Hypoxia and Hypoxia-Inducible Factor-1 Target Genes in Central Nervous System Radiation Injury: A Role for Vascular Endothelial Growth Factor

Robert A. Nordal,1 Andras Nagy,2 Melania Pintilie,3 and C. Shun Wong1

1Department of Radiation Oncology, Sunnybrook and Women’s College Health Sciences Center, 2Department of Pathology and Laboratory Medicine, University of Toronto, Toronto, Ontario, Canada.

ABSTRACT

Purpose: Microvascular permeability changes and loss of blood-brain barrier integrity are important features of central nervous system (CNS) radiation injury. Expression of vascular endothelial growth factor (VEGF), an important determinant of microvascular permeability, was examined to assess its role in CNS radiation damage. Because hypoxia mediates VEGF up-regulation through hypoxia-inducible factor-1α (HIF1α) induction, we studied the relationships of hypoxia, HIF1α expression, and expression of VEGF in this damage pathway.

Experimental Design: Expression of HIF1α, VEGF, and another hypoxia-responsive gene, glucose transporter-1, was assessed in the irradiated rat spinal cord using immunohistochemistry and in situ hybridization. Hypoxic areas were identified using the nitroimidazole 2-(2-nitro-1H-imidazole-1-yl)-(N-(2,2,3,3,3-pentafluoropropyl) acetamide. To determine the causal importance of VEGF expression in radiation myelopathy, we studied the response of transgenic mice with greater (VEGF-Ahi/H11545), reduced (VEGF-Alo/H11545), and wild-type VEGF activity to thoracolumbar irradiation.

Results: In rat spinal cord, the number of cells expressing HIF1α and VEGF increased rapidly from 16 to 20 weeks after radiation, before white matter necrosis and forelimb paralysis. A steep dose response was observed in expression of HIF1α and VEGF. HIF1α and VEGF expressing cells were identified as astrocytes. Hypoxia was present in regions where up-regulation of VEGF and glucose transporter-1 and increased permeability was observed. VEGF-Alo/H11545 mice had a longer latency to development of hindlimb weakness and paralysis compared with wild-type or VEGF-Ahi/H11545 mice.

Conclusions: VEGF expression appears to play an important role in CNS radiation injury. This focuses attention on VEGF and other genes induced in response to hypoxia as targets for therapy to reduce or prevent CNS radiation damage.

INTRODUCTION

Central nervous system (CNS) injury is a major dose-limiting toxicity in radiation therapy for cancer (1). White matter necrosis, demyelination, and vascular changes including increased permeability and blood-brain barrier (BBB) disruption are prominent features in CNS radiation injury (2). Histopathological changes and functional endpoints are well characterized in the rat radiation myelopathy model (3). Animals develop blood-spinal cord barrier (BSCB) disruption, white matter necrosis, and paralysis within 5 months after single doses of 19–25 Gy to the cervical spinal cord.

The BBB restricts the passage of soluble molecules from the circulation into the CNS and is believed to have a protective role. It is also a barrier to the trafficking of leukocytes and thus may protect the brain from inflammatory processes (4). The BBB is composed of the specialized endothelium of the CNS, astrocytes, and pericytes in immediate proximity to endothelium and the basement membrane. The special characteristics of brain capillaries are attributed to the tight junctions between endothelial cells and a paucity of endothelial vesicular transport. Disruption of the BBB is a consistent finding after high-dose radiation (5), and it is observed at both early (hours/days) and late (months) intervals after radiation (6–8). The mechanisms of radiation-induced BBB disruption are not understood.

Radiation-induced BBB disruption precedes development of tissue necrosis, suggesting the importance of the vascular endothelium and factors that affect permeability across the endothelial barrier. This led us to examine the expression of vascular endothelial growth factor (VEGF), a key determinant of vascular permeability. VEGF, also known as vascular permeability factor, was initially described as an agent that increased vascular permeability in the dermis and in tissue surrounding brain tumors (9, 10). VEGF-induced increases in microvessel permeability and edema have been demonstrated in normal brain (11, 12) and in brain microvessel endothelial cell monolayers in culture (13).

