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Evaluation of Combined Bevacizumab Plus Irinotecan Therapy in Brain Tumors Using Magnetic Resonance Imaging Measures of Relative Cerebral Blood Volume

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Abstract

Frequently, bevacizumab is combined with chemotherapeutics such as irinotecan, motivated by studies showing improved clinical outcomes compared to historical controls. However, no systematic studies have been performed to determine if and how these drugs should be combined for optimal therapeutic response. The purpose of this study was to characterize the temporal combinations of bevacizumab and irinotecan by measuring the contrast-agent enhanced tumor volumes and relative cerebral blood volume (rCBV) using dynamic susceptibility contrast (DSC) imaging. The studies, performed in the U87 brain tumor model, show a vascular normalization window with bevacizumab monotherapy and are consistent with clinical indications of no additional benefit in the addition of irinotecan to bevacizumab therapy.

Keywords

glioblastoma; bevacizumab; MRI; irinotecan

Introduction

The most common type and most difficult brain tumors to manage are glioblastomas (astrocytoma grade IV). Median survival of patients with glioblastoma multiforme (GBM) remains 15 months (1, 2). One of the hallmarks of glioblastomas is the high level of new vessel growth or angiogenesis required for progression from low-grade to high-grade tumors. Angiogenesis is influenced by a balance between proangiogenic and antiangiogenic factors. VEGF (vascular endothelial growth factor), one of the most studied proangiogenic factors, has led to the development of therapies to target it and its receptors.

The anti-angiogenic drug bevacizumab has recently been approved by the US Food and Drug Administration for the treatment of recurrent glioblastoma multiforme. Many clinical trials have shown improvement in progression free survival and median survival in patients treated with bevacizumab(3, 4). Frequently bevacizumab is combined with a

chemotherapeutic such as irinotecan, an approach motivated by studies that showed improved clinical outcomes compared to historical controls (5). However, no systematic studies have been performed to determine if and how these drugs should be combined for optimal therapeutic response. Vredenbergh reported a 63% response measured by at least a 50% decrease in cross-sectional area of the enhancing tumor in a phase II clinical trials treating recurrent glioma patients with irinotecan and bevacizumab every other week (6). The combination of bevacizumab every other week and irinotecan every other week in the phase II BRAIN trial showed a 6 month progression free survival of 50.3% in recurrent glioblastoma patients (7).

Clinical trials with the combination therapy of irinotecan and bevacizumab have used the Macdonald criteria to evaluate response (8). Furthermore, it is becoming increasingly clear that standard MRI measures of tumor size, which entail measuring contrast-enhancing tumor volume, are not appropriate for the evaluation of anti-angiogenic drugs since these drugs also decrease contrast extravasation (9). Dynamic susceptibility contrast (DSC)-MRI perfusion imaging is a minimally invasive technology capable of evaluating the vascular effects of therapies. Currently, this technique is used clinically and provides valuable information not obtainable with conventional gadolinium enhanced T1-weighted MRI (10–17). Areas of increased vascularity are observed in DSC-MRI preceding tumor enhancement on conventional MRI (18). We and others have previously used this method to measure morphologic changes in relative cerebral blood volume (rCBV) in brain tumors and demonstrated significant correlation with tumor grade (12, 13, 19).

The present studies were therefore performed to demonstrate the utility of using rCBV (relative cerebral blood volume), derived from DSC imaging, to characterize the response to combinations of bevacizumab and irinotecan in the treatment of a U87 xenograft brain tumor model. To perform these experiments, rats were inoculated with U87 cells and treated with different paradigms of bevacizumab and irinotecan. Indices of tumor vascularity were then evaluated in the control rats and those treated with bevacizumab and irinotecan.

Methods

Cell culture

The U87MG (adult glioblastoma) cell line was purchased from American Type Culture Collection (Manassas, Virginia) and maintained in MEM with Earle's salts, 10% fetal bovine serum (FBS) and 0.1% penicillin/strept in 5% CO₂ at 37°C.

