To evaluate efficacy and MRI findings after intravenous bevacizumab and/or carboplatin in a human glioma animal model, we randomized male nude rats with intracerebral UW28 human glioma xenografts to four groups: (1) controls (n = 9), (2) bevacizumab 10 mg/kg (n = 6), (3) carboplatin 200 mg/m² (n = 6), and (4) bevacizumab + carboplatin (n = 6). MRI was performed on the day of treatment (day 7–10) and 1 week later, and rats were followed for survival. Dynamic MRI was done in three controls and three rats treated with bevacizumab with or without carboplatin before and 24 h after treatment. Median overall survival (OS) was as follows: group 1, 16 days; group 2, 23 days; group 3, 22 days; group 4, 36 days. OS was significantly longer in group 4 than in group 1 (p = 0.0011), group 2 (p = 0.0014), and group 3 (p = 0.0015), and rats had significantly larger tumors. No objective tumor responses were observed on MR images at 1 week after treatment; however, after bevacizumab, dynamic MRI showed reduced gadolinium enhancement intensity and increased time to peak, consistent with decreased vascular permeability. Carboplatin + bevacizumab is effective and superior over bevacizumab or carboplatin monotherapy in this animal model. Increased survival concomitant with increased asymptomatic tumor volume is suggestive that vascular targeting with reduced peritumoral edema and mass effect contributes to the efficacy of bevacizumab. The promising survival data warrant future clinical trials using bevacizumab + carboplatin.

Keywords: bevacizumab, carboplatin, glioma, magnetic resonance imaging, rat model

Despite recent advances in treatment, WHO grade III and IV gliomas still represent a great challenge in oncology, with overall poor outcomes and almost inevitable lethality. A recent study has shown surprisingly high response and survival rates in patients with relapsed grade III and IV gliomas treated with bevacizumab, an antibody against the proangiogenic vascular endothelial growth factor (VEGF), in combination with irinotecan, a topoisomerase I inhibitor. Bevacizumab has shown a significant survival advantage in patients with solid malignancies when administered
with chemotherapeutic agents, especially in colorectal cancer. However, its efficacy as a single agent remains to be substantiated, and it has been postulated that at least part of the bevacizumab efficacy seen in gliomas reflects decreased vascular permeability and therefore mass effect (“dexamethasone effect”) rather than true tumor response. Another postulated explanation for the high efficacy of bevacizumab in gliomas is a synergistic effect due to increased irinotecan delivery to the tumor through bevacizumab-induced vascular normalization, reduced interstitial pressure, and reversal of hypoxia, a known mechanism of tumor resistance to chemotherapy. We questioned whether irinotecan is a necessary component of the treatment regimen or if it can be replaced with a drug with greater efficacy against gliomas, such as cisplatin, carboplatin, or nitrosoureas, given the low response rate of 0%–15% observed with irinotecan monotherapy in relapsed gliomas.

MRI with dynamic contrast enhancement is a new approach to monitoring tumor vasculature and response to therapy. Dynamic MRI allows noninvasive in vivo quantification of tumor vasculature, including cerebral blood volume and the permeability of the blood–brain barrier. These techniques have been shown to be sensitive to changes in the blood–brain barrier in animal models. Bevacizumab has been investigated with dynamic contrast enhanced imaging and has been shown to alter tumor blood volume and perfusion.

Here, we report the efficacy and toxicity of bevacizumab administered in combination with carboplatin in a nude rat model of human glioma and the impact of this treatment on conventional and dynamic MRI. We tested the hypotheses that bevacizumab + carboplatin would result in improved survival compared to control rats, with tolerable toxicity, and that the drug combination would exert a synergistic effect, reflected by improved survival over bevacizumab or carboplatin monotherapy. Furthermore, we investigated whether MR image changes over a 1-week period, as well as intratumoral vascular permeability and blood volume on dynamic MRI, were altered in response to bevacizumab, thereby providing surrogate markers for the antiangiogenic effect of bevacizumab. Finally, the mechanism of increased survival (i.e., targeting the vasculature vs. increasing tumor cell death) was addressed.

