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LABORATORY INVESTIGATION

Combination of anti-VEGF therapy and temozolomide in two experimental human glioma models

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Abstract Anti-angiogenic agents, such as bevacizumab (BEV), can induce normalization of the blood brain barrier, which may influence the penetration and activity of a co-administered cytotoxic drug. However, it is unknown whether this effect is associated with a benefit in overall survival. This study employed intracranial human glioma models to evaluate the effect of BEV alone and in combination with temozolomide (TMZ) and/or radiation therapy (XRT) on overall survival. One hundred eight male athymic rats were intracranially injected with either U251 or U87 human glioma. Ten or eleven days after tumor inoculation, animals bearing U251 and U87, respectively, were treated with: TMZ alone (50 mg/kg for 5 consecutive days, P.O.), BEV alone (15 mg/kg, I.V.), a combination of TMZ and BEV, or a combination of TMZ, BEV, and a single fraction of XRT (20 Gy). Controls received no treatment. The U87 experiment was repeated and the relationship between survival and the extent of anti-angiogenesis via anti-laminin antibodies for the detection of blood vessels was assessed. In both U87 glioma experiments, all of the treatment groups had a statistically significant increase in survival as compared to the control groups. Also, for both U87 experiments the combination groups of TMZ and BEV had significantly better survival when compared to either treatment administered alone, with 75 % of animals demonstrating long-term survival (LTS) (defined as animals alive 120 days after tumor implantation) in one experiment and 25 % LTS in the repeat experiment. In the U251 glioma experiment, all treated groups (except BEV alone) had significantly improved survival as compared to controls with minimal statistical variance among groups. The percent vessel area was lowest in the group of animals treated with BEV alone. The addition of BEV to TMZ and/or XRT had variable effect on prolonging survival in the two human glioma models tested with reduced tumor vascularity in groups treated with BEV. These results indicate that BEV has anti-angiogenic activity and does not seem to hinder the effect of TMZ.

Rachel Grossman and Harry Brastianos are co-first authors and contributed equally to the design and execution of this study as well as to the composition of the manuscript.

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Introduction

Glioblastoma is the most common primary brain tumor in adults and accounts for more than 50 % of all gliomas. It is also the most deadly glioma, with a median survival of 12–14 months [1]. Fewer than 10 % of patients survive 5 years after diagnosis [2]. This is the case even after maximal therapy, which consists of surgical resection, radiation therapy (XRT), temozolomide (TMZ), and locally delivered BCNU wafers (Gliadel) for eligible patients [3].

Glioblastoma is a highly vascularized tumor with irregular blood vessels, extensive vascular proliferation, and increased expression of angiogenic factors, especially vascular endothelial growth factor (VEGF). High expression of VEGF on tumor endothelium results in growth and proliferation of endothelial cells, [4] which correlates with tumor hypoxia, necrosis [5], and a poor overall clinical prognosis [6]. Effective inhibition of VEGF in glioblastoma decreases neovascularization, improves blood vessel integrity, decreases tumor-associated edema, and can result in both improved clinical performance and improved progression-free survival [6–7]. It has been proposed that the optimal approach for controlling glioblastoma growth is to combine cytotoxic therapy with anti-angiogenic therapies [8].

TMZ is the current clinical treatment for newly diagnosed glioblastomas, and bevacizumab (BEV) is the only anti-angiogenic agent with FDA approval for use in recurrent glioblastomas [1]. Well-conducted clinical trials of BEV in patients with recurrent glioblastoma have shown impressive reductions in contrast enhancement and mass effect as well as improvement in progression-free survival—but no clear impact on overall survival [7–9]. In addition, preclinical models have suggested that in the absence of active cytotoxic therapies, BEV monotherapy can contribute to increased tumor invasion and migration [10–11]. These observations suggest that co-administration of cytotoxic and anti-angiogenic therapies are optimal. We explored the influence of BEV alone as well as in combination with TMZ on tumor vascularity and survival using two human glioma xenograft models.