In our initial studies, we found radiation-induced increases in VEGF mRNA and protein in white matter. VEGF expression localized mainly to astrocytes, and the number of positive cells increased steeply with increasing doses above 18 Gy. Evidence of BSCB disruption and tissue hypoxia was present in regions of VEGF expression (14). This suggested that hypoxia may be involved in increased VEGF expression and permeability in this...
damage pathway. There is evidence that hypoxia can produce increased brain microvessel permeability in vivo and in vitro (15, 16). In this study, we hypothesized that hypoxia is a basis for induction of VEGF and other hypoxia-responsive genes and that VEGF plays a role in the mechanisms of CNS radiation injury. We examined the role of the hypoxia-induced transcription factor component HIF1α in up-regulation of hypoxia-responsive genes and studied the importance of VEGF in development of injury and loss of function.

Transmission of important signals induced by hypoxia is mediated by the transcription factor HIF1. HIF1 is a nuclear protein complex that binds to a consensus sequence in enhancers or promoters of target genes (17). The HIF1 heterodimer is composed of the basic helix-loop-helix Per ARNT SIM-domain proteins HIF1α and HIF1β (18). HIF1α is stabilized under hypoxia but is rapidly degraded under normoxia by the ubiquitin-proteasome system. By contrast, HIF1β is stable under normoxia (19). Strong HIF1-mediated up-regulation of VEGF occurs under hypoxia in a number of systems (20–22). Several HIF1-induced genes encode proteins that may be relevant in CNS injury responses, including VEGF, glucose transporter-1 (Glut-1), erythropoietin, heme-oxygenase-1, and several glycolytic enzymes including lactate dehydrogenase-A (23). Glut-1 is a facilitated glucose transporter that mediates glucose transport across the blood-brain barrier. Glut-1 activity in transport of glucose into neurons and glia may promote survival of hypoxic CNS tissue (24).

Areas of hypoxia were demonstrated in the rat by detection of the nitroimidazole EF5. We identified cells responding to hypoxia by HIF1α expression, and evaluated the dose-response, and spatial and temporal distribution of HIF1α, VEGF, and Glut-1 expressing cells to determine whether HIF1 provides the signal for expression of hypoxia-responsive genes in this system. We investigated the relationships between the distribution of hypoxia and HIF1-target genes and regions of albumin leakage attributable to BSCB disruption. In addition, we studied the causal importance of VEGF by determining the cellular effects of spinal cord irradiation in transgenic mice with altered VEGF expression. To do this, we used a clinically relevant mouse radiation myelopathy model that we characterized in preliminary experiments. Mice with reduced, wild-type, or increased VEGF activity were evaluated for development of hindlimb weakness and paralysis after thoracolumbar spinal cord irradiation. We hypothesized that these animals would manifest distinct susceptibilities to spinal cord injury after radiation.

MATERIALS AND METHODS

Animals. Female Fischer 344 rats were used in the studies on HIF1α, VEGF, and Glut-1 expression, tissue hypoxia, and BSCB disruption. Animals were 9–10 weeks old and weighed 150–175 grams at the time of irradiation. Animals were housed two to five animals per cage in the animal facility of the Ontario Cancer Institute under conditions in compliance with approved institutional protocols and the Canadian Council of Animal Care.

To examine the role of VEGF expression in vivo, transgenic mice that manifest altered VEGF expression were used in radiation myelopathy experiments. Mice heterozygous for a novel VEGF allele (VEGF-lac-Z-KI) were created in a CD-1/129 background using a targeting vector containing the bacterial β-galactosidase gene (Lac-Z) ligated to the 3’ untranslated region downstream of wild-type VEGF codons 4–8. Details of the generation and characterization of these animals were described previously (25, 26). Two functional proteins, VEGF and Lac-Z, are produced from the bicistronic allele, and Lac-Z activity serves as a surrogate for VEGF expression. Mice with two distinct phenotypes resulted from differences in the integration site of the targeting construct. These animals are viable as heterozygotes, thereby circumventing the lethality of VEGF haplo-insufficiency (VEGF −/− animals die in utero; Ref. 27). VEGF-Ahu/− animals have increased VEGF transcript stability and increased protein activity (26). VEGF-Ahu/− animals have a COOH terminus recombination-induced mutation in a VEGF splice site and reduced VEGF protein activity because of truncation of the terminal six amino acids. VEGF-Ahu/− animals have approximately 120% of wild-type VEGF activity under the stress of development versus 70% of wild-type activity in VEGF-Ahu/− animals.

