Cancer Therapy: Preclinical

Anti-VEGF Antibodies Mitigate the Development of Radiation Necrosis in Mouse Brain

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Abstract

**Purpose:** To quantify the effectiveness of anti-VEGF antibodies (bevacizumab and B20-4.1.1) as mitigators of radiation-induced, central nervous system (brain) necrosis in a mouse model.

**Experimental Design:** Cohorts of mice were irradiated with single-fraction 50- or 60-Gy doses of radiation targeted to the left hemisphere (brain) using the Leksell Perfexion Gamma Knife. The onset and progression of radiation necrosis were monitored longitudinally by in vivo, small-animal MRI, beginning 4 weeks after irradiation. MRI-derived necrotic volumes for antibody (Ab)-treated and untreated mice were compared. MRI results were supported by correlative histology.

**Results:** Hematoxylin and eosin–stained sections of brains from irradiated, non–Ab-treated mice confirmed profound tissue damage, including regions of fibrinoid vascular necrosis, vascular telangiectasia, hemorrhage, loss of neurons, and edema. Treatment with the murine anti-VEGF antibody B20-4.1.1 mitigated radiation-induced changes in an extraordinary, highly statistically significant manner. The development of radiation necrosis in mice under treatment with bevacizumab (a humanized anti-VEGF antibody) was intermediate between that for B20-4.1.1–treated and non–Ab-treated animals. MRI findings were validated by histologic assessment, which confirmed that anti-VEGF antibody treatment dramatically reduced late-onset necrosis in irradiated brain.

**Conclusions:** The single-hemispheric irradiation mouse model, with longitudinal MRI monitoring, provides a powerful platform for studying the onset and progression of radiation necrosis and for developing and testing new therapies. The observation that anti-VEGF antibodies are effective mitigants of necrosis in our mouse model will enable a wide variety of studies aimed at dose optimization and timing and mechanism of action with direct relevance to ongoing clinical trials of bevacizumab as a treatment for radiation necrosis. *Clin Cancer Res; 20(10); 2695–702. ©2014 AACR.*

Introduction

Radiation is a key component in the treatment of both benign and malignant central nervous system (CNS) tumors, including gliomas, metastases, meningiomas, schwannomas, pituitary adenomas, and other less common neoplasms. Multiple radiation-treatment schemes have been developed to treat various neoplasms in the brain. These treatment protocols use a variety of different fractionation and conformational schemes designed to deliver focused radiation to regions in the brain to maximize control of tumor growth and minimize deleterious effects on normal brain tissue. Outcomes of these clinical protocols may be complicated by radiation effects on nonneoplastic tissue, resulting in a spectrum of phenotypes, ranging from minimal change with no observable clinical symptoms, to delayed radiation necrosis with severe neurologic sequelae. The delayed effects from radiation may produce cerebral edema and necrosis of normal brain parenchyma, resulting in untoward neurologic effects that are difficult to differentiate from recurrent tumor growth.

Radiation necrosis, a delayed radiation neurotoxicity that can occur after radiation treatment of the CNS, can develop between 3 months and 10 years after radiotherapy, with most cases occurring in the first 2 years (1). Necrosis following radiation is not uncommon, occurring in 3% to 24% of patients receiving focal irradiation (1). The incidence may be 3-fold higher with concurrent chemotherapy (2, 3). Currently, only limited options for therapeutic intervention are available for patients with symptomatic radiation necrosis. Surgical resection of necrotic tissue is often not possible due to the location of the necrosis in eloquent regions of the brain. Prolonged treatment with corticosteroids is often used (4), but is complicated by cushingoid side effects, including weight gain, myopathy,
Translational Relevance