Materials and methods

Materials

Temodar (Schering Corporation, a subsidiary of Merck & Co., Inc., NJ, USA) and BEV (Genentech, Inc. CA, USA) were purchased from the Johns Hopkins Hospital pharmacy. Anti-laminin antibodies for the detection of blood vessels were purchased commercially from Life Technologies/Invitrogen. The study was approved by the Johns Hopkins Animal Care and Use Committee (ACUC), and all procedures were conducted within compliance of their regulations.

Cell culture

The U87 and U251 human glioma cell lines (from Dr. Laterra, Johns Hopkins, Baltimore, MD, USA) were grown in MEM with Earle Salts and L-glutamine (MEM 1*Mediatech, Inc.) supplemented with 10 % fetal bovine serum (Gemini Bioproducts, Inc.), 2 mM/L sodium pyruvate (Mediatech, Inc.), 0.1 mmol/L MEM-nonessential amino acids (Mediatech, Inc.), and penicillin–streptomycin (Mediatech, Inc.). The cells were grown at 37 °C in a humidified incubator with 5 % CO_2.

Animals

This study was conducted on athymic male rats weighing 150–200 g, purchased from Harlan Bioproducts (Indianapolis, IN, USA). They were housed in standard facilities and given free access to food and water. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

Anesthesia

Rats were anesthetized with an intraperitoneal injection of 0.6 mL of a stock solution containing ketamine HCl (75 mg/kg, 100 mg/mL), xylazine (7.5 mg/kg, 100 mg/mL), and ethanol (14.25 %) in a sterile 0.9 % NaCl solution.

Intracranial glioma models

For the intracranial implantation of the U87 and U251 glioma cells, 108 athymic male rats were anesthetized. The head was shaved with clippers and prepared with alcohol and Prepodyne solution (West Penetone, Montreal, Canada). A midline scalp incision was made, exposing the sagittal and coronal sutures. With the use of an electric drill with a 2-mm round cutting burr, a small hole was made in the skull centered 3 mm left to the sagittal suture and 5 mm posterior to the coronal suture. Care was taken to avoid the sagittal sinus. The animals were placed in a stereotactic frame, and 1×10^6 U87 glioma cells or 1×10^6 U251 glioma cells were injected over 3 min via a 26-gauge needle inserted to a depth of 4 mm at the center of the burr hole into the rat's striatum. After tumor cell inoculation,

the needle was removed, the site was irrigated with normal saline, and the incision was closed with surgical staples.

Animals were given pharmaceutical grade anesthetic, analgesia, and study agents. Animals were observed for neurologic and systemic toxicity, and survival was recorded. Any animals appearing moribund were humanely euthanized as per Johns Hopkins Animal Care and Use Committee (ACUC) protocol, and the date of death was recorded. At day 120, all surviving rats were deemed long-term survivors (LTS) and were euthanized.

Experimental design

U87 human glioma

Based on a prior study in our laboratory that involved the same concentration of U87 glioma cells, we determined that the mean tumor diameter was 2.8 ± 0.93 mm on day 10 [12]. Therefore, 11 days after tumor implantation, 29 tumor-bearing rats were randomized into four treatment groups. The animals were treated with either TMZ (50 mg/kg) dissolved in water and administered via oral gavage daily (days 11–15) ($n = 5$), BEV (15 mg/kg) injected in a single dose via the tail vein (day 11) ($n = 8$), a combination of TMZ (days 11–15) and BEV (day 11) ($n = 8$), or no treatment ($n = 8$).

In a separate repeat study to confirm our results, 31 rats were injected with U87 tumor and treated as described above. The animals were treated with either TMZ (50 mg/kg) administered via oral gavage daily (days 11–15) ($n = 8$), BEV (15 mg/kg) injected in a single dose via the tail vein (day 11) ($n = 8$), a combination of TMZ (days 11–15) and BEV (day 11) ($n = 8$), or a control group that did not receive any treatment ($n = 8$).

