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Stephen L. Brown  
*Henry Ford Health System, Sbrown1@hfhs.org*

Tavarekere N. Nagaraja  
*Henry Ford Health System, TNagara1@hfhs.org*

Madhava P. Aryal  
*Henry Ford Health System*

Swayamprava Panda  
*Henry Ford Health System*

Glauber Cabral  
*Henry Ford Health System, GCABRAL1@hfhs.org*

*See next page for additional authors*

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Authors
Stephen L. Brown, Tavarekere N. Nagaraja, Madhava P. Aryal, Swayamprava Panda, Glauber Cabral, Kelly A. Keenan, Rasha Elmghirbi, Tom Mikkelsen, David Hearshen, Robert A. Knight, Ning Wen, Jae-Ho Kim, and James R. Ewing

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SHORT COMMUNICATION

MRI-Tracked Tumor Vascular Changes in the Hours after Single-Fraction Irradiation

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Departments of Radiation Oncology, Anesthesiology, Neurosurgery, Neurology and Radiology, Henry Ford Hospital, Detroit, Michigan; Department of Physics, Oakland University, Rochester, Michigan; and Department of Neurology, Wayne State University, Detroit, Michigan

The purpose of this study was to characterize changes in tumor vascular parameters hours after a single radiation exposure in an orthotopic brain tumor model. U-251 human brain tumors were established intracerebrally in rat brains, and tumor blood flow, forward volume transfer constant (Ktrans) and interstitial volume fraction (ve) were measured using magnetic resonance imaging (MRI). Tumors were exposed to a single stereotactic radiation treatment of 20 Gy. Vascular parameters were assessed one additional time between 2 and 24 h after irradiation. After the second MRI session, brain tissue histology was examined for gross changes and apoptosis. In separate studies, cerebral blood flow was measured in nonimplanted controls before radiation exposure and 2 and 24 h after 20 Gy irradiation, and in implanted rats before radiation exposure and at 2 and 24 h after 6 Gy irradiation. Significant changes were observed in tumor-bearing rat brains in the hours after 20 Gy irradiation. Two hours after 20 Gy irradiation, tumor blood flow decreased nearly 80% and ve decreased by 30%. At 4 h, the Ktrans increased by 30% over preirradiation values. Extensive vacuolization and an increase in apoptosis were evident histologically in rats imaged 2 h after irradiation. Between 8 and 12 h after irradiation, all vascular parameters including blood flow returned to near preirradiation values. One day after irradiation, tumor blood flow was elevated 40% over preirradiation values, and other vascular parameters, including Ktrans and ve, were 20–40% below preirradiation values. In contrast, changes in vascular parameters observed in the normal brain 2 or 24 h after 20 Gy irradiation were not significantly different from preirradiation values. Also, tumor blood flow appeared to be unchanged at 2 h after 6 Gy irradiation, with a small increase observed at 24 h, unlike the tumor blood flow changes after 20 Gy irradiation. Large and significant changes in vascular parameters were observed hours after 20 Gy irradiation using noninvasive MRI techniques. It is hypothesized that cellular swelling hours after a high dose of radiation, coinciding with vacuolization, led to a decrease in tumor blood flow and ve. Four hours after radiation exposure, Ktrans increased in concert with an increase in tumor blood flow. Vascular permeability normalized, 24 h after 20 Gy irradiation, as characterized by a decrease in Ktrans. Vascular parameters did not change significantly in the normal brain after 20 Gy irradiation or in the tumor-bearing brain after 6 Gy irradiation.

INTRODUCTION

The use of high-dose radiation therapy (HD-RT), either as a single exposure, termed stereotactic radiosurgery (SRS) or multiple treatment sessions, known as hypofractionated stereotactic radiotherapy (HFSRT), has become an increasingly important treatment option for the management of brain tumors (1, 2). High-dose radiation therapy provides anticancer efficacy comparable to other available approaches, including surgery and conventional whole brain irradiation applied in multiple low-dose fractions, is better tolerated than conventional fractionated radiation therapy (CF-RT), and allows for better dose conformity, allowing surgically inaccessible tumors to be treated (1, 3). Although hypofractionated stereotactic radiotherapy is routinely delivered to a total dose of 18–30 Gy in 2–5 fractions, there remains no clear radiobiological rationale to guide the choice of dose per fraction, and no clinical trial is currently available to provide guidance on the optimum fraction number (i.e. 2–5). In fact, the physiology of tumor response in embedded tumors is not well characterized, and a widely

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1 Address for correspondence: Henry Ford Hospital and Wayne State School of Medicine, 2799 West Grand Boulevard, Detroit, MI 48202-2689; e-mail: sbrown1@hfhs.org.
accepted radiobiological basis for adopting high-dose radiation therapy has not been established.