Radiation necrosis is a severe, but late occurring type of injury to normal tissue, within and surrounding a radiation treatment field, which can lead to significant complications for neurooncology patients. Radiation necrosis is difficult to distinguish from recurrent tumor by either neurologic examination or clinical imaging protocols. Concerns for the development of radiation necrosis often limit therapeutic radiation doses. Current treatment options for radiation necrosis are limited. In the present study, we demonstrate that treatment with anti-VEGF antibodies significantly reduces late-onset necrosis in irradiated brain in a mouse model of radiation necrosis. The animal model highlighted in these experiments can serve as a platform for studies aimed at optimizing the dosing and timing of anti–VEGF-Ab therapy. Findings from this work can provide direct, powerful support for ongoing and future clinical studies with bevacizumab, a humanized anti-VEGF antibody approved by the U.S. Food and Drug Administration, for the treatment of radiation necrosis.

immunosuppression, psychiatric disturbances, and occasionally arthritic sequelae, such as avascular necrosis affecting the shoulders and hips (5). Hyperbaric oxygen treatment has also been considered as a therapeutic modality (6, 7). However, it is cumbersome to deliver, expensive, and available in few medical centers. Its benefit has only been shown in a relatively small number of cases (8).

Two models of the pathogenesis of radiation necrosis have been proposed. These models involve radiation-induced injury to vasculature, radiation-induced injury to glial cells (apoptosis), or a combination thereof (9). In particular, radiation necrosis has been associated with breakdown of the blood–brain barrier, leading to increased vascular permeability and elevated levels of VEGF (1, 10). Elevated VEGF levels can, in turn, damage vascular endothelial cells and, together with subsequent narrowing of vessels due to fibrosis, can result in edema and necrosis (11).

Bevacizumab, a humanized monoclonal antibody against VEGF, was first approved by the U.S. Food and Drug Administration in 2004 for use in treating metastatic colorectal cancer. Since then, it has also been approved for the treatment of non–small cell lung cancer, metastatic breast cancer, and recurrent glioblastoma (12). Bevacizumab has been reported to normalize the vasculature, thereby enhancing the efficient delivery of drugs (13, 14). There is emerging clinical evidence that bevacizumab substantially decreases the effects of radiation necrosis (15–23). A recent randomized double-blind study of bevacizumab therapy for patients with radiation necrosis (19) provided evidence of its efficacy in mitigating radiation necrosis. These studies relied on MRI, and, in particular, T1 postgadolinium enhancement to characterize radiation necrosis, which is complicated by the presence of recurrent tumor. Also, because it is generally not possible to correlate time-course MR observations with histologic findings in patients, these human studies lack information about the mechanisms of action of bevacizumab. Thus, further studies are needed to validate the effects and mechanisms of bevacizumab in the treatment of radiation necrosis.

We have recently developed a mouse model of delayed time-to-onset injury (24) that recapitulates the histologic features observed in patients suffering from CNS radiation necrosis. This model provides a platform for studies aimed at developing methods to identify/detect, monitor, protect against, and mitigate radiation necrosis, and distinguish it from tumor regrowth. In the work reported herein, this model is used to validate the efficacy of both murine and humanized anti–VEGF-A monoclonal antibodies as mitigators of radiation necrosis following high-dose radiation treatment.

Materials and Methods

Animals

All studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and in accordance with protocols approved by the Washington University Division of Comparative Medicine that met or exceeded American Association for the Accreditation of Laboratory Animal Care standards. Female Balb/c mice were used for the study and observed daily and weighed weekly to ensure that interventions were well tolerated.

Irradiation and treatment

Mice were irradiated with the Leksell Gamma Knife Perfexion (Elekta; http://www.eleka.com/), a state-of-the-art unit used for stereotactic irradiation of patients with malignant brain tumors. Mice were supported on a specially designed platform mounted to the stereotactic frame that attaches to the treatment couch of the Gamma Knife. Mice were anesthetized with a mixture of ketamine (25 mg/kg), acepromazine (5 mg/kg), and xylazine (5 mg/kg), injected intraperitoneally 5 minutes before the start of irradiation. A total of 35 female Balb/c mice were irradiated with either 60 Gy or 50 Gy, as described below, and the resulting brain parenchymal changes were characterized by both MRI and histology.

