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EFFECTS OF IRRADIATION ON BRAIN VASCULATURE USING AN *IN SITU* TUMOR MODEL

Janice A. Zawaski, Ph.D.^{*}, M. Waleed Gaber, Ph.D.^{*,†}, Omaila M. Sabek, Ph.D.[‡], Christy M. Wilson, Ph.D.^{*}, Christopher D. Duntsch, M.D., Ph.D.[§], and Thomas E. Merchant, D.O., Ph.D.^{*,¶}

^{*}School of Biomedical Engineering and Imaging, University of Tennessee Health Science Center, Memphis, TN

[†]Department of Pediatrics, Baylor College of Medicine, Houston, TX

[‡]Department of Surgery, Methodist Hospital Research Institute, Houston, TX

[§]Department of Neurosurgery, University of Tennessee Health Science Center, Memphis, TN

[¶]Department of Radiation Oncology, St. Jude Children's Research Hospital, Memphis, TN

Abstract

Purpose—Damage to normal tissue is a limiting factor in clinical radiotherapy (RT). We tested the hypothesis that the presence of tumor alters the response of normal tissues to irradiation using a rat *in situ* brain tumor model.

Methods and Materials—Intravital microscopy was used with a rat cranial window to assess the *in situ* effect of rat C6 glioma on peritumoral tissue with and without RT. The RT regimen included 40 Gy at 8 Gy/day starting Day 5 after tumor implant. Endpoints included blood–brain barrier permeability, clearance index, leukocyte–endothelial interactions and staining for vascular endothelial growth factor (VEGF) glial fibrillary acidic protein, and apoptosis. To characterize the system response to RT, animal survival and tumor surface area and volume were measured. Sham experiments were performed on similar animals implanted with basement membrane matrix absent of tumor cells.

Results—The presence of tumor alone increases permeability but has little effect on leukocyte–endothelial interactions and astrogliosis. Radiation alone increases tissue permeability, leukocyte–endothelial interactions, and astrogliosis. The highest levels of permeability and cell adhesion were seen in the model that combined tumor and irradiation; however, the presence of tumor appeared to reduce the volume of rolling leukocytes. Unirradiated tumor and peritumoral tissue had poor clearance. Irradiated tumor and peritumoral tissue had a similar clearance index to irradiated and unirradiated sham-implanted animals. Radiation reduces the presence of VEGF in peritumoral normal tissues but did not affect the amount of apoptosis in the normal tissue. Apoptosis was identified in the tumor tissue with and without radiation.

Conclusions—We developed a novel approach to demonstrate that the presence of the tumor in a rat intracranial model alters the response of normal tissues to irradiation.

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Reprint requests to: M. Waleed Gaber, Ph.D., Associate Professor Department of Pediatrics, Baylor College of Medicine, Feigin Center Suite 200.04, 1102 Bates Street, Houston, TX 77030. Tel: (832) 824-3170; Fax: (832) 825-4039; gaber@bcm.edu.

Supplemental material is available online.

Conflict of interest: none.

Keywords

Peritumoral; Radiation; Blood–brain barrier; Glioma; Astrogliosis

INTRODUCTION

Radiation therapy (RT) is a primary treatment for brain tumors, used alone or in combination with surgery or chemotherapy. The therapeutic benefit of RT is often limited by damage to the normal tissue. Following RT, brain tumor patients may suffer long-term effects including vasculopathy, stroke, white matter damage, necrosis, memory loss, fatigue, decreased attention and concentration, hearing loss, and endocrinopathy (1). These late effects are preceded by early changes in blood–brain barrier (BBB) permeability, increased leukocyte–endothelial interactions, and astrogliosis (2–4).

The majority of radiation effect studies on normal tissues in animals are performed in the absence of tumor and use a single (often high) dose of irradiation (3, 5–7). These conditions do not reproduce the clinical settings of human oncology patients. The goal of the current study was to develop an animal model that accounts for the abnormal microenvironment created by the presence of tumor to be used to study the effects of RT on normal tissue of the brain. In particular, we consider the peritumoral region to be of particular interest because of its adjacency to the tumor and likely inclusion in the volume that receives the highest doses. In developing the system, we chose to use a regimen of fractionated irradiation to better approximate the clinical radiation setting, a closed cranial window, and intravital microscopy to measure BBB permeability, leukocyte–endothelial interactions, and monitor tumor growth. Vascular endothelial growth factor (VEGF), glial fibrillary acidic protein (GFAP), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining were used to quantify the degree of angiogenesis, astrogliosis, and apoptosis. To the best of our knowledge, this study is the first attempt to evaluate the combination of tumor presence and RT in a rat model evaluating RT-induced damage to normal brain tissue.