One explanation for the positive tumor responses to high-dose radiation therapy is that tumor vasculature is more sensitive to it than has been predicted by conventional radiobiology (4). Unfortunately, there is a paucity of experimental evidence to support this conjecture, and the dynamics of vascular physiology in model tumor systems and surrounding normal tissues in the hours after exposure to high-dose radiation have not been well studied. In part, this lack of data is attributable to the laborious process involved in the techniques that, until recently, have been the only quantitative means available. In the past, preclinical studies that examined the effect of tumor blood flow in response to radiation exposure used techniques that required death of the animal as part of the measurement, making repeated measurements in the same animal challenging. Because of inter-animal variations, sample sizes tended to become large (on the order of 50), making the determination of all but the most evident trends difficult to detect. Recently, noninvasive (thus repeatable) quantitative vascular measures with variances that allow relatively small sample sizes (order of 5–10) to detect changes in tumor vascular physiology have become available (5, 6).

It is important to understand the vascular physiology of the response to radiation, not only for characterizing differences between normal and tumor tissues or helping to determine the influence of vascular changes on tumor response to radiotherapy, but also because radiation therapy is often combined with chemotherapeutics based on delivery via the vasculature. Intriguing observations in preclinical scientific literature indicate that the optimum timing of radiation treatment and pharmacologic agents may be critically dependent on a tumor’s vascular response to radiation (7). For example, in the animal model employed herein the optimum sequence and timing of radiation exposure and the antiangiogenic agent, cilengitide, appears to be drug first, followed by radiation exposure; an alternate sequence of drug/radiation is less effective (8). In contrast, in a related model, when radiation is combined with the vascular targeting agent, arsenic trioxide, the optimum sequence and timing appears to be radiation exposure first followed by pharmacologic agent (9, 10). Currently available information about the vascular response of tumors in the hours after radiation exposure is limited. Of particular note, vascular changes that occur acutely in a tumor after high-dose radiation exposure are not well characterized. Herein, the kinetics of vascular parameters are presented in an orthotopic rat model of human glioma 2–24 h after a 20 Gy single-fraction irradiation and compared with the response of the normal brain. The results are unexpected, demonstrating a profound decrease in tumor perfusion, associated with cell swelling, after a large single dose of radiation. A similar response is not observed in the normal brain.

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FIG. 1. Experimental timeline. The first and second MRI studies were performed approximately 24 h apart. Each group of animals was irradiated after the first study and at one of the following time points before the second MRI: 2, 4, 8, 12 or 24 h.

MATERIALS AND METHODS

The studies were conducted in accordance with a protocol approved by the Henry Ford Hospital Institutional Animal Care and Use Committee (IACUC). Twenty-five athymic rats (strain code: NIH-Foxn1<sup>−/−</sup>; Charles River Laboratories, Wilmington, MA) (~8 weeks old and 180–200g at time of implant) were implanted with U-251 tumor cells intracerebrally and irradiated as described below. MRI vascular parameters were measured twice in each animal, with the two studies separated by approximately 24 h. Figure 1 shows the timing of the MRI studies and radiation exposure. After the initial MRI, irradiations were performed at selected time points, 2, 4, 8, 12 or 24 h, before the second study. Additionally, two control blood flow studies were performed in: 1. Four rats with normal brains (nontumor bearing), treated with 20 Gy of radiation; and 2. Two rats with brain tumors treated with 6 Gy of radiation.

