Cerebral microvascular rarefaction induced by whole brain radiation is reversible by systemic hypoxia in mice

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Whole brain radiation therapy (WBRT) leads to cognitive impairment in 40–50% of brain tumor survivors following treatment. Although the etiology of cognitive deficits post-WBRT remains unclear, vascular rarefaction appears to be an important component of these impairments. In this study, we assessed the effects of WBRT on the cerebrovasculature and the effects of systemic hypoxia as a potential mechanism to reverse the microvascular rarefaction. Transgenic mice expressing green fluorescent protein driven by the Acta2 (smooth muscle actin) promoter for blood vessel visualization were randomly assigned to control or radiated groups. Animals received a clinical series of 4.5 Gy WBRT two times weekly for 4 wk followed by 1 mo of recovery. Subsequently, mice were subjected to 11% (hypoxia) or 21% (normoxia) oxygen for 1 mo. Capillary density in subregions of the hippocampus revealed profound vascular rarefaction that persisted despite local tissue hypoxia. Nevertheless, systemic hypoxia was capable of completely restoring cerebrovascular density. Thus hippocampal microvascular rarefaction post-WBRT is not capable of stimulating angiogenesis and can be reversed by chronic systemic hypoxia. Our results indicate a potential shift in sensitivity to angiogenic stimuli and/or the existence of an independent pathway of regulating cerebral microvasculature.

angiogenesis; hippocampus; capillary; pericyte

In the United States, over 1,500,000 new cases of cancer are expected to be diagnosed this year, with over 22,000 cases expected to be brain and nervous system related (2). Whole brain radiation therapy (WBRT) continues to be one of the most common forms of treatment for metastatic brain tumors located in brain regions that are difficult to reach for surgical removal, as well as for treatment of primary brain tumors following surgical intervention (26). Although this treatment regimen has proven to be effective in reducing or eliminating tumors, some damage to normal brain tissue is inevitable. Previous studies have shown that a relatively large proportion of brain tumor survivors develop cognitive deficits that are evident months to years after treatment (26, 29, 52, 53). These impairments in learning and memory have been confirmed in animal models (6, 46). However, the etiology of WBRT-induced cognitive impairment is not fully understood.

In animal studies, radiation therapy has been shown to induce dose-dependent endothelial apoptosis (30) and disruption of the blood-brain barrier (31), thickening and vacuolation of the vascular basement membrane (25), vascular rarefaction (7), and cognitive deficits (6, 46). To sustain neuronal activity and normal brain function, blood vessel integrity must be preserved to maintain a consistent supply of oxygen, nutrients, and growth factors and provide efficient removal of waste products from cells. Thus it is reasonable to conclude that radiation-induced damage to the cerebral microvasculature contributes at least in part to the impairment in brain function.

The aim of this study was to determine whether the administration of a clinically relevant regimen of WBRT results in microvascular rarefaction within subregions of the hippocampus (an area important for learning and memory) and whether low ambient oxygen levels, a potent physiological stimulus for vessel growth throughout the body, could restore microvascular density following treatment. We report that, by 2 mo post-WBRT, microvascular rarefaction in regions of the hippocampus is readily apparent; however, when mice are challenged with chronic hypoxia (11% oxygen for 28 days), microvascular density was completely restored. This is the first study, to our knowledge, that demonstrates complete recovery of cerebral microvascular density following WBRT and provides evidence that local angiogenic pathways are inhibited or completely suppressed by radiation whereas other pathways are maintained.

MATERIALS AND METHODS

Animals. To enable the direct visualization of blood vessels, a transgenic mouse model utilizing the Acta2 promoter to direct the expression of green fluorescent protein (GFP) was used. These mice were generated by Dr. J. Y. Tsai (47). The design of the Acta2 promoter was described by Wang et al. (51). Homozygous pairs of smooth muscle α-actin (SMA)-GFP mice, a generous gift from Dr. James Tomasek [University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City, OK], were bred, and male offspring were housed 3–5/cage in the rodent barrier facility at OUHSC. Animals were maintained on a 12:12-h light-dark cycle and fed standard rodent chow and water ad libitum. At 10–11 wk of age and 1 wk before radiation treatment, mice were transferred to the conventional facility (OUHSC) and housed under similar conditions. All animal protocols were approved by the Institutional Animal Care and Use Committee of OUHSC.