METHODS AND MATERIALS

Animals and experimental groups

There were six experimental groups in this study (refer to Table E1 in Supplement 1) comprising Male Wistar rats (6–8 weeks) obtained from Harlan (Indianapolis, IN). Rats were anesthetized by an intramuscular injection of ketamine/xylazine (87/13 mg/kg mixture, dose 1 mL/kg). Animal studies were performed in accordance with guidelines established by the University of Tennessee, Health Science Center Institutional Animals Care and Use Committee.

Cell culture

Rat C6 glioma cells (American Type Culture Collection, Manassas, VA) were transfected to express green fluorescent protein (GFP). Cells were monolayer cultured and maintained in Ham's F12K medium, supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in humid air containing 5% CO₂.

Cranial window and implant surgery

As previously described (8), animals were anesthetized and placed in a stereotaxic frame with their body temperature maintained at 37°C. The scalp and underlying soft tissue over the parietal cortex were removed bilaterally. A drill was used to create a rectangular cranial

window centered on the midsagittal suture that extended from the bregma to the lambdoid sutures. The dura mater was excised followed by implantation of C6-GFP cell suspension or basement membrane matrix (BD Matrigel Matrix, BD Bioscience, Bedford, MA). Following implantation, a glass plate was glued to the bone surrounding the cranial window. Animals used for histology underwent burr hole surgery to perform the implant (refer to Supplement 1). All surgical procedures were carried out using sterile technique.

Implantation technique

C6-GFP cells were implanted in the upper right hemisphere of the brain between the bregma and lambdoid sutures avoiding visible vessels. C6-GFP cells were prepared before surgery at a concentration of 2×10^5 cells/ μL of basement membrane matrix. A Hamilton syringe was used to implant 1×10^6 cells in $5 \mu\text{L}$: $2.5 \mu\text{L}$ of cell mixture was injected at a depth of 2.5 mm, $1.25 \mu\text{L}$ of the cell mixture was injected at a depth of 1.5 mm, and $1.25 \mu\text{L}$ of cell mixture was injected at a depth of 0.5 mm. Animals without tumors also underwent implantation to account for the effects of surgery. These animals had BD Matrigel Matrix (BD Bioscience, Bedford, MA) implanted without tumor cells. These animals are referred to as shams animals.

Irradiation

Animals were anesthetized and irradiated using a ^{137}Cs irradiator (Mark 1 Model 25 irradiator, JL Shepard and Associates, San Fernando, CA). Animals received a fractionated regimen of 8 Gy per day for 5 days starting at Day 5 after tumor implant. Irradiation was localized to the cranial region (width of 1 cm) using a model 335 collimator (JL Shepard and Associates). Adequate lead shielding was used to ensure that tissues outside the field received $<3\%$ of the prescribed dose.

BBB permeability measurement

As previously published (3, 8), Texas-Red-dextran 3 kDa (1% solution, 0.4 mL/kg; #D3329, Invitrogen, Carlsbad, CA) was injected intravenously and used as a permeability tracer on Days 11 and 16 postsurgery. Images were acquired using a microscope equipped with a charged-coupled device camera (CoolSnap ES, Photometrics, Tucson, AZ), and GFP, Rhodamine-6G, and Texas-Red filter cubes. Images were analyzed using MetaMorph software (Universal Imaging, Downingtown, PA). To measure the permeability of the pial vessels in the peritumoral tissue, two rectangular regions of interest (ROI) were chosen in the venules (average diameter $70 \pm 20 \mu\text{m}$) and in the adjacent tissue, and their average intensities were recorded to calculate the change in intensity over time. The peritumoral region in our intravital measurements was defined as tissue within <1.5 mm from the edge of the tumor that did not express GFP. For additional information, refer to Supplement 1 ($n = 5-7$ per group).