The U-251 Orthotopic Brain Tumor Model

The U-251 orthotopic tumor model derives from a human glioblastoma. It has been well characterized with respect to tumor growth and radiation response and constitutes a useful model for preclinical studies; its phenotype, lacking a strongly invasive component, resembles that of some metastatic tumors. Athymic rats were inoculated intracerebrally using previously published methods (5, 8, 11, 12). Briefly, animals were anesthetized with intramuscular injections of 80 mg/kg ketamine and 8 mg/kg xylazine. The surgical zone was swabbed with betadine solution, the eyes coated with Lacrilube<sup>®</sup> and the head immobilized in a small animal stereotactic device (David Kopf Instruments, Tujunga, CA). After draping, a 1 cm incision was made 2 mm right of the midline and the skull was exposed. A burr hole was drilled 3.5 mm to the right of bregma, taking care not to penetrate the dura mater. A 10 μL Hamilton syringe with a 26-gauge needle (Model 701; Hamilton<sup>®</sup> Co., Reno, NV) containing U-251MG tumor cells freshly harvested from log phase growth [5 × 10<sup>5</sup> in 10 μl of phosphate buffered saline (PBS)] was lowered to a depth of 3.0 mm and then raised back to a depth of 2.5 mm. Cells were then injected at a rate of 0.5 μL/10 s until the entire volume was delivered. Tumors implanted in animals following this technique routinely grow to about 4 mm diameter by 21 days post-implantation.

Around day 21, and then about 24 h later, two MRI studies were conducted for each animal. Animals were anesthetized with isoflurane (4% for induction, 0.75–1.5% for maintenance, balance N<sub>2</sub>O/O<sub>2</sub> = 2:1) and allowed to spontaneously respire. A tail vein was cannulated for the administration of the contrast agent. Body temperature was maintained at 37°C with a warm air supply monitored by an intrarectal type T thermocouple.
Radiation Exposure

Radiation was delivered using a Novali™ (BrainLab AG, Heimstetten, Germany) stereotactic radiosurgery device operating at 6 MV photons. The location of each tumor was determined before radiation exposure by MRI at the time of the pretreatment blood flow measurement. At this time, with the rat head positioned horizontally, the center of the tumor mass was measured relative to the tumor-implantation drill hole. At the time of irradiation, the stereotactic coordinates of the tumor-implantation drill hole were used to position the radiation field over the stereotactic coordinates defining the tumor center. Rats were anesthetized using intramuscular injections of 80 mg/kg ketamine in the gastrocnemius muscle and 8 mg/kg xylazine in the contralateral leg. Five minutes after anesthesia administration, rats were placed in a stereotactic device with the rat head in the same orientation as when tumor cells were implanted. A total dose of 20 Gy was administered at an approximate dose rate of 8 Gy/min using 2 × 2 cm primary collimation, a 6 mm diameter cone as a secondary collimator, a source-to-surface distance of 75 cm, a 14 mm bolus above the skull for electron equilibrium at the tumor depth and a linear accelerator output rate set at 800 monitor units per min. Tumor dimensions were covered by the 100% isodose line. The treatment beam projected in a single anterior-posterior direction from the top of the skull through the right hemisphere exiting under the jaw. A typical treatment plan is shown in Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13458.1.S1).

In a control study to investigate blood flow changes, 20 Gy was delivered to rats without tumors (n = 4). For two of the rats, a 1 × 1 cm field was positioned on the right hemisphere of the skull, bordering the midline and effectively encompassing half the rat head. The 1 × 1 cm field size was included as the start of an investigation of the effect of radiation volume on vascular parameters (not included in the current article) and to simplify determination of the radiation field on MRIs. For the other two rats, the previously described 6 mm cone was used. In an additional study designed to investigate changes in blood flow in rat tumors exposed to lower-dose radiation, a single 6 Gy dose was administered (n = 2). For all studies of tumor-bearing rats, a 6 mm secondary collimator cone was used. In all additional control studies, animals were imaged approximately 24 h before irradiation, and 2 and 24 h after irradiation.

MRI Studies

Vascular Parameters

The vascular parameters measured using MRI were: blood flow by arterial spin labeling (13, 14); dynamic contrast-enhanced (DCE)-MRI (6, 15) estimating a model-selection paradigm in its analysis (6, 16) to estimate the forward volume transfer constant (Ktrans, a measure of vascular permeability when blood flow does not limit exchange) of a contrast agent; contrast agent plasma volume fraction (vP); and the interstitial volume fraction (vI). All reported vascular measures were within regions that included on average ± SEM, 150 ± 10 voxels.