The two transgenic strains were rederived by embryo transfer. Mice were genotyped by PCR amplification of specific DNA sequences and by detection of lac-Z activity using X-gal substrate staining. Primer sequences for PCR genotyping have been described previously (25, 26). The experimental cohorts were generated from heterozygote matings. Only female animals with nonambiguous genotyping were used in experiments. A total of 1070 pups were genotyped to produce the cohorts used in this study.

Irradiation

Rat Spinal Cord. In the rat myelopathy model, forelimb paralysis occurs five to seven months after single doses exceeding 18 Gy to a 1.8 cm cervical spinal cord segment. Single doses of 17, 18.7, and 20 Gy produce forelimb paralysis within 210 days in 0, 50, and 100% of animals (28). After 20–22 Gy, rats develop paralysis between 19 and 21 weeks. Areas of necrosis, with loss of axons, myelin, and glia are observed in white matter. BBB disruption precedes and is associated with tissue necrosis. Areas of necrosis are initially focal but coalesce and are prominent at 20 weeks after 22 Gy, coinciding with the onset of paralysis.

Rats received graded single fraction doses through anterior and posterior 100 kV X-ray beams to a 1.8-cm cervical spine field extending from C2-T2. Calibration of the X-ray unit output was performed, and doses at depth were calculated. Immobilization was achieved with a polystyrene foam jig and halothane anesthesia. Fields were confirmed by taking X-rays to check positioning.

Mouse Spinal Cord. A mouse model for radiation myelopathy was characterized in initial experiments in our laboratory. Higher doses are required to produce paralysis in mice compared with rats. Paralysis occurred in 50% of animals within 6 months after 55 Gy irradiation through a 1.2-cm aperture, confirming results from earlier reports (29, 30). A Lucite jig and halothane inhalation anesthesia were used for immobilization. A shaped field was used to irradiate the thoracolumbar spinal cord including T10 to L2, with shielding to prevent injury to the kidneys and bowel. Single doses of 55 and 75 Gy were given.
Twenty to 30 female animals (mean 26), 8 to 15 weeks old, were used in each dose group for each of the 3 mouse genotypes. Animals were scored at intervals for loss of hindlimb spread reflex when held inverted by the tail (score 1), hindlimb weakness (inability to suspend body weight by grasping cage rim when held inverted (score 2), or flaccid paralysis (score 3). An occasional animal developing urinary or fecal incontinence before paralysis was censored. The highest score reached by the day of censoring was recorded and used in the analysis.

Histopathology. Animals were anesthetized by i.p. injection of a mixture containing Atravet (Ayerst) 0.05 ml/100 grams body weight and containing ketamine (Vetrephearm) 0.1 ml/100 grams body weight. Animals were killed by transcardiac perfusion with 0.9% saline for 1 min, followed by perfusion with 10% neutral buffered formalin (formalin fixation) or 4% paraformaldehyde (frozen section fixation) for 10 min. The irradiated spinal cord segment was fixed further in 10% formalin for 36 h, followed by preparation of paraffin blocks by standard methods. For frozen section preparation, paraformaldehyde perfusion was followed by immersion in 4% paraformaldehyde in PBS for 24 h at 4°C, followed by 0.5% sucrose for 24 h at 4°C, and snap freezing in frozen section medium (Stephens Scientific) over liquid nitrogen. Slides were prepared by cutting tissues from the mid-plane of the irradiated segment at 3 μm of thickness (paraffin sections), or 5–20 μm of thickness (frozen sections). Except as noted, immunostaining was performed on formalin-fixed, paraffin-embedded sections. Immunostained and in situ hybridization slides were counterstained with H&E and 0.5% methylene green respectively.

Immunohistochemistry

Hypoxia and Hypoxia-Responsive Genes. HIFα expression was assessed using a mouse antirat HIF1α monoclonal antibody (NB100–123; Novus Biologicals, Inc., Littleton, CO). After endogenous peroxidase blocking (3% hydrogen peroxide, 10 min), and microwave antigen retrieval [20 min, high power; 10 mM citrate buffer (pH 6.0)], sequential 15 min incubations in avidin and biotin solutions were performed to block endogenous bovine tissue activity (Vector Labs Avidin/Biotin blocking kit). Sections were incubated with primary antibody at 1:200 dilution in Dako antibody-diluting buffer for 1–2 h, followed by incubation with biotinylated rabbit antimouse IgG (Vector Labs) for one-half h, streptavidin-horseradish peroxidase (HRP) conjugate (Signet ID Labs), and Nova Red chromogen (Vector Labs).