U251 human glioma

Ten days after tumor inoculation, tumor-bearing rats were randomized into treatment groups. The animals were treated with either TMZ (50 mg/kg) dissolved in water and administered via oral gavage daily for 5 consecutive days ($n = 2$), BEV (15 mg/kg) injected in a single dose via the tail vein ($n = 8$), radiation therapy (XRT) given as a single fraction of 20 Gy ($n = 8$), a combination of TMZ and XRT ($n = 7$), or a combination of TMZ, BEV, and XRT ($n = 8$). The control group did not receive any treatment ($n = 8$).

Immunohistochemistry

Groups of rats from the first U87 experiment ($n = 4$ from each treatment group) were sacrificed, perfused with 4 % paraformaldehyde 25 days after tumor inoculation (2 weeks after treatment was started), and de-brained. The remaining rats from each group ($n = 8$) were monitored for survival. To explore the anti-angiogenic effect that is expected with BEV, we studied 20-μm brain sections that were cut and stained with anti-laminin antibodies in order to detect and ascertain the state of vascularization [13]. The tumor blood vessels were quantified as total vessel area relative to tumor cross-sectional area, as previously described [14].

Statistical analysis

Overall survival was the primary end point. The distribution of the intervals until death was determined by the method of Kaplan and Meier. Statistical analysis was completed using Prism 4 software (GraphPad Software, La Jolla, CA, USA). The a priori level of significance was $p < 0.05$.

Results

Survival studies

U87 efficacy results

The U87 glioma control group had a median survival of 19 days. Animals treated with BEV monotherapy had a median survival of 22 days ($p = 0.14$ vs. controls). Animals treated with oral TMZ for 5 consecutive days had a median survival of 63 days ($p = 0.0007$ vs. controls, $p = 0.0056$ vs. BEV). Animals treated with the combination of TMZ and BEV had a median survival of 109 days ($p < 0.0001$ vs. controls, $p < 0.0001$ vs. BEV, $p = 0.0253$ vs. TMZ) with 50 % of the animals living as long-term survivors (LTS) (Fig. 1a; Table 1).

The U87 glioma control group in the repeat study had a median survival of 13 days. Animals treated with the single dose of BEV monotherapy (15 mg/kg) had a median survival of 15 days ($p = 0.0065$ vs. control) and the group receiving oral TMZ (50 mg/kg) had a median survival of 36 days ($p = 0.0032$ vs. control). The combination group that received BEV and oral TMZ did not reach median survival, with 75 % of the animals as long term survivors (LTS) (defined as animals alive 120 days after tumor implantation) ($p = 0.0001$ vs. control; $p = 0.0001$ vs. BEV monotherapy, and $p = 0.0008$ vs. oral TMZ) (Fig. 1b).

U251 efficacy results

The U251 glioma control group had a median survival of 35 days. Animals treated with BEV monotherapy had a