Irradiation and experimental design. After acclimating to the conventional facility for 1 wk, mice were randomly assigned to either control or radiated groups. Animals were weighed and anesthetized via intraperitoneal injection of ketamine and xylazine (100/15 mg/kg). Mice in the radiated group received a fractionated series of WBRT administered as eight fractions of 4.5 Gy/fraction, two times a week.
for 4 wk (total cumulative dose of 36 Gy), at a rate of 1.23 Gy/min while sham animals were anesthetized without radiation. Radiation was administered using a 137-cesium gamma irradiator (GammaCell 40; Nordion International). A Cerrobend shield was used to minimize the dose to the bodies of mice in the irradiated group.

**Dosimetry.** The radiation dose received by the mice was verified using film dosimetry. Radiographic films were placed within a rodent equivalent phantom between the Cerrobend molds in specific locations. Six films were used to measure representations of the anterior and posterior surfaces as well as the centers of the head and body of the mice. The irradiator was then activated for a time known to deliver 4.5 Gy to an empty irradiator chamber. Because the irradiation chamber now contained both rodent and Cerrobend shielding, the actual dose was expected to be different from that delivered to an empty film. Film analysis indicated that the body of the mice received an average of 1.06 ± 0.05 Gy while the head received 4.4 ± 0.2 Gy.

**Hypoxia treatment.** One month following sham or WBRT, mice were further divided into normoxic (21% oxygen) or hypoxic (11% oxygen) conditions. Hypoxia was achieved through the use of a custom-designed Plexiglas chamber (40.5 in. × 24 in. × 10.5 in.) capable of holding six mouse cages. Air flow through the chamber was provided by air pumps and holes drilled into the individual mouse cages. Nitrogen gas and room air were regulated to create a final ambient oxygen level of 11% inside the chamber. Oxygen levels were monitored at least twice daily using an oxygen meter (Extech Instruments, Waltham, MA) inserted through a fitted port. Adjustments to gas flow were made as necessary. Hypoxia was induced gradually by decreasing the levels of oxygen by 1.5% daily for the first 7 days, until the target level of 11% oxygen was obtained. Oxygen levels were then maintained at 11% from day 7 through day 28. Cage changes were performed weekly, exposing animals to room air for short periods (no more than 5 min). Animals in the normoxic group were housed in similar cages as hypoxic animals with oxygen levels maintained at 21%.

**Tissue collection and preparation.** After completion of hypoxia treatment, mice were anesthetized using ketamine and xylazine (100/15 mg/kg). Animals in the hypoxic groups were exposed to room air for no more than 1 h before death to minimize changes that may occur with reoxygenation. Blood was collected via cardiac puncture with reoxygenation. Blood was collected via cardiac puncture for determining hematocrit levels. Animals were transcardially perfused using cold, heparinized sodium phosphate buffer. Brains were removed, left hemispheres were postfixed in 4% paraformaldehyde overnight; and right hemispheres were dissected into hippocampus and cortex, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

**Vascular endothelial growth factor measurement.** Protein was isolated from the right hippocampus using a Protein and RNA Isolation System kit (PARIS; Ambion, Austin, TX) following the manufacturer’s protocol. Protein concentration was measured using the DC Protein Assay (Bio-Rad). Equal amounts of protein from the nuclear extracts were loaded and electrophoresed in 4–20% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes using semidry transfer. After being blocked with 4–20% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes using semidry transfer. After being blocked for 1 h in Odyssey Blocking Solution (Li-Cor, Lincoln, NE), membranes were incubated in 1:500 rabbit polyclonal antibody to VEGF-R2 and rabbit polyclonal antibody to b-tubulin (Abcam, Cambridge, MA) at room temperature for 1 h. Membranes were then incubated with 1:10,000 goat anti-rabbit AlexaFluor 680 secondary antibody at room temperature for 1 h. Membranes were scanned using an Odyssey system (Li-Cor), and bands were analyzed using Odyssey Analysis software. Protein expression levels were represented as a ratio of VEGF-R2 to tubulin.