On the day before the scheduled irradiation, rats underwent MRI to determine their suitability for exposure based on their tumor size. If the maximum extent of their tumor was 3–4 mm, baseline vascular parameters were collected. If tumors were smaller than 3 mm, rats were imaged on a subsequent day. If the tumors were too large, the rats were excluded from further study (and not included in the data presented). Studies were performed in a 7 Tesla, 20 cm bore system with a DirectDrive spectrometer and console (Agilent Technologies, Santa Clara, CA). Gradient maximum strengths and rise times were 250 mT/m and 120 μs. All MRI image sets were acquired with a 32 × 32 mm² field of vision.

High-resolution T1-weighted images were acquired pre- and post-contrast agent with the following parameters: matrix = 256 × 192, 27 slices, 0.5 mm thickness, no gap, NE = 1, NA = 4, TE/TR = 16/800 ms. A dual-echo three-slice gradient-echo (2GE) MRI sequence was used for the DCE-MRI study (5, 6). Prior to the 2GE sequence, and immediately after, two Look-Locker sequences were run so that a voxel-by-voxel estimate of T1 in the tissue could be made pre- and post-contrast agent administration. Look-Locker sequence parameters were as follows: matrix = 128 × 64, five 2.0 mm slices, no gap, NE = 24 inversion-recovery echoes, TR = 2000 ms. The 2GE sequence that was run between the paired Look-Locker acquisitions had the following parameters: 150 acquisitions at 4.0 s intervals, matrix = 128 × 64, three 2.0 mm slices, no gap, tip angle = 27°, NE = 2, NA = 1, TE = 2.0, 4.0 ms, TR = 60 ms, SW = 150 kHz. The contrast agent (0.25 mM/kg) (Magnevist®; Bayer Healthcare Pharmaceuticals, Wayne, NJ) bolus injection was performed by hand push at image 15. Total run time was 10 min.

Spin-echo arterial spin-labeled cerebral blood flow estimates were acquired in a single central slice, as in previous investigations (14). MRI parameters were as follows: matrix = 128 × 64, one 2.0 mm slice, NA = 4, TE/TR = 20/1050. Arterial labeling = 1 s, with alternating gradients and frequency offsets in combinations of 4. Total time = 18 min. Results are generally reported as percentage changes from pretreatment values (14, 17).

Histology

Immediately after the final imaging session anesthetized rats were perfused by cardiac infusion with 4% neutral buffered paraformaldehyde and brains removed for histological examination. Paraffin-embedded tissue sections were processed for H&E staining for gross examination and TUNEL staining for assessment of the presence of apoptosis.

Hematoxylin and Eosin Staining

Formalin-fixed, paraffin embedded brain tissue was H&E stained using previously published techniques (11, 12).

TUNEL Staining

The TUNEL (ApopTag® Plus In Situ Apoptosis Detection Kit; Millipore, Billerica, MA) method was applied to formalin-fixed, paraffin embedded brain tissue. After dewaxing and hydration, the sections were washed in PBS, pH 7.6, for 5 min, then treated with proteinase K (20 μg/ml) diluted in PBS at room temperature for 15 min and then washed in distilled water for 5 min. TUNEL incubation solution, comprised of terminal deoxynucleotidyl transferase (TdT) buffer, cobalt chloride, TdT and biotin-16dUTP, was prepared according to the manufacture’s protocol. The sections were incubated in TdT buffer for 1 h at 37°C. After incubation, stop solution was applied to brain sections for 10 min, after which they were washed twice (5 min) in the PBS. The sections were then incubated with digoxigenin antibody peroxidase conjugate at room temperature for 30 min. Finally, brain sections were incubated in 3,3′-diaminobenzidine solution for 1 min. The fragmented DNAs were visualized as a brownish color inside nuclei. The sections were counterstained with methyl green before being dehydrated and cleared through graded alcohols and xylenes, and protected with a glass coverslip. Images were captured using a Nikon™ DXM1200C camera attached to a Nikon £00M microscope (Nikon Corp., Tokyo, Japan).

It is estimated that the histological sections corresponding to time after irradiation of 2, 4, 8, 12 and 24 h are actually 3.5, 5.5, 9.5, 13.5 and 25.5 h after irradiation since the MRI vascular studies took place about 1.5 h before the animal was euthanized for histology. Consequently, the histology labels indicate the animal groups and not the actual times of death postirradiation.