Clearance index

Following intravenous injection of Texas-Red-dextran 3 kDa (0.4 mL/kg), images of the brain were acquired at low magnification using intravital microscope at 5, 10, 15, and 20 min after dye injection on Day 16 postimplant. An ROI was drawn in the tissue of interest, and the average intensity was recorded and normalized to the intensity at 5 min. For tumor tissue, the ROI encompassed the whole tumor. The edges of the tumor were delineated from the normal tissue by the GFP fluorescence. The peritumoral ROI was drawn adjacent to the tumor and outside of the tumor tissue expressing GFP, and was $300 \mu\text{m}$ wide. In the irradiated sham implant and unirradiated sham implant, the ROI was drawn in one side of the cranial window. ROIs were carefully drawn to avoid areas of dura regrowth ($n = 5-6$ per group).

Leukocyte-endothelial interactions

Rhodamine-6G (0.5% solution, 0.4 mg/kg; Sigma, St. Louis, MO), a lipophilic positively charged molecule that selectively stains 99.9% leukocytes mitochondria, was injected intravenously (8). Leukocyte–endothelial interactions were measured in the same vessels as the permeability measurement averaging 70 μm in diameter and in the immediate vasculature on Days 10 and 15 postimplant. A leukocyte was counted as adherent if it was stationary for 30 seconds and was plotted as the number of adherent leukocytes per 100 μm of vessel length. Leukocytes that were rolling within this length over 30 seconds were counted ($n = 5\text{--}8$ per group). Vessels were visually inspected to ensure normal flow and measurements were not performed within two diameters of a vascular branch.

Tumor surface area and volume measurements

Images of the cranial window were acquired using a GFP filter cube and fixed exposure. A threshold was applied that was 150 intensity units above the average background. The area (mm^2) was calculated from the pixels within the threshold limit in the tumor area. This technique was used for *in vivo* tumor surface area measurements and the *ex vivo* measurements of tumor dimensions used in the ellipsoidal volume formula to calculate tumor volume ($\text{volume} = 0.52 * (\text{major axis}) * (\text{minor axis})^2$; Tumor + RT $n = 7$, Tumor only $n < 16$).

Histology

Animals that had undergone burr hole surgery and implantation were anesthetized on Days 10 and 15 postsurgery and perfused with heparinized saline. The brain was then extracted, sectioned in the coronal plane through the tumor/implantation site, and either snap frozen using liquid nitrogen and stored at -80°C or placed in formalin and paraffin embedded. Refer to Supplement 1 for VEGF, GFAP, or TUNEL staining procedures.

Statistical analysis

Statistical analyses were performed using SigmaStat software (SPSS Science, Chicago, IL). Analysis of variance and multiple comparison procedures (Tukey's test) were used to determine the significance differences among the means of the treated and sham-treated groups. Data are presented as mean $\pm$ SEM, and differences between the means were considered to be statistically significant at the $p < 0.05$ level.

RESULTS

Animal survival

Without irradiation, 78% of the tumor-implant animals fulfilled the criteria for euthanasia, $>10\%$ weight loss established by the Institutional Animals Care and Use Committee, from tumor progression within 30 days of implantation. All of the animals with irradiated tumor implants survived until sacrifice at 30 days after implantation (Fig. 1A).

Tumor surface area and estimated volume measurements

Tumor surface area was visualized through the cranial window (Fig. 1B). The mean normalized surface area of the tumors in the unirradiated group was 6.46 ± 0.79 , 11.63 ± 0.89 , 11.26 ± 2.76 normalized surface area at 10, 15, and 20 days, respectively. Irradiation significant reduction in tumor surface area compared with the unirradiated tumor group. The respective values on Days 10, 15, and 20 were 3.81 ± 0.34 ($p < 0.05$), 2.37 ± 0.69 ($p < 0.001$), and 1.40 ± 0.85 normalized surface area ($p < 0.05$). The surface area of the tumor correlated ($R^2 = 0.64$) with tumor volume estimated from the coronal section of the tumor postsacrifice ($p = 0.031$, data not shown).

Peritumoral permeability

No change in BBB permeability was observed at 11 days after implant, but by Day 16, a significant increase was observed in unirradiated peritumoral tissue ($31.75 \pm 0.85 \times 10^{-7}$ cm/s; $p < 0.05$), irradiated peritumoral tissue ($36.7 \pm 1.88 \times 10^{-7}$ cm/s; $p < 0.001$; Fig. 2), and irradiated shams ($34.97 \pm 1.02 \times 10^{-7}$ cm/s; $p < 0.001$) compared with unirradiated sham implant animals ($27.46 \pm 1.17 \times 10^{-7}$ cm/s).