VEGF IHC was performed using a rabbit polyclonal IgG reactive with rat VEGF (SC-507; Santa Cruz Biotechnology). Microwave antigen retrieval and 3% H2O2 blocking was performed as described above, followed by blocking with 5% normal goat serum and incubation with the primary antibody at 1:200 dilution. Sections were incubated with a biotinylated secondary antibody, streptavidin-HRP and diaminobenzidine (DAB). Detection of the VEGF-vascular endothelial growth factor receptor (VEGFR-2 complex (activated VEGFR-2)) by IHC was used to evaluate VEGF activity in transgenic and wild type mouse strains. The monoclonal antibody GV39M, which displayed much greater affinity for receptor-bound VEGF than for free VEGF in competitive ELISA (31), was supplied by Drs. R. Brekken and P. Thorpe, University of Texas. Animals were sacrificed 9 weeks after 75 Gy. Antigen retrieval was performed with 0.4% pepsin digestion (pH 2) for 5 min at 42°C. Incubation with the biotinylated primary antibody at 1:100 dilution overnight was followed by streptavidin-HRP (Signet ID Labs), and color development using Nova Red substrate (Vector Labs).

Lac-Z activity was detected in transgenic mice using 3% H2O2 blocking, microwave antigen retrieval, blocking with normal mouse serum, and anti-β-gal antibody (Z378A; Promega) at 1:10,000 dilution. Incubation with an antimouse secondary antibody was followed by streptavidin-HRP, and DAB substrate. Lac-Z enzymatic activity was assessed by incubation of spinal cord tissue with 1 mg/ml Xgal substrate in N-N-dimethyl formamide, after brief (15 min) fixation in 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), 2 mM MgCl2, 2 mM sodium phosphate (pH 7.3).

Glut-1 IHC was performed on formalin-fixed sections after 3% H2O2 blocking, microwave antigen retrieval, and serum protein blocking reagent (Signet). A rabbit anti-Glut-1 primary antibody (Chemicon) reactive with rat Glut-1 was used at 1:40,000 dilution, followed by a goat antirabbit IgG at 1:200 dilution, streptavidin-HRP, and DAB substrate.

Cell Type Identification. Detection of glial fibrillary acidic protein (GFAP) was used to identify astrocytes. GFAP IHC was performed after 3% H2O2 treatment using a rabbit polyclonal antibody (Dako) diluted 1:100 in PBS. Slides were washed in PBS and incubated in goat antirabbit IgG conjugated to FITC (Jackson Immunoresearch Laboratories) at 1:100 dilution in PBS.

Ricinus communis agglutinin-1 lectin (RCA-1) labeling was used to identify microglia. Pepsin pretreatment was performed after 3% H2O2, followed by incubation with a biotin-RCA-1 conjugate at 1:1000 dilution (Vector Labs) for 1 h, incubation with streptavidin-HRP (Signet) for 30 min, and color development with DAB substrate or streptavidin-fluorescein for fluorescent detection.

Blood-Spinal Cord Barrier Disruption. BSCB disruption was demonstrated by detection of endogenous serum albumin in spinal cord tissue. Albumin is normally excluded from the CNS parenchyma but leaks across vascular walls in BSCB disruption. Slides were pretreated with 3% H2O2, 0.4% pepsin (pH 2) for 5 min and incubated with a polyclonal sheep antibody reactive with rat albumin (Biomed, Poole, United Kingdom) at 1:6400 dilution for 1 h. After washes, incubations with biotin-antishape IgG and streptavidin-HRP (Signet) were performed for 30 min each, followed by incubation with DAB.
**In Situ Hybridization.** Total RNA was isolated from rat spinal cord using RNeasy mini kit (Qiagen). cDNA was generated by reverse transcription using random primers (Life Technologies, Inc.). VEGF and VEGFR-specific primers were used to amplify the sequence of interest in a pool of cDNAs, and the resulting product was cloned into the pCR 3.1 vector for transcription of specific RNA probes. Primers, cDNA, and probe preparation, and hybridization conditions have been described previously (32, 33). Rat lung sections were used as a positive control.