Animals

Care of the animals before and during the experimental procedures was conducted in accordance with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Male Athymic rats weighing approximately 250 g were obtained from Charles River Laboratories (Wilmington, Massachusetts) and housed in pairs in individually ventilated cages. Animals were fed autoclaved laboratory diet; food and RO hyperchlorinated water were available *ad libitum*.

Intracranial xenograft transplantation

Male Athymic nude rats were anesthetized with ketamine (60 mg/kg), acepromazine (0.9 mg/kg) and xylazine (6 mg/kg) IP. Heads were immobilized and using an aseptic technique a 1 mm burr hole was drilled in the skull 1mm anterior and 2 mm lateral to bregma on the

right side as we previously described (20). A 10 μ l gas-tight syringe (Hamilton Company, Reno, Nevada) was used to inject 2×10^5 cells into the right frontal lobe at a depth of 3 mm relative to the dural surface. The injection time was 5 minutes, after which the needle was retracted slowly for an additional 5 minutes. The skin was closed with cyanoacrylate.

Therapies

One group of animals (n=7) was treated iv under isoflurane anesthesia with bevacizumab (Avastin, Genentech, South San Francisco, CA) monotherapy at day 10 (5 mg/kg). Another group (n=7) was treated iv under isoflurane anesthesia with Irinotecan (20.83 mg/kg) (Camptosar, Pfizer, New York, NY) monotherapy at day 10. Bevacizumab, given iv at day 10 (5 mg/kg), was combined with Irinotecan (20.83 mg/kg) administered iv at days 8 (n=7), 12 (n=7) or 14 (n=7) post-inoculation. Control animals were administered saline at day 10 (n=8).

MRI Studies

At 10 days after tumor cell inoculation, baseline MRI studies were performed on a 9.4T Bruker AVANCE Scanner fitted with a linear transmit coil and a surface receive coil of 2 cm² area. Imaging was performed on days 10, 12, 14, and 16, which are days T0, T2, T4 and T6 post-bevacizumab. The rats were anesthetized with 1.5% isoflurane and immobilized with a fiberglass bite-bar. Temperature was monitored and maintained at $37^\circ\text{C} \pm 1.5^\circ\text{C}$ throughout the experiment. A RARE (rapid acquisition rapid echo) imaging sequence (TE/TR = 8ms/4ms; matrix = 256×256 ; FOV = 3.5 cm, slice = 17.5 mm) was used to acquire sagittal scout images. A T1-weighted spin-echo image was acquired (TE/TR = 11 ms/500ms; matrix = 256×256 ; FOV = 3.5 cm; slice 2mm). Five axial (rat coronal) imaging slices were chosen based on the RARE images and the tumor inoculation site. A loading dose of Gadodiamide (0.1 mmol/kg) was administered 10 minutes before the DSC (dynamic susceptibility contrast) scan in 0.6 ml of saline using a MR-compatible power injector at a rate of (Harvard Apparatus, Holliston, MA). A GRE-EPI (gradient-echo echo planar imaging) sequence (TE = 18.8 ms, TR = 500 ms, 5 NEX) was used to acquire the DSC data. Specifically, GRE-EPI images were collected continuously for a total of 2 minutes, for 1 minute before, and then during and after a bolus injection of a 0.1mmole/kg Gd contrast agent. Finally, a T1-weighted spin-echo image was acquired (TE/TR = 11 ms/500ms; matrix = 256×256 ; FOV = 3.5 cm; slice 2mm) to delineate enhancing tumor.

Leakage-corrected rCBV (relative cerebral blood volume) parameters were determined as previously described (19). Briefly, the signal-time courses were converted into concentration-time curves on a voxel-wise basis. From these the rCBV is likewise determined on a voxel-wise basis all of which are corrected for any contrast agent leakage effects as previously described (21–23). The tumor region of interest (ROI) was initially determined from the contrast enhancing region on the post-contrast T1 weighted image. The ROIs were modified to ensure only areas of increased rCBV are included. Subsequently, the rCBV maps were standardized using a two-step piecewise linear transformation method as previously described (24). Enhancing tumor volumes (reported in mm³) were determined from the post-contrast T1w images, in all slices showing enhancing tumor. At each imaging time point the mean percent change from baseline (day 10 or T0) are determined for enhancing tumor volume for the standardized tumor ROI.