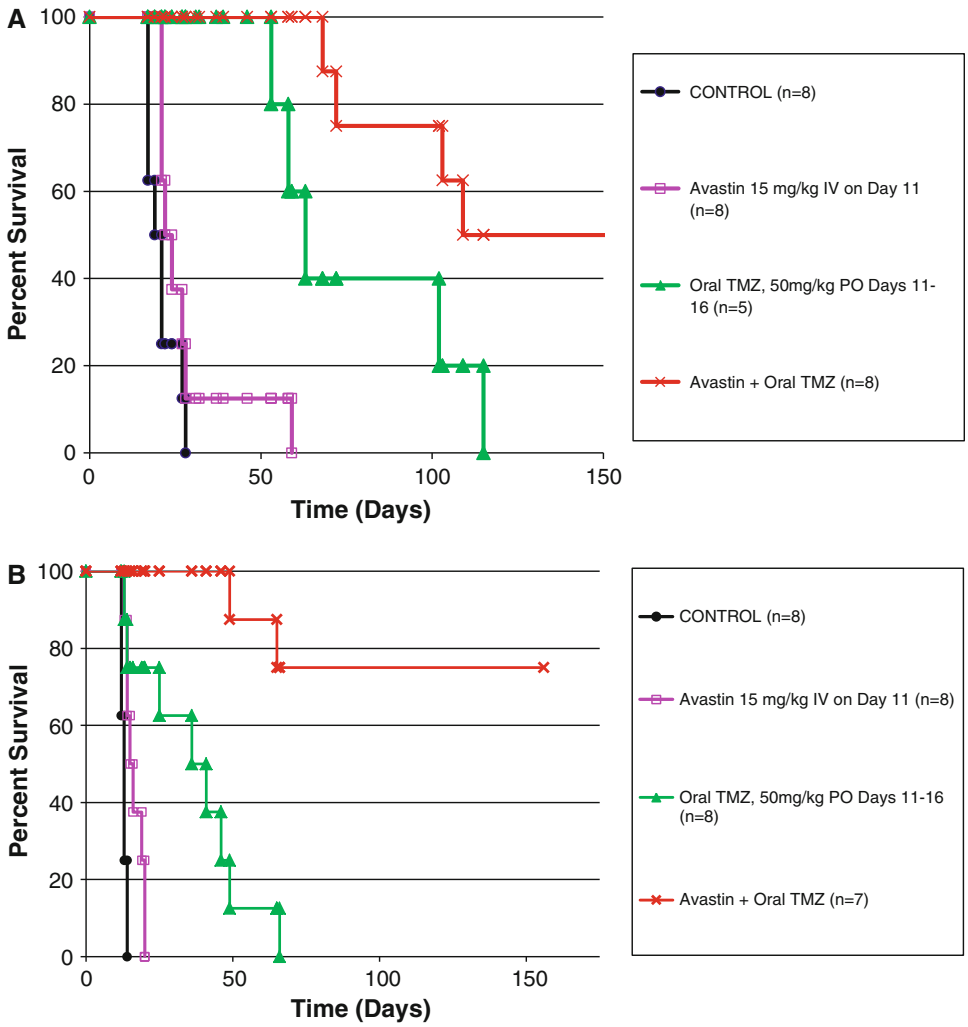

Fig. 1 Kaplan–Meyer survival curve of rats bearing U87 intracerebral glioma. **a** Combination drug therapy of TMZ and BEV improved overall survival of rats bearing U87 intracerebral glioma over either therapy alone, with 25 % of the treated animals surviving more than 120 days (i.e., long term survival (LTS)). **b** A second efficacy study with combination drug therapy of TMZ and BEV resulted in improved overall survival of rats bearing U87 intracerebral glioma over either therapy alone, with 75 % LTS

median survival of 50 days ($p < 0.0001$ vs. control). Animals treated with oral TMZ for 5 consecutive days had a median survival of 93 days. Animals treated with a single fraction of 20 Gy XRT did not reach median survival, with 87.5 % LTS, ($p = 0.003$ vs. BEV, and $p = 0.0003$ vs. controls). Animals treated with the combination of TMZ and XRT, and those who were treated with a combination of TMZ, XRT, and BEV, did not reach median survival, with 86 and 87.5 % LTS, respectively (Fig. 2; Table 2).

Immunohistochemistry

Quantitative analysis of tumor blood vessels demonstrated that the mean (±SD) percent vessel area in the U87 tumor of control animals was 8.05 ± 2.94 %, compared with 6.59 ± 2.67 % in the TMZ group, and 3.63 ± 1.45 % in the BEV group. The percent vessel area was lowest in the group of animals that were treated with combination of TMZ and BEV with a vessel area of 2.29 ± 2.36 % ($p < 0.05$ compared to controls, and TMZ) (Fig. 3).