Leukocyte adhesion

The number of adherent leukocytes per 100 μm of vessel length was found to be significantly increased only in the irradiated peritumoral group (1.27 ± 0.46 adherent leukocytes/100 $\mu\text{m}/30$ s, $p < 0.05$) at 10 days compared with the unirradiated sham group (0.53 ± 0.21 adherent leukocytes/100 $\mu\text{m}/30$ s). At Day 15 postsurgery, there was no significant change in the number of adherent leukocytes between any of the experimental groups (Fig. 3A).

Leukocyte rolling

Rolling leukocytes were counted at the same time as adhered leukocytes using the same vessels. Following RT at Day 10 postsurgery, there was a significant increase in rolling leukocytes in the irradiated sham implant group (8.00 ± 0.93 rolling leukocytes/100 $\mu\text{m}/30$ s, $p < 0.001$) compared with the unirradiated sham implant group (2.73 ± 0.54 rolling leukocytes/100 $\mu\text{m}/30$ s). At 10 days, the irradiated sham implant group had a significantly higher number of rolling leukocytes than the unirradiated peritumoral group (3.17 ± 0.55 rolling leukocytes/100 $\mu\text{m}/30$ s, $p < 0.001$). On Day 15 postsurgery, the same pattern was observed. The number of rolling leukocytes was higher in the irradiated sham implant group (7.76 ± 0.41 rolling leukocytes/100 $\mu\text{m}/30$ s) compared with the unirradiated sham implant group (2.23 ± 0.18 rolling leukocytes/100 $\mu\text{m}/30$ s; $p < 0.001$) and the unirradiated peritumoral group (1.46 ± 0.46 rolling leukocytes/100 $\mu\text{m}/30$ s, $p < 0.001$). The irradiated sham implant group also had significantly higher number of rolling leukocytes than the irradiated peritumoral group ($p < 0.05$). The irradiated peritumoral group also had significantly higher number of rolling leukocytes at 15 days than the unirradiated peritumoral group ($p < 0.01$; Fig. 3B).

Clearance index

Clearance in tumor, peritumoral, and sham tissues (irradiated and unirradiated) was measured at Day 16 postimplant. At 20 min after dye injection, the unirradiated tumor tissue had retained more dye than all other tissue regions (1.02 ± 0.02 normalized intensity, $p < 0.01$) indicating a change in clearance of the dye from the tumor. In addition, the unirradiated peritumoral tissue (0.98 ± 0.02) had significantly higher intensity at 20 min after dye injection compared with the unirradiated sham tissue (0.85 ± 0.03 normalized intensity, $p < 0.01$; Fig. 4). It is important to note that treatment with irradiation resulted in a clearance index similar to that of the unirradiated sham implant animals.

Astrogliosis after tumor implantation

The magnitude of astrogliosis in normal tissue was measured at Day 15 postimplant in the unirradiated sham and irradiated and unirradiated tumor implant groups. For the tumor-bearing groups, measurements were taken in the contralateral cerebral cortex because of tumor shrinkage. Astrogliosis was significantly greater in the irradiated tumor-implanted group (256.5 ± 11.7 astrocytes/ mm^2) compared with the unirradiated tumor-implanted group (223.3 ± 4.8 astrocytes/ mm^2 ; $p < 0.05$) and the unirradiated sham-implanted group (192.3 ± 5.3 astrocytes/ mm^2 ; $p < 0.001$; Fig. 5A and 5B–D).

VEGF protein expression

VEGF was detected in the peritumoral region and in the tumor (not shown) at 10 and 15 days after implantation (Fig. 6). Following tumor implant and RT at Day 15 postimplant, no VEGF protein was detectable (images not shown).

Apoptosis

Apoptotic cells were not detected in the brain tissue on Days 10 and 15 regardless of whether the animal's brain was irradiated. In the tumor tissue at 10 days with and without RT, there were single apoptotic cells identified (Fig. 7B and 7D). At 15 days postimplant, there was a high degree of apoptosis in the tumor that did not change even after RT (Fig. 7F and 7H).