Statistical Analyses

Error bars on graphs (and the percentage errors reported in the text) represent the percentage error of the mean value calculated as the sum, added in quadrature, of the standard deviation for the preirradiation
measurements expressed as a percentage relative to the mean preirradiation value (i.e. unity) and that of the measurement at 2, 4, 8, 12 or 24 h postirradiation.

In practice, our experience in a control population (5) has been that intra-subject variation is less than that of inter-subject variation, thus making test–retest comparisons, with the animal as its own control, more sensitive to physiological changes than grouped comparisons of the set of animals before irradiation, compared to postirradiation. To make the effect of an intervention easily understood, it has been our usual practice to express test–retest differences as percentage changes. This is a practice that will be followed herein.

Vascular measurements at the time points after irradiation were compared with preirradiation values from the same rats, computed as percentage differences. Significant percentage differences were determined using the Wilcoxon signed-rank test. The null hypothesis was that there was no likelihood of finding a preponderance of percentage differences in one direction. Differences of \( P \leq 0.05 \) were considered statistically significant.

In a sample of 17 control animals tested previously (5), but without a therapeutic intervention, it was found that paired differences in \( v_p \), \( K^{\text{trans}} \) and \( v_e \) had standard errors of the mean that were on the order of 3–4% of the sample mean value. Thus, in a sample of 4–5 animals treated with an intervening therapeutic intervention, it should be expected that paired differences in parameters of more than about \( \pm 20\% \) demonstrate a significant effect of the intervention.

Representative blood flow distribution parameters are presented in Supplementary Table S1 (http://dx.doi.org/10.1667/RR134581.S1), including upper value, lower value, range, median, mean, variance, skewness and kurtosis. Similar values characterizing the distributions of \( K^{\text{trans}} \) and \( v_e \) are presented in Supplementary Table S2 (http://dx.doi.org/10.1667/RR134581.S1).

RESULTS

In 25 animals prior to irradiation, tumor blood flow was characterized by arterial spin labeling as follows: mean ± SEM = 83.1 ± 7.0 ml/100 g min and median 83.2 ml/100 g min. The DCE-MRI vascular parameters in tumor prior to radiation exposure were (mean ± SEM and median): \( \nu_p = 1.18 \pm 0.12\% \) and 1.09%; \( K^{\text{trans}} = (4.01 \pm 0.21) \times 10^{-2} \) min\(^{-1}\) and 4.06 \( \times 10^{-2} \) min\(^{-1}\); \( v_e = 13.74 \pm 0.70\% \) and 13.50%. Blood flow across animals was normally distributed prior to irradiation (Supplementary Tables S1 and S2; http://dx.doi.org/10.1667/RR134581.S1) with mean values similar to those previously reported (6, 11, 18).

Figure 2A shows that 2 h after 20 Gy irradiation, tumor blood flow exhibited a marked initial decrease to between 70 and 80% of preirradiation levels. Blood flow values at 2 and 4 h after 20 Gy irradiation were significantly below preirradiation values (\( P < 0.05 \)), followed by a return to preirradiation values over the subsequent 8 h. At 8 and 12 h postirradiation, tumor blood flow was on average not significantly different from preirradiated levels. Subsequently, tumor blood flow increased. At 24 h postirradiation, tumor blood flow exceeded preirradiation levels, on average, by 40% (\( P < 0.05 \)).

Parameters of representative blood flow distribution are shown in Table 1. The blood flow patterns within individual animals were normally distributed even at low blood flow values (see, for example, the 2 h time point after 20 Gy), the mean values were similar to the median values and both the skewness and kurtosis were usually small.

The kinetics of other vascular parameters are shown in Fig. 2B. The mean of the forward volume transfer constant, \( K^{\text{trans}} \), in the tumor region of interest demonstrated a bimodal response, increasing at 4 h to 35% above preirradiated values (\( P < 0.05 \)) and declining at 12 and 24 h postirradiation. At 24 h, \( K^{\text{trans}} \) values were 20–40% below the preirradiation level (\( P < 0.05 \)).