**Materials and Methods**

**Tissue Culture**

UW28 human glioma cells (from Dr. Ali-Osman, Duke University, Durham, NC, USA) were grown in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Cells were harvested immediately prior to intracerebral implantation and were used only if viability exceeded 90%. For in vitro analysis of toxicity, live cell number was determined with the WST-1 Cell Proliferation Assay Kit (Chemicon International Inc., Temecula, CA, USA). Cells were plated in 96-well tissue culture plates and treated with carboplatin (10–200 µg/ml). The WST-1 reagent was added 3 days after carboplatin, and absorbance at 450 nm was determined using a microplate reader.

**Tumor Inoculation**

The care and use of animals were approved by the institutional Animal Care and Use Committee and were supervised by the Oregon Health and Science University (OHSU) Department of Animal Care. For tumor implantation, male athymic nude rats (nu/nu) were anesthetized with ketamine (60 mg/kg i.p.) and diazepam (7.5 mg/kg i.p.). Animals’ heads were held in a stereotaxic device, a midsagittal incision was performed, and a 2-mm burr hole was drilled in the skull. Animals received 1.2–1.5 × 10⁶ UW28 cells (>90% viable) in a volume of 12–15 µl over 5 min using a 27-gauge needle. Cells were stereotaxically injected in the right caudate-putamen (vertical, bregma 6.5 mm; lateral, bregma 3.1 mm). The needle was initially advanced to a depth of 7 mm and then withdrawn to a depth of 6.5 mm to limit reflux up the needle track. After inoculation, the needle was slowly removed, and the skin incision was closed.

**Treatment Study Design**

For the treatment study, drugs were injected intravenously into the femoral vein in isoflurane-anesthetized rats on days 7–10 after inoculation. The time point of treatment was chosen based on our experiences with earliest evidence of tumor on histology from a previous pilot study (n = 5 rats) and the availability of MRI to evaluate the presence of tumor. A second MRI scan was done 1 week after treatment (days 14–17). On the day of the first MRI, rats were randomized into four groups: (1) control group (no treatment; n = 9), (2) bevacizumab 10 mg/kg i.v. (n = 6), (3) carboplatin 200 mg/m² i.v. (n = 6), and (4) bevacizumab 10 mg/kg i.v. plus carboplatin 200 mg/m² i.v. (n = 6). The bevacizumab dose was chosen based on a recently published study in relapsed glioma patients, and the carboplatin dose was selected due to its proven tolerable toxicity in previous rat studies from our group and its use in human studies. Rats with no evidence of tumor as determined by T1 enhancement on both pre- and posttreatment MRI scans were not included in the study, nor were rats with atypical tumor location (e.g., at the skull base). In some rats, the first MRI showed enhancement at the cortex that could not be distinguished from tissue damage (Fig. 1A); these were included in the analysis if the second MRI showed clear evidence of tumor growth in the caudate nucleus inoculation site (Fig. 1B). Excluded animals were sacrificed following the second MRI. The animals were examined daily and weighed weekly from inoculation until sacrifice. Animals were followed for survival and sacrificed using intracardiac thiopental injection (25 mg) according to standardized criteria, including (1) occurrence of severe clinical signs or symptoms that made survival
rats, one rat treated with bevacizumab alone, and two rats treated with bevacizumab + carboplatin, at two different time points 24 h apart, between days 9 and 11. One control group rat could only be scanned once due to technical issues with the MR scanner. In the three treated rats, the first dynamic scan was done before and the second 24 h after treatment. Rats were anesthetized using medetomidine (0.6 mg/kg i.p.) and ketamine (15 mg/kg i.p.) and imaged on a 12-T MRI scanner (Bruker BioSpin, Ettlingen, Germany) using a custom rat head volume coil for transmitting and a surface coil for receiving radiofrequency signal. For quantitative Gd permeability measurement, a catheter in the jugular vein was preloaded with 50 µl Omniscan, and the contrast agent was administered at a 1 ml/min flow rate followed by saline flush. During injection rapid, repeated (1.8 s/image) T1-weighted FLASH (fast low-angle-shot) acquisitions were started on a single coronal slice and acquired for 5 min (TR, 25 ms; TE, 1.7 ms).