![Fig. 1](image)

*Fig. 1* Expression of hypoxia-inducible factor-1α (HIF1α) and HIF1-target genes. HIF1α immunoreactivity (cells with reddish-brown staining, examples indicated by arrows) is detected in the irradiated rat spinal cord at 19 (A) and 20 (B) weeks after 20 Gy. Expression precedes and is seen around areas of developing white matter necrosis. Vascular endothelial growth factor (VEGF) mRNA expression (purple staining, examples indicated by arrows) detected by *in situ* hybridization (ISH) is seen in white matter in a pattern similar to HIF1α (E-G). Like HIF1α, expression of VEGF increased at 19 (E) and 20 (F) weeks after 20 Gy, as did glucose transporter-1 (Glut-1) immunoreactivity. [Glut-1 immunohistochemistry (IHC): 19 (I) and 20 (J) weeks after 20 Gy]. HIF1α (C) and VEGF (G) expression localized to nuclei/perinuclear areas (20 Gy, 20 weeks, high power; arrows indicate cells positive for the marker). Glut-1 expression in white matter (20 Gy, 20 weeks) localized to cells with glial morphology (K; high power). D and H show absence of marker-specific staining for HIF1α and VEGF (ISH), respectively, in nonirradiated spinal cord (20 weeks). Glial cells in H&E-stained sections (D) exhibit blue nonspecific staining only. In nonirradiated control sections, Glut-1 expression is seen in its usual distribution in microvessels only, with no areas of concentrated expression (L). Original magnifications, ×100; except C, G, and K (×650).
Quantification of VEGF and HIF1α Expression. Cells expressing HIF1α or VEGF were counted in transverse spinal cord sections. Three sections were counted per animal for a minimum of three animals at each dose and time point. Sections from rats sacrificed 20 weeks after 0–22 Gy was used to study the dose-response. Sections from animals sacrificed 0 to 20 weeks after 20 Gy were used to determine the time course of HIF1α and VEGF expression. The average number of positive cells per section and SEs were calculated for each dose and time point at ×200 magnification. In all IHC experiments, the procedures were repeated with omission of the primary antibody as a negative control. The no-treatment controls consisted of nonirradiated specimens. Normal adult rat small bowel served as negative control tissue for HIF1α, VEGF, and VEGFR IHC.

Statistics. Transgenic mouse myelopathy data were analyzed by Kaplan-Meier analysis with SAS software (SAS Institute Inc., Cary, NC). The log-rank test of significance was used to evaluate differences between groups.

RESULTS

Histopathology. H&E-stained spinal cord sections from rats sacrificed 16–24 weeks after irradiation showed no gross histological changes after single doses of 8–17 Gy. Progressive abnormalities in white matter were present in sections from animals receiving 18–22 Gy, beginning at 19 weeks. No pathology was evident in gray matter. Areas of necrosis, with cell loss, debris, and vacuolization, were distributed randomly throughout the white matter. Necrotic areas were generally focal at 19 weeks, and more extensive by 20 weeks. Areas of focal and confluent demyelination were also seen at these time points (data not shown). Inflammatory cells and infiltrates were absent. No gross vascular lesions were apparent. Examination of H&E-stained mouse sections from paralyzed animals showed areas of necrosis, scattered foci of demyelination, and a lack of inflammatory cells. The vasculature appeared normal, except for occasional evidence of vessel dilation.

HIF1α Expression. HIF1α immunoreactivity was first seen in white matter 16 weeks after 20 Gy. Some positive cells seen at 16 and 17 weeks were in histologically normal-appearing areas. At later times, most positive cells were near focal or confluent areas of necrosis in white matter (Fig. 1, A–C). HIF1α-positive cells had glial morphology. A nuclear staining pattern was observed (Fig. 1C). Only occasional HIF1α-positive cells were seen in gray matter. HIF1α-positive cells were not seen in nonirradiated tissues (Fig. 1D). The number of HIF1α expressing cells per section increased markedly at 19 and 20 weeks after 20 Gy (Fig. 2A) and demonstrated a steep dose response at 20 weeks (Fig. 2B).