Assessment of tumor vascularity

Brains were obtained for histological analysis from rats at post-inoculation day 16 following the final scan. The rats were deeply anesthetized, and the brain was removed and placed in formalin solution. The tissue was paraffin embedded and cut in 5 μ m sections. For determination of vWF-positive cells, immunohistochemistry was carried out using a human

primary antibody that was obtained from Dako. Following deparaffinization and rehydration, tissue was prepared as follows: 1) endogenous peroxidase activity was blocked by incubation in 3% H₂O₂, 2) an antigen retrieval step was performed by heating tissues in 0.1 M citrate buffer, pH 6.0, for 30 min in a water bath, and 3) endogenous biotin blocked with sequential incubations with avidin and biotin (Avidin-Biotin Blocking kit, Zymed), and 4) nonspecific sites blocked by incubation in blocking solution (Zymed kit). Human primary antibodies specific for the vWF were applied at a concentration of 1:1000 for 16 h at 4°C in blocking buffer. Detection was performed using a streptavidin-biotin immunoperoxidase technique with diaminobenzidine as a substrate. The specificity of staining was verified by performing control experiments in which tissues were incubated with an equal concentration of nonimmune rabbit IgG. Sections were visualized using a Nikon Eclipse 80i microscope equipped with a MicroPublisher 3.3 RTV color video camera (Q Imaging, Surrey, BC, Canada). The images were captured on-line using Metamorph imaging software (Version 7.0, Universal Imaging). At least two images of the hot spot staining in tumor were stored using a $\times 20$ objective lens and a field dimension of 0.26 mm². The number of vessels were counted and averaged by a study group member who was blinded to the experimental groups.

Statistical analysis

Data are presented as means $\pm$ SEM. Generalized estimating equations (GEEs) were used to test the effects of dose, time and their interaction. GEEs are an alternative to repeated measures ANOVA when either the data is non-normal, some observations are missing (here 6–8 points available per time point), or the correlation structure over time needs to be accounted for (here the rats were observed on 4 days). The GEE analysis used the percent change from baseline as the outcome. A two-tailed Mann-Whitney test was used to evaluate the data. The 95% confidence interval was considered significant.

Results

In Figure 1 are shown representative post-contrast T1 weighted images and the rCBV maps obtained in one rat treated with saline (a) and another treated with bevacizumab combined with irinotecan 2 days later (b). On the post-contrast T1 weighted images, it is apparent that the tumor volumes increase for both treated and untreated animals, but to a greater extent in the untreated rat (Fig 1a). The blood volume increases more rapidly in the untreated animal, as apparent on the rCBV maps. The yellow contour shows the enhancing tumor and rCBV ROIs drawn for each study date.

Figure 2 shows the enhancing tumor volumes as a function of time for the six different groups of rats. Initial tumor volumes were similar in each group at treatment onset (6–15.5 mm³). In untreated rats, tumor growth as measured by enhancing tumor volume increased 204% from days T2 to T6 (Fig 2a). Bevacizumab monotherapy treatment administered at day T0, significantly inhibited tumor growth relative to controls at all time points (Fig 2b) ($p < 0.02$). Significant inhibition did not result when treating with irinotecan alone (Fig 2d), but did result on some days when irinotecan was given at days 2 (T2) and 4 (T4) post bevacizumab (Fig 2e,f). Combination of irinotecan days 2 and 4 post bevacizumab was not significantly different than bevacizumab monotherapy. At day T2 the combination of irinotecan 2 days before bevacizumab was not as effective as bevacizumab monotherapy. The GEE analysis showed a significant time effect ($p < 0.001$), and a significant treatment effect with all treatments ($p < 0.03$) except Irinotecan given 2 days before bevacizumab ($p = 0.058$).