The rate of Gd extravasation in the tumor was measured as the time to peak enhancement and compared before and after treatment, to quantify the changes of the vascular permeability.16

Histology
Brains were excised and fixed in 10% buffered formalin for vibratome sectioning, 100 µm in the coronal plane. For tumor volumetrics, every sixth brain section was stained with hematoxylin and then imaged at high resolution (30-µm pixel diameter) on an Epson 1640XL flatbed scanner using Adobe Photoshop software. Tumor volumes were assessed using NIH ImageJ software, as we have previously validated.13

Statistical Considerations
No power calculations were made a priori or post hoc. For T1-Gd and T2 volumes and for hematologic toxicities, the data were not normally distributed, so non-
parametric Kruskal-Wallis one-way analysis of variance (ANOVA) models were fitted to the data. Pearson (parametric) and Spearman (nonparametric) correlations between posttest and percent change in T1-Gd and T2 volumes were estimated. Statistical analyses of the qualitative dynamic MRI results were not performed due to the small sample number in each group. For histologic volumes, a one-way ANOVA model was fit with Tukey-adjusted pairwise multiple comparisons. The Wilcoxon test was also used to compare the four groups. Tumor volumes were also assessed with the Kruskal-Wallis test, which does not assume normality. Overall survival (OS) was calculated from the day of inoculation to sacrifice. OS was estimated using the Kaplan-Meier product limit method, and differences among the groups were assessed with the generalized Wilcoxon test. Pairwise comparisons among groups were made with the generalized Wilcoxon test using the Bonferroni adjustment. The generalized Wilcoxon test is presented rather than the log rank since the graphs suggested the proportional hazards assumption might not be met for these data; however, the results using the log rank test were similar. The significance level was 0.05 (two-sided) for all statistical tests.

Results

Radiographic, Pathologic, and Clinical Characterization of the UW28 Glioma Model

A total of 49 rats were inoculated; 43 of 48 (90%) evaluable rats had evidence of tumor on at least one of the two MRI scans. Two MRI scans were performed in 40 rats. Only one scan was done in eight rats and none in one rat, either because of technical issues or because the rats had to be sacrificed prior to the second scan. Typically, 3-T MRI demonstrated T2/FLAIR signal changes and Gd enhancement in the proximity of the burr hole (cerebral cortex) and/or the inoculation site (caudate-putamen) and/or along the needle track. Pre-Gd T1-weighted images showed diffuse slightly elevated signal intensity in the injected hemisphere in large tumors. Gd enhancement was typically of moderate intensity, consistent with moderate leakiness of the intratumoral vasculature. In animals with evidence of tumor on the first scan, tumor growth was observed in all and mass effect with midline shift to the left and ventricle compression in most of the animals on the second MRI scan (Fig. 1).

Twenty-seven of the 43 tumor-bearing rats (63%) met the inclusion criteria and were randomized for inclusion in the in vivo treatment study. Median tumor volumes on the first 3-T MRI (T2-weighted sequences) were as follows: untreated controls, 19.0 (range, 2–211) mm3; bevacizumab, 32.8 (range, 22.5–187) mm3; carboplatin, 21.5 (range, 9–27) mm3; bevacizumab + carboplatin, 9.8 (range, 2.5–131) mm3. Table 1 shows the volumetric results from the pre- and posttreatment T2-weighted and T1 + Gd MRI. Two control animals died from their brain tumor prior to the second MRI. Qualitative MRI characteristics (e.g., intensity of Gd enhancement) did not differ between the four groups. There was no statistical difference in tumor sizes between the four treatment groups on the first or on the second scan. There was excellent correlation between the T2-weighted MRI and the T1 + Gd scans for both the pretreatment scans (Pearson r = 0.995) and the posttreatment scans (Pearson r = 0.992) (Fig. 1C). Percent change from baseline was also highly correlated between T1 + Gd and T2 (Pearson r = 0.969). Spearman correlations were all similar and are not reported. T1 + Gd volumes averaged 80% of the T2 volumes in the same animal.