Right cortices were processed using a nuclear extraction kit (Panomics, Fremont, CA) according to the manufacturer’s instructions. Protein concentration was measured using the DC Protein Assay (Bio-Rad). Equal amounts of protein from the nuclear extracts were loaded and electrophoresed in 4–20% gradient gels. Membranes were incubated in 1:500 rabbit polyclonal antibody to hypoxia-inducible factor (HIF)-1a (Novus Biologicals, Littleton, CO) followed by 1:10,000 goat anti-rabbit 680 antibody. Membranes were then stripped using Nitro Stripping Buffer (Li-Cor) and probed using 1:2,000 rabbit polyclonal antibody to a-tubulin followed by 1:10,000 goat anti-rabbit 680 antibody and scanned using the Odyssey system. Protein expression was represented as target band intensity/tubulin band intensity.

**Quantification of capillary density.** Postfixed left hemispheres were cryoprotected in a series of graded sucrose solutions (10, 20, and 30%) and frozen in Cryo-Gel (Electron Microscopy Sciences, Hatfield, PA) for sectioning. Cononal sections of 70 μm were cut through the hippocampus and stored free-floating in cryoprotectant solution (25% glycerol, 25% ethylene glycol, 25% 0.1 M phosphate buffer, and 25% water) at −20°C. Sections washed in TBS (×3), transferred to slides, and covered with a cover slip. Image stacks were captured using confocal microscopy (Leica Microsystems, Urbana, IL) using a ×40 oil objective.

**Capillary density in the CA1, CA3, and dentate gyrus (DG) of the hippocampus (shown in Fig. 1) was quantified as the length of blood vessels <10 μm in diameter per volume of tissue using NeuronLucida with AutoNeuron (MicroBrightField, Williston, VT). The computer software allows for the tracing of blood vessels in three-dimensional space through acquired image stacks. The total length of capillaries.
RESULTS

General. Changes in body mass were monitored for the duration of the study. No differences in body mass were evident before radiation (control 27.4 ± 0.5 g, radiated 27.7 ± 0.4 g); however, as expected, midway through and following completion of the WBRT series, animals in the radiated group experienced significant weight loss (P < 0.01) (Table 1). During the 1-mo recovery period, animals in the radiated group demonstrated recovery of body mass but remained significantly lower than the controls (P < 0.01). The radiated animals continued to regain weight until the end of the study when no difference in body mass compared with their control treatment-matched groups (P > 0.05) was observed.

Hematocrit, VEGF, VEGF-R2, and HIF-1α protein levels confirm the hypoxic state of animals. At the end of the hypoxic study, hematocrit was measured to verify that animals demonstrated normal physiological responses to hypoxia. Hematocrit was slightly reduced in the radiated normoxic group (38.9 ± 2.12) compared with the control normoxic (44.79 ± 2.77), but this difference did not reach statistical significance (P = 0.06; Fig. 2A). Following hypoxia (11% O2 for 28 days), both radiated and control groups had elevated hematocrit values (65.19 ± 1.41 in the controls and 62.84 ± 1.36 in the radiated groups), representing a 31.3 and 38.1% increase, respectively. The right hippocampus was processed for VEGF protein expression by ELISA. Following WBRT, there was no change in VEGF expression (258.65 ± 21.2 pg/mg protein in controls and 224.86 ± 16.9 pg/mg protein in the radiated group; Fig. 2B) under normoxic conditions. However, in response to chronic hypoxia, VEGF levels were significantly increased in the hippocampal tissue of both groups (377.03 ± 17.3 pg/mg protein in controls and 413.52 ± 15.9 pg/mg protein in the radiated group, P < 0.001), representing a 31.4 and 45.6% increase, respectively.