The mean \( v_e \) was below preirradiation values throughout the study, pointing to a significant loss of interstitial volume fraction. At 2 h after irradiation, \( v_e \) was 30% below preirradiated values (2 h \( v_e \), \( P < 0.05 \)). \( v_e \) temporarily approached preirradiated values 4 h after irradiation, but subsequently decreased to 30% below preirradiated levels at 24 h postirradiation (24 h \( v_e \), \( P < 0.05 \)).

Over the 24 h period after irradiation, plasma volume, \( v_p \), was unchanged. In fact, \( v_p \) fluctuated above and below
preirradiated values. However, the associated error bars were large and the trends unremarkable over the 24 h period (data not shown).

Compared to the large and significant vascular changes observed after 20 Gy irradiation in brain tumors, observed changes in blood flow were smaller in normal rat brains \((n = 4)\) after 20 Gy irradiation. Normal brain blood flow at 2 h after 20 Gy irradiation decreased by 23 ± 5% compared with preirradiation values. Similarly, at 24 h blood flow was somewhat elevated above the preirradiation value by 15 ± 27%, but not significantly different from preirradiation levels. The blood flow changes in the normal brain after 20 Gy did not differ using either the 6 mm secondary collimator cone or the 1 × 1 cm field size. Consequently, the changes in the normal brain blood flow after 20 Gy irradiation were judged to be unremarkable.

Interestingly, 2 h after 6 Gy irradiation within the region of interest of the tumor, there was little evidence of change in blood flow compared with preirradiation blood flow values. However, at 24 h blood flow appeared to be increased by 30 ± 6% compared with preirradiation blood flow values. These findings should be weighted by the small sample size, and further investigation in the normal brain as well as other tumor types should be conducted.

The acute vascular changes observed in the tumor after 20 Gy irradiation coincided with profound histological changes within the tumor. Examination of brain sections demonstrated gross injury within the tumor even at the earliest time point after 20 Gy irradiation. Figure 3 shows the contrast between irradiated and nonirradiated rat brains and is representative of all animals in the group. Regions of the tumor exhibit vacuoles and rounded nuclei and occasionally double nucleoli.

To determine if the vascular changes observed were accompanied by changes in cellular apoptosis, brain sections underwent TUNEL assay. Figure 4 shows large regions of the tumor characterized by an increase in apoptosis at the earliest time point after 20 Gy irradiation, whereas nonirradiated, control brain showed minimal levels of apoptosis. Some, but not the majority, of cells staining positive for apoptosis were endothelial cells.

**DISCUSSION**

To the best of our knowledge, this is the first report of a differential effect of high-dose radiotherapy on the acute vascular response of tumor and normal tissue. In an orthotopic tumor model, tumor blood flow was found to decrease after high-dose irradiation, remained low for several hours, but ultimately exceeded baseline values after 24 h. Normal brain blood flow was relatively unaffected during this timeframe after a high-dose radiation exposure. Others have reported tumor blood flow changes after irradiation using subcutaneously implanted tumors \((19–21)\), including an acute decrease in tumor blood flow (i.e. ischemia) followed by an increase (i.e. hyperemia). However, the potential significance of acute changes in vascular physiology for additional cell killing in tumor, but not in normal tissue has not been previously recognized. In particular, to our knowledge this is the first report in which: 1. The decrease in tumor blood flow observed at 2 h after 20 Gy irradiation was significantly different from normal brain; and 2. Unlike the overshoot in the blood flow of the tumor at 24 h postirradiation, there was no such overshoot in the normal brain. Furthermore, unlike that observed after 20 Gy irradiation, the differential in blood flow between tumor and normal brain after the lower 6 Gy radiation dose was not observed, although we caution that the sample size was small.

The mechanism that causes acute blood flow in the tumor after irradiation to differ from that of normal tissue is not yet fully understood, although it appears that cell swelling and flow are at least covariant. Nevertheless, this physiological
change is not without its consequences; striking differences between normal parenchyma and tumor have been observed histologically (Fig. 3). Tumor tissue vacuolization was easily observed, even hours after a 20 Gy radiation exposure, whereas the normal brain was histologically unremarkable. As noted, coinciding with a decrease in tumor blood flow, a significant decrease in interstitial volume fraction determined by DCE-MRI was observed. Cellular swelling was not measurable in the normal brain, and such measures using DCE-MRI may not be possible, since they require extravasation of contrast agent. Nevertheless, there is no histological evidence that swelling in the normal brain occurred.