VEGF inhibitory monoclonal antibody

Bevacizumab (Genentech/Roche) is a humanized mAb (monoclonal antibody) that inhibits VEGF-A. Anti-VEGF antibody B20-4.1.1 (Genentech), heretofore referred to as B20-4.1.1, is a cross-species reactive, function-blocking mAb targeting both human and murine VEGF-A.

Experimental outline

As part of the overall study, two different sets of experiments, designated as “A” and “B” were performed. In experiment “A,” two cohorts of mice (n = 10 each) received a single 50-Gy dose (50% isodose) of Gamma Knife radiation. At this dose, the onset of radiation necrosis typically
occurs approximately 4 weeks after irradiation. Mouse cohort #1 was an irradiated, non-antibody (Ab)-treated, control group; cohort #2 received B20-4.1.1 (10 mg/kg), twice weekly, from 4 to 13 weeks after irradiation. In experiment “B,” three cohorts of mice received a single fraction of 60-Gy radiation (50% isodose). At this dose, the onset of radiation necrosis typically occurs approximately 3 weeks after irradiation. Mouse cohort #1 (n = 5) was an irradiated, non-Ab-treated antibody, control group; cohort #2 (n = 5) received B20-4.1.1 (10 mg/kg), twice weekly, from 3 to 10 weeks after irradiation; cohort #3 (n = 5) received bevacizumab (10 mg/kg, twice weekly) from 3 to 10 weeks after irradiation. Antibodies were administered intraperitoneally.

MRI
Images were collected in an Agilent/Varian 4.7-T small-animal MR scanner equipped with a DirectDrive console. The scanner is built around an Oxford Instruments (Oxford, United Kingdom) 33-cm, clear-bore magnet equipped with 21-cm inner diameter, actively shielded Agilent/MagneX gradient coils (maximum gradient, 28 G/cm; rise time, approximately 200 ms), and Oy International Electric Company model A-240 amplifiers (300 V and 300 A).

MRI data were collected using an actively decoupled coil pair: 1.5-cm outer diameter surface coil (receive) and a 9-cm inner diameter Helmholz coil (transmit). Before the imaging experiments, mice were anesthetized with isoflurane/O2 [3% (vol/vol)] and maintained on isoflurane/O2 [1% (vol/vol)] throughout the experiments. Mice were restrained in a laboratory-constructed Teflon head holder with ear bars and a tooth bar. To maintain the body temperature of the mice at approximately 37°C, mice were placed on a water pad circulating warm water. Mice were injected intraperitoneally with 0.5 mL Omniscan (gadodiamide; GE Healthcare) contrast agent, diluted 1:10 in sterile saline.

Non-Ab-treated mice treated with 50 Gy of radiation were imaged 4, 8, and 13 weeks after irradiation. Ab-treated mice that had received 50 Gy of radiation were imaged 4, 5, 7, 9, 11, and 13 weeks after irradiation. Mice that had received 60 Gy of radiation were imaged weekly from 3 to 10 weeks after irradiation (non-Ab-treated mice were not imaged at week 9). Multislice, T2-weighted, spin-echo transaxial images were collected beginning approximately 3 minutes following gadodiamide administration with the following parameters: time to repetition (TR) = 1.5 s; time to echo (TE) = 0.05 s; field of view (FOV) = 1.5 × 1.5 cm²; slice thickness = 0.5 mm; 21 slices to cover the whole brain; total acquisition time = 12 minutes. Multislice, T1-weighted, spin-echo transaxial images were then collected over 5 minutes with the following parameters: TR = 0.65 s; TE = 0.02 s; FOV = 1.5 × 1.5 cm²; slice thickness = 0.5 mm; 21 slices to cover the whole brain.