VEGF-R2 has been reported to be upregulated in hypoxic conditions (50). Therefore, to confirm the hypoxic state of the tissues, we used Western blotting to determine the relative protein expression of VEGF-R2 in the hippocampus. WBRT induced a 2.3-fold increase in the relative expression of VEGF-R2 (P = 0.038 compared with controls; Fig. 2C). Chronic hypoxia stimulated a 2.7-fold increase in receptor expression in the controls (P = 0.012 compared with the control normoxic group). An increased expression of VEGF-R2 in response to WBRT alone is consistent with the hypothesis that the reduction in vessel density (see below) produced a hypoxic condition within the hippocampus of WBRT-treated animals.

It is well established that the transcription factor, HIF-1α, is stabilized in low oxygen levels (24) and is therefore an excellent hypoxic marker. To determine the level of tissue hypoxia present following WBRT and chronic hypoxia treatment, HIF-1α protein levels were measured in the cortical nuclear fractions using Western blot analysis. Under normoxic conditions, WBRT induced a 2.8-fold increase in nuclear HIF-1α compared with control animals (P = 0.009; Fig. 2F), further supporting the conclusion that the brains of radiated animals were hypoxic.

Microvascular rarefaction in hippocampal subregions and recovery of vascular density following hypoxia. Capillaries were identified by their expression of CD31, using a lumen diameter of 10 μm or less as a standard identifier (40, 55). Capillary density was quantified in CA1, CA3, and DG of the mouse hippocampus and was represented as the total length of capillaries (mm) per volume of tissue sampled (mm^3). Figure 3 shows a representative image of SMA-positive vessels (Fig. 3, A–D) and CD31 (Fig. 3, E–H) and merged (Fig. 3, I–L) staining of blood vessels in the CA3 region of the hippocampus. Following WBRT, CD31-stained capillary density decreased in the radiated group compared with controls in CA1 (347.97 ± 96.5 vs. 728.21 ± 53.7, P = 0.002; Fig. 4A), CA3 (291.57 ± 85.4 vs. 593.65 ± 75.3, P = 0.01; Fig. 4B), and DG (331.74 ± 81.1 vs. 873.17 ± 79.6, P = 0.003; Fig. 4C). Chronic hypoxia was able to completely

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Table 1. Changes in body mass of animals

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Control</th>
<th>Radiated</th>
<th>Significance (P value)</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>27.4 ± 0.5 (29)</td>
<td>27.7 ± 0.4 (16)</td>
<td>NS (0.590)</td>
</tr>
<tr>
<td>18 Gy</td>
<td>28.7 ± 0.5 (29)</td>
<td>25.7 ± 1.2 (16)</td>
<td>(0.008)**</td>
</tr>
<tr>
<td>36 Gy</td>
<td>29.0 ± 0.5 (29)</td>
<td>25.3 ± 1.0 (16)</td>
<td>(&lt;0.001)***</td>
</tr>
<tr>
<td>1 mo post-WBRT</td>
<td>29.3 ± 0.5 (29)</td>
<td>26.2 ± 1.3 (16)</td>
<td>(0.006)**</td>
</tr>
<tr>
<td>End of study</td>
<td>Normal</td>
<td>30.0 ± 0.7 (13)</td>
<td>28.3 ± 1.0 (8)</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>28.6 ± 0.6 (16)</td>
<td>27.1 ± 0.4 (8)</td>
</tr>
</tbody>
</table>

Data represent means ± SE; for control and radiated groups, n. of animals is in parentheses. Animals were selected for body mass measurement at each major stage of the experiment. Time points shown represent: before commencement of whole brain radiation therapy (WBRT) (baseline), after a cumulative dose of 18 Gy, after completion of the cumulative series of irradiation (36 Gy), 1 mo post-WBRT, and at the end of the hypoxia or normoxia treatment. NS, not significant. **P < 0.01 and ***P < 0.001.

