732.
10 Eirola N, Holtkamp N, von Deimling A. Involvement of HIF-1 in desferoxamine-induced invasion of glioblastoma cells. Clin Exp Metastasis 2007; 24: 57–66.
11 Lu KV, Jong KA, Rajasekaran AK, Cloughesy TF, Michels PS. Upregulation of tissue inhibitor of metalloproteinases-1 (TIMP-2) promotes matrix metalloproteinase (MMP-2) activation and cell invasion in a human glioblastoma cell line. Lab Invest 2004; 84: 8–20.
12 Verhaeghe RG, Haeldy KA, Purdom E, Wang V, Qi Y, Wilkerson MD et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRα, IDH1, EGFR, and NF1. Cancer Cell 2010; 17: 98–110.
13 Amelik P, Lathia JD, Rasmussen R, Wu Q, Bartkova J et al. Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. J Exp Med 2012; 209: 507–520.
14 Lee J, Yu H, Choi K, Choi C. Differential dependency of human cancer cells on vascular endothelial growth factor-mediated autocrine growth and survival. Cancer Lett 2011; 309: 145–150.
15 Kargiotos O, Chetty C, Gondi CS, Tsung AJ, Dinh DH, Gujrati M et al. Adenovirus-mediated transfer of sRINa against MMP-2 MRNA results in impaired invasion and tumor-induced angiogenesis, induces apoptosis in vitro and inhibits tumor growth in vivo in glioblastoma. Oncogene 2008; 27: 4830–4840.
16 Lamfers ML, Gianni D, Tunc H, Iedema S, Schagen FH, Carette JE et al. Tissue inhibitor of metalloproteinase-3 expression from an oncolytic adenovirus inhibits matrix metalloproteinase activity in vivo without affecting antitumor efficacy in malignant glioma. Cancer Res 2005; 65: 9336–9340.
17 Zhang W, Fukui G, Buthelezi D, Stegemann-Rachamimov AO, Chen JW, Wojtowicz GR et al. Bevacizumab with angiostatin-armed oHSV increases antiangiogenesis and decreases bevacizumab-induced invasion in U87 glioma. Mol Ther 2011; 19: 37–45.
18 Guse K, Ranki T, Ala-Opas M, Bono P, Sarkioja M, Rajekj M et al. Treatment of metastatic renal cancer with casp-id-modified oncolytic adenoviruses. Mol Cancer Ther 2007; 6: 2728–2736.
34 Gutermann A, Mayer E, von Dehn-Rothfelser K, Breidenstein C, Weber M, Muench M et al. Efficacy of oncolytic herpesvirus NV1020 can be enhanced by combination with chemotherapeutics in colon carcinoma cells. *Hum Gene Ther* 2006; 17: 1241–1253.

35 Guse K, Sloniecka M, Diaconu I, Ottolino-Perry K, Tang N, Ng C et al. Anti-angiogenic arming of an oncolytic vaccinia virus enhances antitumor efficacy in renal cell cancer models. *J Virol* 2010; 84: 856–866.

36 Fuh G, Wu P, Liang WC, Ultsch M, Lee CV, Moffat B et al. Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab. *J Biol Chem* 2006; 281: 6625–6631.

37 Ulasov IV, Sonabend AM, Nandi S, Khramtsov A, Han Y, Lesniak MS. Combination of adenoviral virotherapy and temozolomide chemotherapy eradicates malignant glioma through autophagic and apoptotic cell death in vivo. *Br J Cancer* 2009; 100: 1154–1164.

Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)