Figure 3 illustrates the mean rCBV for the three treatment days for the different treatment paradigms. Contralateral rCBV was less than tumor rCBV at all time point for all treatments

(2216–3342 standardized units). In control rats, tumor blood volume increased from 4761 at baseline to 7204 at day T6 (Fig 3a). Treatment with Bevacizumab (Fig 3b) alone had a delayed effect on rCBV with a significant decrease at day T4 (3941) compared with control (untreated) case (6370). This decrease may indicate a vascular normalization window, which closed at day T6 as rCBV increased. In the case for which irinotecan is combined 2 days before treatment with Bevacizumab (Fig 3d) there is a significant decrease in of tumor angiogenesis at day T2 compared to the untreated. The GEE analysis showed a significant time effect ($p=0.001$) and a significant treatment effect with Irinotecan combined 2 days before ($p=0.004$) or after ($p=0.015$) Bevacizumab. In figure 4, the number of voxels within the rCBV tumor ROIs are graphed, showing the extent of hypervascularity. Treatment with bevacizumab alone or after irinotecan transiently decreased the extent of hypervascularity.

To characterize the vascular density, we carried out immunohistochemical analysis of a marker for endothelial cells, vWF. Immunohistochemical staining for vWF was prominent in tumors. The number of vessels/field are significantly lower for animals treated with irinotecan before bevacizumab compared to untreated controls. The bevacizumab monotherapy rats had vessel densities comparable to untreated controls, suggesting the end of the vascular normalization window.

Discussion

This is the first study to systematically examine the timing of bevacizumab and irinotecan therapies for therapeutic response. Bevacizumab monotherapy had the most inhibition of enhancing tumor volume at all time points compared to controls. Irinotecan given 2 or 4 days after Bevacizumab inhibited enhancing tumor volume at day T6 and T8 compared to controls. Bevacizumab monotherapy had a delayed response in tumor rCBV with a decrease at day T4 that returned to baseline levels, possibly indicating a vascular normalization window. Irinotecan administered 2 days before Bevacizumab had an earlier response, resulting in a decrease in tumor rCBV at day T2. The vessel density measured at day T6 showed no decrease for bevacizumab monotherapy, consistent with the end of vascular normalization. The discrepancy between vessel density and rCBV is expected as rCBV takes into account that there are not as many dilated vessels. This study demonstrates enhancing tumor volume and rCBV provide different information. rCBV likely is better and more direct indicator since it is not confounded by the effects of Bevacizumab on leakage. Previous studies have shown rCBV correlates with survival (15, 25). Studies from our laboratory showed that rCBV can predict the response to bevacizumab more reliably than standard MRI in recurrent high grade gliomas (25).

New therapies for malignant gliomas are targeting the angiogenic pathways and vasculature of tumors. Of these pathways, vascular endothelial growth factor (VEGF) and its receptors have been the main focus of cancer therapies. Winkler and colleagues have shown that combination therapy of DC101, a VEGFR2-specific monoclonal antibody, and radiation significantly delayed tumor growth an effect that was more than additive when using either therapy alone. In their study, mice inoculated with U87 intracranial xenografts were treated with DC101 (40 mg/kg) on days 0, 3, and 6 with radiation (7 Gy) days 4–6, resulting in decreased tumor growth and hypoxia (26). Similarly, studies by Vrendenbergh et al. reported a 63% response measured by at least a 50% decrease in cross-sectional area of the enhancing tumor in recurrent glioblastoma patients treated with bevacizumab and irinotecan (8). In the present study, significant inhibition of the U87MG degree of vascularity and enhancing tumor volume depended on the timing of the irinotecan and bevacizumab treatment. Enhancing tumor volume is dependent on the vascular permeability, which may be altered by the direct effect of anti-angiogenic therapies.