DISCUSSION

The aim of this research was to extend our previous observations in evaluating normal brain tissue responses to irradiation and determine the effect of tumor presence. The presence of tumor resulted in quantifiable changes in BBB permeability, leukocyte–endothelial interactions, clearance, astrogliosis, and VEGF expression in peritumoral normal tissue. Furthermore, RT caused a significant change in BBB permeability, altered the kinetics and degree of the leukocyte–endothelial interactions, and caused astrogliosis. However, RT did not alter VEGF expression or apoptosis. Our findings indicate that RT-induced increase in the peritumoral permeability is caused by inflammatory damage to the astrocytes and not by elevated levels of VEGF. (Results are summarized in Supplement 1, Table E2).

In this study, we used a fractionated regimen of 8 Gy/day for 5 days. In patients, hypofractionation doses of 2.8–8 Gy is used for palliative treatments, and doses higher than 8 Gy are considered ablative (9). Park *et al.* (10) also reported that in stereotactic body radiotherapy, the effective dose strongly depends on the total dose rather than the number of fractions and argued that 40 Gy delivered in 5 fractions is equivalent to 60 Gy in 30 fractions. The difference in dose is due to the shoulder (curve) in the LQ model, which is overestimated at higher doses and does not fit the experimentally measured survival data.

Our fractionated irradiation scheme significantly hindered tumor growth, leading to tumor regression and a 100% survival at 30 days. The reason for such effective response to RT could be in our use of high dose per fraction (8 Gy). Also, the tumor size at initiation of RT might be a critical factor, and the immunogenicity of C6 cells could invoke a host immune response (11).

There was a lack of correlation between the increase in permeability and adherent leukocytes in the irradiated peritumoral group at both time points. Yuan *et al.* (12) showed that following fractionated RT (2 Gy/day for 20 days), changes in BBB permeability were delayed until Day 90 after start of treatment, whereas changes in leukocyte–endothelial interactions were undetectable. This is similar to our findings and seems to indicate that the inflammatory response following fractionated RT is different from a single dose of radiation (2). This lack of correlation in our model may be due to the tumor's ability to downregulate leukocyte–endothelial interactions. This is supported by our finding that leukocyte–endothelial interactions in the unirradiated peritumoral group were not significantly higher at any time point compared with the unirradiated sham implant group. Wu *et al.* (13, 14) showed that when tumor-bearing tissue was irradiated, the number of leukocyte interactions in the microvessels either did not change in the peritumoral/tumor vasculature or it decreased. Even though there were changes in leukocyte–endothelial interactions following radiation, this study demonstrates the tumor's ability to alter the kinetics and magnitude of interactions.

As expected, unirradiated tumor and peritumoral tissue had the poorest clearance index. The leakiness of the tumor vasculature could lead to an increase in fluorescent probe extravasation, which results in an increase in signal intensity (15, 16). Similarly, an increase in oncotic pressure would result in a slow fill and emptying of the tissue bed, also resulting in elevated signal intensity. Indeed, our model was able to detect a change in vascular permeability in the unirradiated peritumoral vasculature on Day 16 after tumor implant as well as a decrease in peritumoral and tumor tissue clearance. These findings agree with previously reported clinical and experimental observations (16, 17).

Following irradiation, the degree of astrogliosis increased significantly in the contralateral cerebral cortex of the tumor-bearing brain. Furthermore, with an uncorrected p value, we observed a significantly higher degree of astrogliosis in the contralateral cerebral cortex of the tumor implant group compared to sham (t test, $p = 0.014$), but the statistical significance was lost when correcting for multiple comparisons. This was an unexpected result given that there were no signs of tumor in the contralateral region and supports our hypothesis that the presence of tumor alters the normal tissue. This is important because activated astrocytes have been shown to secrete inflammatory agents (18, 19) that may amplify microvascular damage. We have also observed an increase in gliosis in the peritumoral region at 500 μm from the tumor edge (data not shown). This increase in gliosis might render difficult the identification of tumor margins for surgical and RT planning (20). Together our findings point to RT-induced astrogliosis as an important factor in acute radiation response. We also investigated whether apoptosis was responsible for increase in permeability following RT. We did not find any apoptotic response 24 hours post-RT, which indicates that it did not play a direct role in our observed increase in permeability. Ch'ang *et al.* from the laboratory of Kolesnick and Fuks (21) found at a dose of 8 Gy that they measured a little more than 10% of the maximal effect of radiation-induced endothelial apoptosis in the intestinal crypt using whole body radiation. This is different from our findings, but others have also been unable to reproduce the degree of apoptosis found by Fuks and Kolesnick in the crypt (22, 23). In the central nervous system, their group demonstrated that endothelial apoptotic response peaks at 12 hr post-RT (doses 5–100 Gy) and is still slightly elevated at 24 hours post-RT (24). Experiments done by Pena *et al.* at 10 Gy resulted in very few apoptotic cells in the brain. There were no visible apoptotic cells in the peritumoral region, whereas the tumor had heterogeneous apoptosis.