Histology
Immediately after the last MRI session (13 weeks after irradiation for mice receiving 50-Gy irradiation, 10 weeks after irradiation for mice receiving 60-Gy irradiation), all the mice were perfused intracardially with 0.1 mol/L phosphate-buffered saline and formalin. The heads were then dissected and immersed in formalin for 24 hours. All the brains were removed from skulls and embedded in paraffin. A single, 8-μm-thick coronal tissue section was taken from each brain near the radiation center and stained with hematoxylin and eosin (H&E) according to standard protocols. Histologic comparison of tissue sections was facilitated by the accurate targeting of radiation, ensuring that all histologic slices reflected the same anatomic features.

Statistical analysis
For experiment “A,” Laird and Ware’s growth curve method (25) was used to compare the differences in the rates of progression of radiation necrosis, because the Ab-treated and non-Ab-treated irradiated cohorts were imaged at different time points. For experiment “B,” in addition to the overall rates of progression, two-way ANOVA for repeated measurement data was also used to compare the MRI-derived necrotic volumes between Ab-treated and non-Ab-treated irradiated cohorts, followed by ad hoc multiple comparisons for differences at specific time points. All the tests were two-sided and a P value of 0.05 or less was taken to indicate statistical significance. The statistical analysis was performed using SAS 9.3 (SAS Institutes).

Results
MRI detects radiation necrosis as image hyperintensity in T2-weighted images
We have established a robust murine model of radiation necrosis using a Gamma Knife Perfexion to hemispherically target mouse brain. Representative T2-weighted spin-echo images of control, bevacizumab-treated, and B20-4.1.1–treated mice, covering the same anatomic region of the brain and collected at 3, 6, and 10 weeks following a single 60-Gy dose of radiation, are shown in Fig. 1. Hyperintense areas in these images correspond with regions of radiation necrosis in the brain. Significant hyperintense regions are clearly seen in non-Ab-treated, control mice at 6 weeks after irradiation and these regions expand significantly in extent by 10 weeks. The images of mice treated with B20-4.1.1 show minimal hyperintensity, even at 10 weeks after irradiation, whereas the images of bevacizumab-treated mice describe an intermediate situation, showing no hyperintense regions at 6 weeks but small such regions at 10 weeks after irradiation.

Necrosis volumes can be measured quantitatively from MR images
An important aspect of the noninvasive MRI is the ability to measure quantitatively the progression of radiation necrosis. For each set of T2-weighted spin-echo images, regions of interest were drawn around the entire brain in several contiguous image slices, chosen to include the entire hyperintense region. Each brain was divided along the midline into left (irradiated) and right (nonirradiated) hemispheres. The image intensity for each individual pixel...
in the left hemisphere was normalized by the average of the 25 pixels (5 × 5 square) including and immediately surrounding its mirror-image pixel in the right hemisphere, and histograms of normalized intensity for the irradiated hemisphere were constructed, as shown in Fig. 2. The same analysis was performed on a cohort (n = 10) of nonirradiated mice. The histogram of average intensity distribution for nonirradiated subjects, shown in Fig. 2A, is symmetric; 99% of the pixels are distributed in the intensity range 0.6 to 1.4 about a normalized mean of 1.0. Therefore, an intensity threshold of 1.4 was chosen as the cutoff for normal brain tissue. For images of irradiated mice with/without treatment at different weeks after irradiation, the number of pixels exceeding this threshold serves to measure the necrotic volume at each time point. Figure 2B shows image-pixel intensity histograms for B20-4.1.1– and bevacizumab-treated mice at 6 and 10 weeks following a single 60-Gy dose of radiation. The rates of progression of radiation necrosis over the designated time period can be derived from the slopes of least-squares fits of number of pixels versus time over the designated period.

**Anti-VEGF antibodies slow the progression of radiation necrosis in irradiated brain tissue**

Treatment with anti-VEGF antibodies is an important potential strategy for mitigating the development of radiation necrosis. Figure 3 shows the progression of the mean volumes of MRI-derived necrotic regions for non–Ab-treated and B20-4.1.1–treated mice in experiment A, in which all the mice received a single 50-Gy dose of radiation. There was almost no progression of radiation necrosis in the B20-4.1.1–treated cohort (P < 0.0001, week 13) compared with non–Ab-treated control, indicating a significant mitigative effect due to the treatment.