The lack of complete inhibition of tumor growth and vascularity with bevacizumab and irinotecan in our model may be explained by the presence of additional angiogenic factors not targeted by bevacizumab. Platelet derived growth factor (PDGF) is an important signaling pathway in angiogenesis (27–30). The importance of it is highlighted in studies where pericytes provide an escape mechanism and recurrent tumor growth through the PDGF pathway despite blockade of the VEGF2 receptor (27, 29). Such effects may be mediated by basic fibroblast growth factor (bFGF) a growth factor upregulated with suppression of VEGF, that is capable of inducing angiogenesis (31, 32). However, in these studies, unlike the clinical population, treatment with anti-VEGF therapy does not eliminate enhancing tumor volume (33). This phenomenon may be explained by the presence of rodent VEGF, as Avastin only binds to human VEGF produced by the implanted human tumor cells. There is an anti-VEGF antibodies that binds to rat and human VEGF allowing a more complete inhibition of VEGF, and this antibody will be the focus of follow up studies (34).

There are many factors that affect MRI measures of angiogenesis including vessel density and caliber (35). One may therefore correctly argue that no one factor fully characterizes angiogenesis. However, we believe it is correct to say that each factor gives some indication of angiogenesis and this is our implication with rCBV measurements performed here. But with each factor we must always take into consideration the fact that each is an incomplete measure of angiogenesis under given conditions. rCBV has previously been shown to correlate with survival (15, 25) with lower rCBVs predicting better overall survival. Lowest rCBV does in fact suggest best combination and timing under given conditions. In this context where rCBV was decreased most is suggestive of a therapy or combination that is most effective. Additional studies are necessary to fully evaluate overall survival and indicate optimization. In addition, quality of life is also an important factor to consider as a measure of treatment success. In this regard studies need to be performed to also correlate imaging markers such as rCBV with other measures of outcomes such as progression free survival or other quality of life metrics.

In summary, these data support the clinical research showing no improvement with the addition of irinotecan to bevacizumab therapy. Our results suggest a vascular normalization window seen with monotherapy measured using tumor blood volume. Our results demonstrate the utility of rCBV in characterizing the temporal therapeutic paradigms in preclinical studies and may likewise assist in more effective design treatment paradigms for malignant gliomas in patients. Combination therapy with temozolomide or radiation will be studied in subsequent rCBV studies using our xenograft model.

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Fig. 1. Panel A: Representative images of post contrast T1 weighted image and rCBV maps from an untreated animal shown longitudinally (top to bottom). Panel B: Representative images of post contrast T1 weighted images and rCBV maps from an animal treated with bevacizumab combined with irinotecan 2 days later shown at days T0, T2, T4, and T6. The enhancing tumor and rCBV ROIs, drawn on each image or map, are indicated by the yellow contours.

Figure 2.

The effect of bevacizumab and irinotecan treatment on enhancing tumor volume. Tumor volume was measured as enhancing region on post-contrast T1 weighted images. Animals were imaged at days T0, T2, T4, and T6. Data are presented mean $\pm$ SEM. * $P < 0.05$ Saline vs. Treated by two-tailed Mann-Whitney test. # $P < 0.05$ Bevacizumab vs. Combination Irinotecan and Bevacizumab by two-tailed Mann-Whitney test.

Figure 3.

The mean rCBV values measured for different combinations of bevacizumab and irinotecan using ROIs drawn only in areas of increased rCBV. Data are presented mean $\pm$ SEM

* $P < 0.05$ Saline vs. Treated by two-tailed Mann-Whitney test. The decrease in rCBV was significant compared to untreated controls only at day T4 for bevacizumab monotherapy.

Figure 4.

The extent of hypervascularity is measured by the number of voxels in the rCBV masks. Data are presented mean $\pm$ SEM * $P < 0.05$ Saline vs. Treated by two-tailed Mann-Whitney test.

Figure 5. Effect of bevacizumab and irinotecan on vascular density. Shown are vWF stained sections from a control (a) and animal treated with bevacizumab at T0 and irinotecan T2 (b). c: Quantification of vascular density. Data are means $\pm$ SEM of vessels per field. * $P < 0.05$ Saline vs. Treated by two-tailed Mann-Whitney test.