We evaluated whether the percent change in tumor volume 1 week after treatment provided any information about the efficacy of treatment. One tumor in the control group and one in the bevacizumab + carboplatin group showed no change between pre- and posttreatment scans; all other tumors had progressed on the second MRI, 1 week after treatment. Comparisons across the four groups approached statistical significance for percent change in volumes using T1 + Gd (p = 0.0693) and T2 (p = 0.077). The sample size was likely inadequate.

Table 1. MR volumetrics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Volume</th>
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<tbody>
<tr>
<td></td>
<td>Before Treatment (1 Week)</td>
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<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Arm</td>
<td></td>
</tr>
<tr>
<td>T2 MRI</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.0</td>
</tr>
<tr>
<td>BEV</td>
<td>32.8</td>
</tr>
<tr>
<td>Carbo</td>
<td>21.5</td>
</tr>
<tr>
<td>BEV + Carbo</td>
<td>9.8</td>
</tr>
<tr>
<td>T1 + Gd MRI</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.8</td>
</tr>
<tr>
<td>BEV</td>
<td>28.3</td>
</tr>
<tr>
<td>Carbo</td>
<td>15.3</td>
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<tr>
<td>BEV + Carbo</td>
<td>8.0</td>
</tr>
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</table>

Abbreviations: BEV, bevacizumab; Carbo, carboplatin.
Jahnke et al.: Bevacizumab and carboplatin in a glioma model

Equate to detect differences among the groups given the variability of these measures.

Hematologic toxicity was evaluated immediately prior to sacrifice by complete blood counts. At these late time points after chemotherapy, blood counts did not significantly differ across the treatment arms (nonparametric ANOVA; results not shown).

**Dynamic MRI**

In the two control rats chosen for dynamic MRI that were scanned 24 h apart, no differences in the intensity of contrast enhancement on postcontrast T1 sequences and no differences in blood volume or vascular permeability could be detected. The control rat that was scanned only once had MRI features identical to those of the other control rats. In the three rats treated with bevacizumab alone or in combination with carboplatin, post-Gd T1 signal changes were reduced after treatment compared to the baseline scan, and the time to maximum enhancement was increased (Fig. 2). The time to peak enhancement ranged from 52 to 77 s in untreated animals, while the time delay in the bevacizumab + carboplatin–treated rat (Fig. 2D) was increased to 118 s. These data indicate that vascular permeability was reduced by the combination of bevacizumab + carboplatin.

**Treatment Efficacy**

UW28 cells were sensitive to carboplatin in vitro. A 3-day treatment with carboplatin killed the cells with half-maximal toxicity at 100 µg/ml and 90% lethality at 200 µg/ml, as determined by the WST-1 cell proliferation assay (data not shown). Bevacizumab was ineffective in culture.

Median OS in the four treatment groups was as follows: (1) control (no treatment), 16 (range, 10–22) days; (2) bevacizumab alone, 23 (range, 17–30) days; (3) carboplatin alone, 22 (range, 19–24) days; (4) bevacizumab + carboplatin, 36 (range, 31–39) days. The survival time of individual animals is shown in Fig. 3A, and the Kaplan-Meier survival curves are shown in Fig. 3B. OS was significantly different among the four groups. Based on Bonferroni-adjusted comparisons between pairs of groups, OS of the combination treatment rats (group 4) was significantly different from group 1 (p = 0.0011), group 2 (p = 0.0014), and group 3 (p = 0.0015). No other statistically significant differences were observed comparing other pairs of groups, although the comparisons between groups 1 and 2 (p = 0.0116) and groups 1 and 3 (p = 0.0230) approached significance but were not significant compared to the Bonferroni-adjusted significance level.