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In this document, the author discusses the effects of whole brain radiation therapy (WBRT) on body mass, hematocrit, VEGF, VEGF-R2, and HIF-1α protein levels, and capillary density in the hippocampus. The study shows that WBRT induced a significant reduction in body mass, while chronic hypoxia increased hematocrit and VEGF expression. VEGF-R2 and HIF-1α expression were also increased under hypoxic conditions, confirming the hypoxic state of the animals. Capillary density was decreased following WBRT, and chronic hypoxia partially reversed this rarefaction.
restore capillary density in the radiated groups within all regions assessed \( (P < 0.01\) each) to levels found in nonradiated hypoxic animals.

Capillaries expressing SMA were quantified in the CA1, CA3, and DG of the mouse hippocampus. Following WBRT, SMA capillary density in the CA1 (Fig. 4D) and CA3 (Fig. 4E) did not show a significant reduction \( (P > 0.05)\) compared with the control normoxic groups. However, in response to hypoxia treatment, SMA capillary density was significantly increased in both the control and radiated groups \( (P < 0.01)\). In the DG (Fig. 4F), WBRT induced a significant reduction in capillary density in the normoxic-treated radiated group \( (738.15 \pm 49.0\) to \(418.94 \pm 70.7, P = 0.01)\). In response to hypoxia, SMA capillary density increased in both groups \( (P < 0.01)\). Analysis of capillary diameter showed no difference between groups or treatments in CA1 (Fig. 4G) and CA3 (Fig. 4H); however, capillary lumen diameter was reduced in the normoxic, WBRT group compared with the control normoxic group \( (P = 0.044)\) in the DG (Fig. 4I).

**Capillary coverage by pericytes in the mouse hippocampus.** Although there is no definitive immunohistochemical marker for pericytes \( (14, 15)\), pericytes were identified by their expression of SMA and general morphology (round nucleus and long cytoplasmic projections as described in Refs. 14 and 15) and confirmed by their expression of NG2 and/or PDGFR-\(\beta\). SMA\(^+\) putative pericytes were observed along CD31-stained endothelial cells of capillaries with the described morphology (Fig. 5, A–D). These cells were also positive for PDGFR-\(\beta\) (Fig. 5, E–G) and NG2 (Fig. 5H). The percent coverage of capillaries by pericytes was quantified by using a ratio of

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**Fig. 2.** Confirmation of hypoxic status of animals. Hematocrit (A) and vascular endothelial growth factor (VEGF, B) protein levels increased in response to hypoxia treatment in both groups \( (n = 8–16\) animals/group). C: representative Western blot for VEGF receptor (R) 2 levels. D: quantification of Western blot analysis showed that VEGF-R2 protein levels increased in the control animals in response to hypoxia and also increased in response to radiation \( (n = 6\) group). E: representative Western blot for cortical hypoxia-inducible factor (HIF)-1\(\alpha\) in nuclear fractions. F: quantification of the Western blot showed that, in the normoxic-treated radiated group, nuclear HIF-1\(\alpha\) is increased compared with controls \( (n = 4\) group). CN, control normoxia; CH, control hypoxia; RN, radiated normoxia; RH, radiated hypoxia. \(*P < 0.05\) and \(***P < 0.001\) vs. the normoxic group. \(#P < 0.05\) and \(##P < 0.01\) vs. the control normoxic group.

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SMA\(^+\) capillary length to CD31\(^+\) capillary length. In the CA1 region (Fig. 5I), the percent coverage by pericytes increased significantly in response to hypoxia in the controls (from 73.02 ± 5.4 to 138.40 ± 10.9\%, \(P < 0.001\)) and also increased in both the radiated normoxic (\(P = 0.012\)) and hypoxic (\(P = 0.033\)) groups compared with the control normoxic group. In the CA3 region (Fig. 5J) of the hippocampus, a significant increase in the percent coverage was observed only in the control hypoxic group (\(P < 0.001\)). There were no significant differences between groups or treatments in the DG (Fig. 5K).