Figure 4A shows the progression of the mean volumes of necrotic region for non–Ab-treated, bevacizumab-treated, and B20-4.1.1–treated cohorts in experiment B, in which all the mice received a single 60-Gy dose of radiation. The overall slope of the progression curve for B20-4.1.1–treated mice is slightly negative, demonstrating the mitigative effect of B20-4.1.1 where the treated cohort had a greatly diminished volume of necrosis at weeks 6 to 10 after irradiation compared with non–Ab-treated controls (P < 0.0001, weeks 6, 7, 8, and 10). Bevacizumab also slows the overall rate of progression (P < 0.0001, weeks 6, 7, 8, and 10). Considering the initial 3- to 7-week period after irradiation, bevacizumab has the same mitigative effect as B20-4.1.1 (P = 0.8, week 7). However, for the latter 7- to 10-week period, radiation-induced necrosis developed at a higher rate than in the B20-4.1.1–treated cohort (P < 0.0001, week 10), indicating that the mitigative effect of bevacizumab was...
weaker than B20-4.1.1 in the late period following the
initiation of treatment.

These effects are evident in Fig. 4B, in which the rates
of progression of radiation necrosis for the three cohorts
are plotted. The progression rate for the B20-4.1.1–
treated cohort was substantially reduced relative to the
non–Ab-treated cohort over both the 3- to 7-week
($P < 0.0001$) and 7- to 10-week periods ($P = 0.0002$) after
irradiation. Although bevacizumab treatment slowed the
rate of progression relative to the non–Ab-treated cohort
during the initial 3- to 7-week period ($P < 0.0001$), its
effect was lost over the latter 7- to 10-week period ($P =
0.2$). It is apparent that in mice, B20-4.1.1 was a more
potent mitigator of radiation necrosis compared with
bevacizumab.

Histology reveals that anti-VEGF antibodies mitigate
radiation necrosis in irradiated brain tissue

To validate these imaging findings, histologic studies
were performed on perfused mouse-brain tissue. Figure
5A shows representative $2 \times$ (top), $10 \times$ (middle) H&E
histologic images and corresponding T2W MR images (bot-
tom) for non–Ab-treated, bevacizumab-treated, and B20-
4.1.1–treated mice at 10 weeks following a single 60-Gy
dose of radiation. Corresponding histologic images and T2-
weighted images of control and B20-4.1.1–treated mice
following a single 50-Gy fraction of radiation are shown
in Fig. 5B. The irradiated hemispheres of the control mice
demonstrated many of the classic histologic features of
radiation necrosis, including fibrinoid vascular necrosis
(black arrow), vascular telangiectasia (yellow arrows), hem-
orrhage (red arrow), and loss of neurons and edema (blue
arrows; ref. 26). In contrast, the irradiated hemisphere of
the bevacizumab-treated mouse showed only modest tissue
damage and the irradiated hemisphere of the B20-4.1.1–
treated mouse displayed no visible tissue damage. These
histologic findings support the MR data shown in Figs. 3
and 4, demonstrating a significant mitigative effect of
anti–VEGF-A therapy.

Discussion

Surgery, chemotherapy, and/or radiation are modalities
used in treatment protocols for patients with brain neo-
plasms. The risk of late-onset radiation necrosis significantly
limits the dose of radiation that can be used in these
protocols. The identification of agents that mitigate the
delayed effects of radiation-induced changes on normal
tissue, while not reducing the therapeutic efficacy of rad-
iation on tumor tissue, could significantly increase the
therapeutic ratio. Currently, the clinical therapeutic options for treating radiation necrosis are limited.