Discussion

It is increasingly evident that multi-agent therapy will be required to improve survival in patients with glioblastoma. A strong preclinical rationale for VEGF inhibition, as well as the recent success with BEV in clinical trials for patients with recurrent glioblastoma, has generated a great deal of interest in an anti-angiogenic approach [7, 15]. However, despite remarkable radiographic responses and the improved clinical performance seen with some anti-angiogenic therapies, there is not yet evidence of improved overall survival [7]. Moreover, there is concern that anti-angiogenic therapies may contribute to the development of a more infiltrative glioma phenotype [10]. In order to minimize the potential for tumor infiltration in the setting of VEGF inhibition, it has been proposed that concurrent effective cytotoxic therapy is required [10]. It has been hypothesized that anti-angiogenic therapies enhance delivery of chemotherapy by improving blood perfusion throughout the tumor via normalization of erratic blood

Table 1 Treatment of the U87 experimental malignant glioma models with TMZ and BEV

Group	Median survival (range)	Long term survivors (%)	*p* value
Experiment 1 (Fig. 1a)			
Control ($n = 8$)	19 (17–31)	0	–
BEV, I.V. ($n = 8$)	22 (21–63)	0	0.14 vs. controls
TMZ, P.O. ($n = 5$)	63 (53–115)	0	0.0007 vs. controls, 0.0056 vs. BEV
BEV, I.V. + TMZ, P.O. ($n = 8$)	109 (68 to >120)	50	<0.0001 vs. controls, < 0.0001 vs. BEV, 0.025 vs. TMZ
Experiment 2 (Fig. 1b)			
Control ($n = 8$)	13 (12–14)	0	–
BEV, I.V. ($n = 8$)	15 (13–20)	0	0.0065 vs. control
TMZ, P.O. ($n = 8$)	36 (13–46)	0	0.0032 vs. control, 0.0215 vs. BEV
BEV, I.V. + TMZ, P.O. ($n = 7$)	120 (49 to >120)	75	0.0001 vs. control, 0.0001 vs. BEV, 0.0008 vs. TMZ

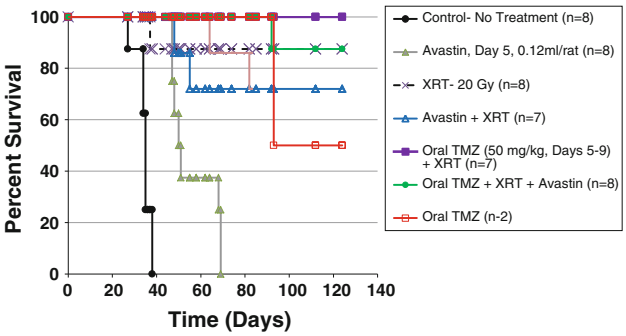

Fig. 2 Kaplan–Meyer survival *curve* of rats bearing U251 intracerebral glioma. Combination drug therapy of TMZ, XRT, and BEV resulted in 87.5 % LTS

Table 2 Treatment of the U251 experimental malignant glioma model with TMZ, BEV, and XRT

Group	Median survival (range)	Long-term survival (%)	*p* value
Control ($n = 8$)	35	0	–
I.V. Bevacizumab ($n = 8$)	50	12.5	$p < 0.0001$ vs. control
P.O. TMZ	93	50	$p < 0.0001$ vs. control
XRT 20 Gy ($n = 8$)	Did not reach median	87.5	$p = 0.0003$ vs. control
P.O. TMZ + XRT ($n = 7$)	Did not reach median	86	$p = 0.0004$ vs. control
I.V. Bevacizumab + XRT ($n = 7$) bevacizumab	Did not reach median	58	0.001 vs.
P.O. TMZ + I.V Bevacizumab + XRT ($n = 8$) TMZ + XRT	Did not reach median	87.5	$p = 0.07$ vs.

vessels and reduction of intratumoral pressure [16, 17]. These properties have been demonstrated in solid tumors outside the BBB with anti-angiogenic therapies [16, 17]; however, it is not clear whether these effects are equally beneficial for drug delivery in brain tumors, where modulation of the BBB has to be considered.