A decrease in interstitial volume fraction suggests that cellular swelling occurs in tumors and not normal tissue almost immediately (hours) after high-dose irradiation. Cellular swelling may result from apoptosis (22), a process characterized by nuclear pyknosis, and could cause an acute reduction in blood flow by compression of cerebral microvessels. Indeed, ceramide-dependent apoptosis has been proposed after high-dose irradiation (23). Ceramide-dependent apoptosis has been shown to govern endothelial cell survival after irradiation, although it is of note that the same investigators show the effect to be present in both tumor (23) and normal tissue (24). Likewise, Park et al. proposed that vascular changes lead to tumor response (4). However, their rationale is that prolonged hypoxia causes cell death. Again, their explanations offer no reason for a differential effect between tumor and normal tissue. Finally, arguments put forth to explain radiation injury based on immunological response to high-dose exposures (25–27) do not consider differences between tumor and normal tissue. The data shown in this article represent the first report of a differential tumor/normal tissue biological effect observed hours after high-dose irradiation.

The cause of the dramatic early damage to the tumor, observed in tumor histological sections, is not known, although there is certainly a high level of DNA fragmentation characteristic of apoptosis in the U-251 tumor soon after high-dose irradiation, as shown in Fig. 4. The transient relative increase in $K^{\text{trans}}$ 4 h after a large dose of radiation (Fig. 2) is consistent with damage to tumor vasculature and is not a new observation. A transient increase in vascular permeability after a large dose of radiation was previously demonstrated qualitatively in subcutaneously transplanted tumors grown in the feet of mice (28). As discussed, in the model presented herein, the change in $K^{\text{trans}}$ preceded 2 h earlier by a decrease in interstitial volume fraction. The effect of 20 Gy irradiation on interstitial volume fraction was significant and immediate, remaining below preirradiation values for at least 24 h. The initial large decrease in $v_c$ suggests an immediate postirradiation cellular swelling. As noted, this phenomenon might be consistent with the hypothesis that endothelial cells undergo apoptotic death.

FIG. 4. Signs of apoptosis in the hours after irradiation. Left-side panels show nonirradiated tumor and right-side panels show irradiated tumor. A higher level of DNA fragmentation is evident in the tumor in the hours after 20 Gy irradiation. The left-side panels show low background staining and sparse apoptotic bodies detected by DNA fragmentation using the terminal end of nucleic acids, so-called terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The right-side panels show both a higher level of DNA fragmentation throughout the irradiated tumor soon after exposure to 20 Gy, as well as more apoptotic bodies. The rat represented in the right-side panels was imaged 2 h after irradiation and euthanized within 1–2 h thereafter.
after single high-dose radiation exposure (4) or perhaps more likely, cancer cells throughout the tumor undergo this process. The latter scenario of overall cellular swelling due to tumor cell apoptosis in the first hours after radiation exposure is consistent with this observation, since such swelling might be expected to compress the vascular supply of the tumor, thus reducing blood flow. As interstitial volume fraction returned toward preirradiation levels, presumably due to resolution of cellular swelling, and vascular permeability decreased, thus probably decreasing tumor interstitial pressure, tumor blood flow also improved. We observed increased blood flow coincident with an initial increase in forward volume transfer constant, $K_{\text{trans}}$, as expected since $K_{\text{trans}}$ is the product of flow and extraction fraction. In this model of an embedded cerebral tumor, plasma volume trends were unremarkable, characterized by large errors and remaining relatively unchanged throughout the 24 h measurement period. At about 8 h postirradiation, all vascular parameters, including blood flow, approached preirradiation values. However, by 24 h postirradiation, the blood flow had overshot preirradiation values by 40%. Other vascular parameters, i.e., $K_{\text{trans}}$ and $v_e$, were decreased by 20–30%.

The exposure time used in the current study is shorter than that typically used for radiosurgery in humans. Further study is needed to understand the importance of total dose, dose rate and exposure time. Decreased blood flow developing over the time of a more protracted exposure time could have implications for tumor response.