**DISCUSSION**

In this study, we assessed microvascular changes induced by WBRT and the effect of chronic hypoxia in the mouse hippocampus, a brain region closely associated with learning and memory. WBRT induced profound microvascular rarefaction within 2 mo in CA1, CA3, and DG of the mouse hippocampus. Surprisingly, following 1 mo of hypoxia, a complete recovery of microvascular density was observed in the radiated group, indicating that, although WBRT induced vascular rarefaction and tissue hypoxia (established by HIF-1α and VEGF-R2 levels), other mechanisms capable of inducing vascular regrowth remain intact and can be induced by generalized hypoxia. This is the first study to demonstrate that cerebral microvascular rarefaction occurs in subregions of the hippocampus following WBRT and that a complete recovery of capillary density can be achieved in response to low ambient oxygen levels. Although we cannot exclude the possibility that radiation results in a reduced angiogenic response to localized hypoxia, the present data suggest that more generalized vasculogenic pathways, not affected by WBRT, can be induced by a generalized hypoxic stimulus and restore brain microvasculature.

Radiation and vascular changes. It is well known that radiation has profound effects on the vasculature. Previous studies have assessed changes in endothelial cells as well as vascular structure and density following several different radiation regimens. For example, one study reported that microvascular rarefaction occurs in the rat brain as early as 10 wk following fractionated WBRT, and a partial recovery is observed at 20 wk (7). Nevertheless, localized changes within hippocampal subregions were not assessed, and significant changes were only observed when larger brain regions were combined for analysis. The present study utilized a mouse model combined with a rigorous analytical approach to assess three-dimensional microvascular changes in subregions of the hippocampus, making it relevant to the cognitive changes induced by radiation treatment. Importantly, significant changes in all regions of the hippocampus were observed within 2 mo of completion of the fractionated doses of radia-
Other studies have utilized single doses of 5–200 Gy to the brain and observed a 15% decrease in endothelial cell number within 1 day of irradiation that was maintained for up to 1 mo (33). Finally, decreased endothelial cell density within the CA1 region of the hippocampus has been reported 12 mo posttreatment after single doses as low as 0.5 and 2 Gy high-linear energy transfer radiation (35). Taken together, these studies demonstrate that endothelial cells are highly sensitive to radiation and are consistent with our findings of a profound capillary rarefaction that occurs within the mouse hippocampus following WBRT.

**Significance of capillary density and maintenance of vascular integrity.** As expected, previous studies indicate that capillary density is highly correlated with regional blood flow (16, 56), and our results suggest that the density of the brain microvasculature is one critical factor that contributes to the reduction in blood flow to brain tissues after radiation. The brain is a highly metabolic organ requiring consistent oxygen levels for normal function. Maintaining an adequate supply of oxygen, nutrients, and trophic factors to subregions of the brain is accomplished by highly organized capillary networks that minimize the diffusion distance between the blood vessels and neurons/glia. For example, one study found that regional uptake of loperamide was significantly correlated with local capillary density (56). In addition to the loss of nutrient exchange that undoubtedly occurs from capillary rarefaction, there is a reduced ability to remove the waste products of cellular metabolism (including CO₂) from brain tissues. Capillary diameter is also an important indicator of diffusion characteristics, and a large range of diameters has been reported for capillaries. For example, capillary diameters ranging from 3 to 8 μm in the rat cortex (55), 3.2 to 6.9 μm in rabbit