We measured VEGF protein expression to investigate whether the increase in permeability was caused by angiogenesis following RT. VEGF protein was found in tumor and peritumoral tissues on Day 10 postimplant but was undetectable in these regions at Day 15 postimplant, and there was no observed change following RT. These observations contradict any significant role for VEGF signaling in the observed RT-induced increase in permeability in these regions in our experiment, but others have observed such change in tumors *in vitro* and *in vivo* (25–27), which might be explained by the difference in experimental design.

In conclusion, our work showed that the presence of the tumor, in a rat brain tumor model, altered radiation-induced effects in normal tissue. Our observed change in peritumoral permeability following irradiation may be related to the observed astrogliosis and not VEGF expression or apoptosis. Furthermore, the fractionated tumor model developed in this study can be used to investigate the mechanisms of acute and long-term radiation-induced damage in the peritumoral/normal tissue of the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 (A) Survival of tumor-bearing animals with and without radiation therapy (RT): 8 Gy/day $\times$ 5, starting Day 5 after implantation of tumor. (B) Surface area of the tumor $\pm$ RT. * p < 0.05, ** p < 0.001, compared with unirradiated tumors on the given days.

Fig. 2. Peritumoral permeability on Day 16 after implant. Radiation therapy (RT): 8 Gy/day $\times$ 5. * p < 0.05, ** p < 0.001, compared with unirradiated sham implant.

Fig. 3. (A) Number of adhered leukocytes and (B) rolling leukocytes at Days 10 and 15. * $p < 0.05$ vs. sham, ** $p < 0.001$ vs. sham, # $p < 0.05$ and ## $p < 0.001$ compared with peritumoral, \$ $p < 0.05$ vs. peritumoral + radiation therapy (RT).

Fig. 4. Tumor perfusion 16 days after tumor or sham implant, as measured by intensity of dye following intravenous injection of Texas-Red-dextran 3 kDa, as a function of minutes after injection. * $p < 0.01$, ** $P < 0.001$ compared with unirradiated sham implant. RT = radiation therapy.

Fig. 5.

(A) Astroglial staining on Day 15 in normal tissue in unirradiated sham and in the cerebral cortex contralateral to the tumor implant in irradiated and unirradiated brains. * $p < 0.01$ compared with the unirradiated sham implanted. # $p < 0.05$ compared with unirradiated tumor implant groups. (B–D) Astroglial staining in histological sections from (B) unirradiated sham-implanted animal, (C) contralateral to the tumor, and (D) in an irradiated tumor-bearing brain contralateral to the tumor. Bar represents 100 μm . RT = radiation therapy.

Fig. 6.

Immunofluorescent staining of vascular endothelial growth factor (VEGF) expression in the tumor brains at (A, B) Day 10 postimplant, (C, D) after radiotherapy on Day 10 postimplant, and (E, F) at Day 15 after tumor implant. Staining for VEGF expression was performed (A, C, E) contralateral to the tumor, (B, D, F) in the peritumoral region. Blue = 4', 6-diamidino-2-phenylindole (DAPI) staining, red = VEGF, green = C6-GFP cells. Bar represents 50 μm .

Fig. 7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of apoptotic cells in the brains at (A, B) 10 days postimplant, (C, D) after radiotherapy on Day 10 postimplant, (E, F) 15 days postimplant, and (G, H) after radiotherapy 15 days postimplant. Images were A, C, E, G acquired in the brain tissue, and images B, D, F, H were acquired in the tumor tissue. Brown = TUNEL, gray = nuclei. Bar represents 25 μm .