**Tumor Volumes**

Immunohistochemistry was used to evaluate tumor growth pattern and biology. All animals in the study showed tumor in the brain adjacent to the burr hole and/or within the caudate-putamen. All tumors were well circumscribed without major infiltration into the surrounding brain. Necrotic areas were observed within...
tumors in two of eight evaluable brains in the control group, as determined by tissue damage on hematoxylin staining. This finding was more frequent in the treatment groups, including six of six in the bevacizumab + carboplatin group (Fig. 4A, B).

Tumor sizes on histology ranged from 41 to 357 mm³ at 10–39 days after tumor implantation. Median tumor volumes on histology were (1) controls, 132 (range, 41–183) mm³; (2) bevacizumab, 223 (range, 112–357) mm³; (3) carboplatin, 155 (range, 126–287) mm³; (4) bevacizumab + carboplatin, 255 (range, 206–290) mm³. Mean histologic tumor volumes are shown in Fig. 4C. A one-way ANOVA model demonstrated a significant difference among the groups with respect to histologic volume ($p = 0.0158$). The differences between groups 1 and 2 ($p = 0.0374$) and groups 1 and 4 ($p = 0.0243$) were significant using a Tukey adjustment for multiple comparisons; no other pairs of means differed from one another. The Kruskal-Wallis test, which avoids the assumption of normality, was not significant ($p = 0.07$ for both volumes). Thus, all rats had large tumors at death, but the rats receiving bevacizumab, with or without carboplatin, had larger tumors than did controls or rats receiving carboplatin alone. A comparison of survival time and histologic tumor volume is shown in Fig. 5 for the control and bevacizumab + carboplatin groups.
Longer survival in the bevacizumab + carboplatin rats was accompanied by larger tumor volumes.

**Discussion**

This study investigated the efficacy of bevacizumab and carboplatin in a human glioma animal model. The UW28 glioma model in nude rat brain yielded consistent results with high tumor take and reproducible findings on MRI and histology. The treatment study provides evidence for our initial hypothesis that the combination of bevacizumab and carboplatin is effective and also has greater efficacy than either drug administered alone, with acceptable hematologic toxicity.

Although no objective tumor responses were observed on MRI, dynamic MRI showed reduced tumor vascular permeability in rats treated with bevacizumab shortly after treatment. Rapid decreases in Gd enhancement on posttreatment MRI has also been reported in patients with malignant gliomas who receive bevacizumab as part of their treatment regimen. By normalizing structurally and functionally abnormal tumor vasculature and decreasing VEGF-mediated vascular permeability, bevacizumab decreases interstitial tumor pressure, hypoxia, and acidosis, thus increasing delivery and efficacy of cytotoxic agents. This synergism may provide an explanation for the superior survival in rats treated with bevacizumab in combination with carboplatin over either agent alone. Bevacizumab also has direct effects on stem-cell-like glioma cells and significantly reduces tumor-associated edema and mass effect, which may explain the slightly better survival of rats treated with bevacizumab over the control group. In this study, bevacizumab effects on MRI seemed to be transient. We did not observe decreased Gd enhancement on MRI 1 week after treatment, as opposed to 24 h after treatment on dynamic MRI. However, dynamic MRI was not done 1 week after treatment, so persisting changes in tumor vascular permeability could have been missed. Nevertheless, being consistent with our preclinical data, bevacizumab monotherapy will most likely not result in sustained clinical tumor control since it only targets one member of the VEGF family (i.e., VEGF-A), and it can be postulated that other angiogenic factors could compensate for the loss of VEGF-A activity. As demonstrated in this study, an additional cytotoxic agent will most likely be necessary to achieve prolonged tumor responses in patients, as already postulated by others.

Cytotoxic chemotherapy in malignant gliomas has been limited due to innate chemoresistance and limitations of drug delivery across the blood–brain barrier. The most active agents are nitrosoureas, cisplatin, carboplatin, procarbazine, and temozolomide. As a second-generation platinum analog, can partially cross the blood–cerebrospinal fluid barrier. As opposed to irinotecan, it is not metabolized by cytochrome P450, and its pharmacokinetics are not influenced by corticosteroids and anticonvulsants. Furthermore, it has superior in vitro activity against glioma cell lines compared to nitrosoureas. As a single agent, carboplatin has mainly been used for relapsed malignant glioma and has shown tumor control rates from 40%–50% without major toxicities. These favorable features provided a rationale to evaluate carboplatin in this study.