The pattern of profound decrease in tumor blood flow, followed by a robust overshoot, suggests that an ischemia-reperfusion mechanism (29–32) may be a factor in the overall efficacy of high-dose radiation in the treatment of embedded tumors. Despite an extensive knowledge base in a number of human pathologies, including heart attacks and stroke, there is a dearth of recent studies in ischemia-reperfusion in tumors. Parkins and colleagues, in a series of articles, documented the effects of ischemia-reperfusion in solid tumors (29–32). Animal tumors were temporarily clamped physically to show that little or no cell kill was detectable in tumors exposed to up to 3 h of ischemia when tumors were excised immediately before reperfusion. However, when reperfusion was permitted, extensive cell kill was evident 24 h later. The investigators attributed damage to oxygen radicals, because intravenous administration of superoxide dismutase or catalase, at the time when vascular reperfusion occurred, resulted in significant protection against tumor cell kill (29). Because these experiments involved only ischemia, the combined effect of radiation exposure and ischemia-reperfusion could not be described, and remains to be examined in depth, now that a technique for noninvasively estimating acute changes in tumor physiology is available.

Since the mid-1980s it has been established that oxygen-derived free radicals hold a major role in post-ischemic tissue injury (33). During ischemia native xanthine dehydrogenase is converted to superoxide-producing xanthine oxidase by a calcium-triggered, protease-dependent process. Also during ischemia, ATP is degraded; a breakdown product of adenine is hypoxanthine. Consequently, the catabolic degradation of ATP during ischemia provides an oxidizable substrate, hypoxanthine. On reperfusion, molecular oxygen is resupplied and a burst of superoxide production ensues, resulting in extensive tissue damage. We hypothesize, based on radiation-induced acute changes in tumor blood flow that a similar mechanism occurs in solid tumors after high-dose irradiation. If this is true, xanthine dehydrogenase is rapidly converted to the oxidase during the period of nonperfusion in the tumor shortly after it is irradiated at a high dose. Superoxide is not particularly reactive by itself, but undergoes multiple reactions into unstable and highly toxic reactive oxygen species and reactive nitrogen species (ROS/RNS). The production of ROS/RNS after ischemia-reperfusion is shown schematically in Fig. 5.

In addition, other mechanisms may be at work. For example, SDF-1, a major antigen for leukocytes and macrophages, is known to be expressed in tumors and increases its expression after hypoxia (34, 35). Leukocytes and macrophages are known to produce damaging ROS/RNS, however, this is a relatively unexplored pathway in the context of ischemia-reperfusion.

In summary, measures of acute changes in tumor physiology may help explain the remarkable clinical tumor responses to high-dose radiation. Hypofractionated stereotactic radiotherapy is generally delivered using doses per fraction varying between 5 and 9 Gy, substantially higher than the doses per fraction of 1.8–2.0 Gy commonly used with conventional fractionated radiation therapy. It is unknown if these doses are high enough to produce vascular changes in a pattern similar to that observed in the current study. As mentioned, several other processes may be at work. First, it has been suggested that tumor vasculature is sensitive to radiation above a threshold dose due to a ceramide-dependent apoptosis of endothelial cells (23). Second, Song and colleagues have long studied the effects of radiation exposure on tumor blood flow, and believe that high-dose radiation causes prolonged ischemia that leads to additional cell killing, which otherwise would not occur (4). Third, Demaria and colleagues and McBride and colleagues have implicated the immune system in the tumor response to high-dose radiation (27). Of note, all of these postulates share the same limitation that they do not explain the differential effect of high-dose radiation on the tumor and surrounding normal tissue.

Further studies are needed to examine the effect of single high doses of radiation. Tumor vascular dynamics should be considered in the context that changes in blood flow after radiation exposure depend on dose, dose rate, tumor volume, tumor type, microenvironment and immune response. Measurements of tumor oxygenation have been reported in other animal models (36, 37) and would be
important to repeat in an orthotopic brain tumor model hours after irradiation. Vascular changes that are reported in the first 24 h postirradiation may have utility for understanding the acute response of tumors to radiotherapy alone, as well as when radiation is combined with vascular targeting agents, antiangiogenic agents and other treatment modalities.

SUPPLEMENTARY INFORMATION

Table S1. Representative blood flow distributions.
Table S2. DCE-MRI vascular parameter distributions.
Fig. S1. Example of a radiation treatment plan.

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