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**Fig. 4. Changes in capillary density within subregions of the hippocampus.** Sections were stained using CD31 antibody and imaged using confocal microscopy. Capillaries were defined as blood vessels <10 μm in diameter. Capillaries were traced using Neurolucida and quantified as length of vessel per volume of tissue (as described in MATERIALS AND METHODS). CD31 capillary density in CA1 (A), CA3 (B), and DG (C). SMA capillary density in CA1 (D), CA3 (E), and DG (F). Capillary diameter in CA1 (G), CA3 (H), and DG (I). Data indicate profound capillary rarefaction in all regions assessed in response to WBRT and complete recovery of density with hypoxia. Data represent means ± SE. ***P < 0.01 and ****P < 0.001 vs. the normoxic group and #P < 0.05 and ##P < 0.01 vs. control-normoxic; the number of animals per group is indicated in parentheses.
tenuissimus muscle (5), and 2.87 μm in reperfused and 4.08 μm in control gerbil brains have been observed (18). In this study, there was no difference in capillary diameters between treatment groups in CA1 or CA3 but a slight, significant decrease in the radiated normoxic group in the DG compared with the control normoxic group. Whether this decrease in capillary diameter is a contributing factor in impaired brain function remains to be established. However, more importantly, we observed a 40% decrease in vessel density, strongly supporting the hypothesis that vascular rarefaction occurring postradiation may have a significant role in the reported decline of cognitive performance (6, 8, 12).

Hypoxia-induced angiogenesis. Angiogenesis is a multistep process that results in the formation of new blood vessels from a preexisting vascular network (11). Alternatively, vasculogenesis, a process of de novo vessel formation, involves the mobilization of several populations of cells from the bone marrow (1, 9, 22, 28, 54). The processes of angiogenesis and vasculogenesis are recognized as the two primary mechanisms necessary to develop, maintain, and/or restore vascular integrity after disruption of vascular networks (44). Previous results indicate that prevention of glioblastoma recurrence can only be accomplished when vasculogenesis, and not angiogenesis, is inhibited (27), demonstrating that these processes are activated by different stimuli and utilize independent pathways.

Hypoxia is a potent angiogenic stimulus (10, 23), acting through the stabilization of HIF-1α and activation of downstream angiogenic factors such as VEGF and erythropoietin (13, 32, 41). Recent studies have shown that radiation induces tissue hypoxia, HIF-1α expression, and oxidative stress as early as 4 wk posttreatment (42). In this study, we hypothesized that WBRT-induced vascular rarefaction was the result of diminished local angiogenic capacity. In response to WBRT, profound vascular rarefaction occurred in the presence of tissue hypoxia. The absence of significant angiogenesis in the presence of hypoxia indicates that local vascular repair is impaired in radiated animals. However, we also subjected these animals to a low ambient oxygen level of 11% oxygen for 1 mo and then reassessed capillary density. In response to hypoxia, vessel density was completely restored in radiated animals, providing prima facia evidence that a general limitation of vessel growth is not the primary factor in the chronic vessel rarefaction after radiation therapy.

Although the present study was not designed to distinguish between angiogenesis and vasculogenesis in the process of vessel repair, our results are consistent with the hypothesis that radiation suppresses local angiogenesis, whereas vessel density can be reestablished through hypoxia-induced vasculogenesis. Udagawa et al. (48) reported that radiation-induced suppression of angiogenesis is independent of restoration of hematopoietic function. This is consistent with our findings of vascular rarefaction occurring postradiation with diminished local angiogenic capacity.