This study has several limitations. Animal models do not completely reflect the situation in humans and often lack reproducibility due to changes in cell cultures over time. We tried to overcome the latter issue by completing this study within a relatively short time period of 3 months and by strictly randomizing rats to the four treatment groups. Despite remarkably differing survival times across the study groups, we did not observe tumor responses on MRI. The fact that we used T-cell–deficient nude rats with reduced innate immunity may have led to only short-lived tumor responses or decreases in tumor growth rates that may have been missed on the second MRI scan, which was done arbitrarily 1 week after treatment. Larger sample size or different time frame may have detected differences between groups that were not detected in the present study. We observed higher tumor volumes on histology in the rats that received bevacizumab, either alone or in combination with carboplatin, as opposed to controls and those that were treated with carboplatin alone. One may speculate that due to reduced mass effect and peritumoral edema following the normalization of blood–brain barrier vasculature by bevacizumab, as evidenced by reduced contrast enhancement on MRI, rats were able to tolerate larger tumor volumes even without evidence of objective tumor responses. In the intracerebral injection tumor model, there may be partial destruction of the blood–brain barrier around the needle track with concomitant local increased permeability. One may speculate that there was elevated penetration and efficacy of chemotherapy in this model compared to de novo tumors. However, MRI prior to treatment showed minimal Gd permeability in the caudate nucleus area of tumor inoculation in most animals. Therefore, we argue that possible high
permeability in the implanted model does not explain the superiority of the bevacizumab + carboplatin arm in terms of survival and tolerance of high tumor volumes without clinical symptoms.

In conclusion, the combination of carboplatin and bevacizumab was effective in a new UW28 rat glioma model and superior over bevacizumab or carboplatin monotherapy, suggesting that besides bevacizumab effects on tumor vasculature, actual tumor cell death contributes to the efficacy of this regimen. In a recent phase II trial on the effect of bevacizumab alone (n = 85) or in combination with irinotecan (n = 82) on 6-month progression-free survival in recurrent, treatment-refractory glioma patients, the combination arm demonstrated a better 6-month progression-free survival (50.2%) and overall response rate (32.9%) than the bevacizumab-alone arm (35.1% and 20%, respectively). Median OS was slightly better in the bevacizumab monotherapy arm (9.7 months) compared with the combination treatment arm (8.9 months), thereby challenging the assumption that bevacizumab in combination with chemotherapy is more effective than bevacizumab alone. However, the authors did not report further treatment after progression on the study treatment regimens; for example, it is unclear whether patients were switched to bevacizumab plus additional chemotherapy after progression on the bevacizumab monotherapy arm. The promising survival data in our study warrant future clinical trials using bevacizumab in combination with carboplatin. Due to its known favorable pharmacokinetics and tumor control rates in patients with malignant glioma, carboplatin may prove to be a better combination partner for bevacizumab than irinotecan; however, this needs to be verified in future clinical trials. Surprisingly, the main synergistic effect of bevacizumab + carboplatin may be due to decreased intracranial pressure and/or increased compliance. Although there was more necrosis by histology in rat brain tumors treated with both agents, tumor volumes increased linearly compared with controls. Increased survival was accompanied by asymptomatic increased tumor size. Thus, bevacizumab + carboplatin may be targeting the vasculature more than killing glioma cells, resulting in symptom control and increased survival due to decreased edema rather than actual tumor response. Another theory for lack of sustained response may be a change in tumor phenotype to a more invasive type or tumor growth with restored blood–brain barrier under bevacizumab treatment. The latter assumption is supported by clinical data on glioma patients under bevacizumab treatment in whom recurrence pattern analysis revealed a significant increase in the volume of infiltrative tumor relative to enhancing tumor in bevacizumab responders.

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