We examined combination therapy by using two experimental human glioma models. Both models showed that combination of BEV and TMZ does not reduce the efficacy of either drug. However, it seems that treatment with TMZ leads to impressive effect in U251, thus it was impossible to demonstrate in this glioma model the additive value of BEV in combination therapy. In the U87 glioma model, however, the effect of BEV on tumor vascularity as shown by laminin expression (Fig. 3) was indeed translated into a survival advantage in two consecutive survival studies as shown in Fig. 1a, b. The discrepancy between these two glioma models reflects the limitation of using these models.

Also, although we noted an increase in survival in our two animal models this beneficial impact on overall survival has not been reproduced in patients. Two large clinical trials have been completed testing the combination of BEV, TMZ, and radiation therapy in patients with newly diagnosed glioblastoma: RTOG and NCT00943826. Results from these trials confirm the positive impact of such combination therapy on progression-free survival, but not on overall survival [18]. While the U.S. Food and Drug Administration (FDA) has yet to authorize the use of BEV in newly diagnosed glioblastoma, it is worth noting that this practice has already been approved in Japan on the grounds that progression-free survival is promising for patients with such poor prognoses [19]. However, the optimal dosing of BEV in the clinical care of patients with glioblastoma remains uncertain and additional modeling would be helpful to help guide clinical studies.

In this series of studies we demonstrated that the human monoclonocal antibody BEV has the expected effect on

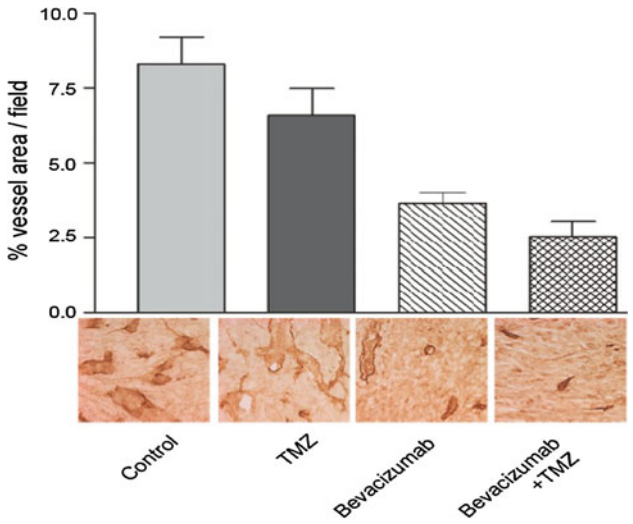

Fig. 3 Intracranial U87 xenografts were established and treated with either BEV, TMZ, their combination, or control on day 10 after inoculation. Post-inoculation day 25 tumors were examined for tumor blood vessels by immunohistochemistry using anti-laminin antibodies. The % vessel area per high-powered microscopic field was quantified by computer-assisted image analysis [14]. The % vessel area was lowest in the group of animals that were treated with a combination of TMZ and BEV ($p < 0.05$ compared to controls, and TMZ)

murine vessels. It is important to note that by using BEV (human specific monoclonal antibody) in human glioma models in the athymic rat, BEV acts only on the tumor-derived VEGF and not on the rodent endogenous endothelial cells.

Our data suggests that in human glioma models the combination of BEV and TMZ does not reduce the efficacy of either drug, but can improve overall survival. A limitation of this study is the limited dosing duration. Hence, this treatment design does not resemble the clinical protocols in use. However, it does allow for assessment of vascular changes in tissue in a short duration to allow modeling of treatment combinations to assist in creating the optimal strategy for combining BEV and TMZ for patients with newly diagnosed glioblastoma.

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