It has been suggested that radiation necrosis results from local tissue injury characterized by disruption of the blood–brain barrier and consequent tissue edema. Endothelial cell death, which results in breakdown of the blood–brain barrier, edema, and hypoxia and enhanced expression of VEGF has been described as an important step in the development of radiation necrosis (10). The mitigative potential of anti-VEGF therapy using bevacizumab in the treatment of radiation necrosis has been shown in several retrospective human studies and a recent prospective human study in a
small number of patients (17–19, 22), though these studies lacked statistical power. MRI monitoring of the onset and progression of radiation necrosis, in concert with Gamma Knife irradiation, offers an attractive strategy for validating and optimizing anti–VEGF-Ab therapy. We have established an animal model that faithfully reproduces the histology of radiation necrosis observed in patients. Herein, we demonstrate that anti-VEGF antibodies can delay the onset of radiation necrosis and believe that the results of mitigation studies in the mouse will provide essential dosing and mechanism-of-action information, with direct relevance to ongoing clinical trials of bevacizumab.

In this study, the onset and progression of radiation necrosis in mouse brain were characterized by the volume of hyperintense regions on T2-weighted images. Characterization using contrast-enhanced T1-weighted images yielded essentially equivalent findings (data not shown). The greatly reduced progression of radiation necrosis in treated mice, as measured longitudinally by in vivo MRI, and the much lighter tissue damage observed in H&E-stained tissue sections for bevacizumab- and B20-4.1.1–treated mice, demonstrated the efficacy of anti–VEGF-A therapy. B20-4.1.1 has a better mitigative effect than bevacizumab in the mouse model of radiation necrosis. The mitigative effects of bevacizumab disappeared approximately 4 weeks after the start of treatment (Fig. 4). This may be due to an antidiotypic immunogenic response (27–29), whereby murine antibodies directed against the antigen-specific part of bevacizumab are produced, thereby inhibiting its binding to mouse VEGF. The greater therapeutic efficacy of B20-4.1.1 is likely due to its higher affinity for mouse VEGF-A (30).

Although the results of the present study demonstrate that bevacizumab and B20-4.1.1 can significantly reduce the progression of radiation necrosis, the mechanism of the mitigative effect of these VEGF inhibitors remains underdetermined and requires further investigation. Outstanding questions also remain about the ideal dosing schemes and the treatment periods for anti–VEGF-Ab therapy. Multiple dosing schemes, ranging from 5 to 10 mg/kg have been reported in the treatments of tumor and radiation necrosis in both human (17, 19) and animal studies (31). Typically, bevacizumab, with a half-life of approximately 20 days in humans, is administered once every 2 or 3 weeks in patients (17, 19, 32), and once or twice weekly in mice (31, 33). For our mitigation study, a high-end dosing scheme (10 mg/kg, twice weekly) was chosen and demonstrated promising results in both bevacizumab and B20-4.1.1 on radiation necrosis. By reducing the development of necrosis following irradiation, anti–VEGF-Ab therapy may overcome the deleterious effects of focal irradiation to effectively treat lesions with fewer side effects.

Efforts to measure the effects of using lower doses of B20-4.1.1 in our mouse model are ongoing. Also, in the current studies, irradiated mice were treated with bevacizumab or B20-4.1.1 for 7 or 8 weeks, beginning with the first radiographic sign of radiation necrosis. However, the mitigative effectiveness found upon initiating the treatment earlier (e.g., immediately following irradiation) or stopping the treatment after a fixed period of time (e.g., 4 weeks) remains to be investigated. Future studies that address mechanism of action will include dynamic contrast-enhanced MRI, an imaging method for quantitatively measuring vascular permeability (34, 35) and specific histologic stains targeting permeability, such as Evans Blue (36).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Jiang, J.A. Engelbach, L. Yuan, J. Cates, R.E. Drzymala, R.E. Schmidt
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Jiang, F. Gao, K.M. Rich, R.E. Schmidt, J.R. Garbow
Writing, review, and/or revision of the manuscript: X. Jiang, L. Yuan, J. Cates, F. Gao, R.E. Drzymala, D.E. Hallahan, K.M. Rich, R.E. Schmidt, J.J.H. Ackerman, J.R. Garbow
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