Fig. 5. Coverage of capillaries by pericytes. Sample capillary covered by SMA+ pericyte (A), CD31 staining (B), and merged image captured at ×100 magnification (C). Scale bar represents 10 μm. D: ×20 magnification image showing putative pericytes covering CD31-stained endothelial cells. The general morphology is consistent to that of a pericyte with the centralized nucleus (arrow) and long cytoplasmic projections (arrowhead) covering a capillary. Scale bar represents 20 μm. SMA-positive cells are also positive for platelet-derived growth factor receptor (PDGFR)-β (E–G) and neuron/glia-type 2 antigen (NG2, H). Scale bar represents 10 μm. The density of CD31+ capillaries was represented as a percentage of the density of SMA+ capillaries to obtain the percent of coverage. The percent of coverage of capillaries by pericytes in CA1 (I), CA3 (J), and DG (K) are shown. Data represent mean% ± SE. ***P < 0.001 vs. normoxic group, #P < 0.05 vs. control, and $P < 0.05 vs. control normoxic group.
poietic cells, in that, although hematopoietic reconstitution occurred following radiation, vessel density was not restored but remained lower than the nonradiated controls. The relative contribution of each of these mechanisms to the restoration of microvascular density observed in our study is uncertain. However, the lack of local angiogenesis in the radiated group suggests that the radiation-induced tissue hypoxia alone is not an adequate stimulus to restore vessel density. On the contrary, the introduction of a generalized hypoxic stimulus is necessary to initiate vessel growth. Our data indicate that at least two independent processes contribute to vessel growth. We hypothesize that inhibition or a shift in sensitivity of local angiogenic processes occurs following WBRT, whereas other processes induced by hypoxia (e.g., vasculogenesis) remain intact and are able to compensate for the effects of radiation.

**Pericyte outgrowth in the microvasculature.** Pericytes are a heterogeneous population of mural cells associated with the microvasculature (3, 34) having important roles in endothelial proliferation (45), blood-brain barrier integrity (19), contraction of capillaries, and regulation of capillary blood flow (43). Several different markers have been used to identify pericytes, including SMA (19, 21, 36, 37, 39), NG2 (21, 38, 49), desmin (21), endostatin (49), and PDGF-β (17, 38, 49). In this study, putative pericytes were identified by their morphology and expression of SMA, NG2, and PDGF-β, as well as their association to blood vessels with a diameter <10 μm. Pericytes have been shown to guide and precede proliferating endothelial cells during embryonic angiogenesis (49) and also function in stabilizing newly formed blood vessels, maintaining the endothelial cells in a quiescent state (32). In tumor vasculature, entire endothelial-free vessel tubes consisting only of pericytes have been observed (38). Importantly, blood vessel coverage by pericytes has been postulated to be key in understanding the nature of the blood vessel. The absence of SMA-positive pericytes covering endothelial cells has been suggested to indicate a “window of plasticity” for remodeling of the microvasculature (4). In addition, assessment of the ratio of pericytes to endothelial cells has been reported to show organ-specific distributions. Finally, the pericyte-to-endothelial cell ratio has been reported to be highest in the central nervous system (34), specifically the retina with a 1:1 ratio (45).

In this study, we found a significant increase in SMA capillary density in response to exposure to low oxygen levels. Also, when CD31 capillary density was expressed as a percentage of SMA capillary density, we found that the control group had <100% coverage under normoxic conditions but increased to >100% in hypoxic conditions. The increase in pericyte coverage with hypoxia treatment supports the conclusion that pericytes have a role in guiding the development of new vessel growth during angiogenesis. However, in the radiated group, 100% coverage of capillaries by SMA was observed under normoxic conditions and did not increase further with hypoxia. These observations suggest that pericyte growth remains intact after radiation and may stabilize blood vessels potentially as a protective mechanism. Although our data related to pericyte coverage are of interest, further research will be required to assess the significance of these findings.

In summary, WBRT results in capillary rarefaction in sub-regions of the hippocampus, a brain region highly associated with cognitive function. We have demonstrated that microvascular rarefaction post-WBRT induces local tissue hypoxia, but the hypoxic state within the tissue is not adequate to stimulate an angiogenic response. Nevertheless, a generalized stimulus of low ambient oxygen levels completely restores brain microvascular density. Our data suggest that WBRT inhibits angiogenesis through a yet to be determined local mechanism resulting in either a shift in the sensitivity for hypoxia-induced angiogenesis or a permanent impairment in local angiogenesis. Nevertheless, the cerebral vessel rarefaction can be overcome by a generalized hypoxic stimulus.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

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