VEGF Expression. In situ hybridization revealed up-regulation of VEGF in white matter but not in gray matter (Fig. 1, E–G). The staining localized to the nuclear area (Fig. 1G). A few scattered cells were observed in histologically normal-appearing white matter, but most positive cells were located around areas of demyelination or necrosis. There were no positive cells in nonirradiated sections (Fig. 1H) or sense strand control sections (data not shown). The number of VEGF-positive cells per spinal cord section increased rapidly in the weeks immediately preceding paralysis (Fig. 1, E and F). VEGF expressing cells were first seen 17 weeks after 20 Gy (Fig. 2C), were not seen below a threshold dose of 18 Gy, and increased sharply at higher doses (Fig. 2D) to approximately 120–130 cells/section 20 weeks after 22 Gy. These in situ hybridization data were in agreement with previous data for VEGF protein expression present by immunohistochemistry (IHC) is first detected 16 weeks after 20 Gy (A) versus 17 weeks for VEGF (C). The steep dose-response in the number of cells expressing VEGF [D, VEGF in situ hybridization (ISH)] parallels that for HIF1α (B) at 20 weeks. Error bars represent SEs of mean. Sections were obtained from three to four animals for each dose and time point (three or more sections per animal).
expression obtained using IHC (33), showing the same distribution, timing, and relationship to developing areas of tissue damage. In situ hybridization using our own probe was more consistent from experiment to experiment in our more recent work, compared with IHC using antibodies from an external supplier. In situ hybridization also exhibited less background, which aids in counterstaining and assessment against other markers.

**Glucose Transporter-1 Expression.** Nonvascular Glut-1 expression was present 19–20 weeks after 20 Gy in areas of white matter damage in the spinal cord (Fig. 1, I–K). Expression was only seen after myelopathic doses of ≥19 Gy. This increased expression was seen in white matter and localized to cells with glial morphology (Fig. 1K). The expected vascular Glut-1 immunostaining was seen in both white and gray matter of nonirradiated spinal cord (Fig. 1, I–L), and no change in this component of expression after 18–22 Gy was apparent.

**Identification of HIF1α and VEGF Expressing Cells.** Dual IHC for HIF1α and GFAP or RCA-1 was used to identify cells responding to hypoxia with HIF1α expression. For sections obtained 20 weeks after 22 Gy, 43% of HIF1α-positive cells were GFAP positive (Fig. 3, A and B). There was no apparent difference in the proportion of cells positive for GFAP in different regions of white matter. No expression of the microglial marker RCA-1 was detected in HIF1α-positive cells (data not shown). Similar to our previous report (33), most VEGF expressing cells obtained 20 weeks after 22 Gy were GFAP positive (data not shown). Dual fluorescent IHC for HIF1α and VEGF was combined with 4,6-diamidino-2-phenylindole nuclear staining to determine whether cells coexpressing both markers were present. Cells expressing both proteins were identified in white matter sections obtained 20 weeks after 22 Gy, numbering approximately 3–5 per spinal cord section (Fig. 3, C–E). Because of the technical challenges of this technique, this may be an underestimate of the true number of dual-positive cells.

**Relationships among Hypoxia, HIF1-Target Gene Expression, and BSCB Disruption.** To study the spatial relationship between regions with increased vascular permeability, hypoxia, and hypoxia-responsive gene expression, we examined these processes in serial 5–6 μm sections in the rat spinal cord. Most regions showing albumin leakage attributable to BSCB breakdown had some evidence of hypoxia and of VEGF and Glut-1 expression in adjacent sections. Examples are shown in Fig. 3, where staining for EF5 (F), albumin (G), VEGF (H), and Glut-1 (I) colocalize in adjacent spinal cord white matter frozen sections 20 weeks after doses of 22 Gy. Whereas EF5 detection identifies hypoxic regions, HIF1α expression reveals a key cellular response to hypoxia and the presence of a key inducer of hypoxia-responsive genes. Cells responding to hypoxia by HIF1α expression were also found to colocalize with BSCB disruption, VEGF expression, and Glut-1 expression in adjacent sections of the same spinal cord region (Fig. 3, J–M). Regional expression of in situ hybridization signal for VEGF and Glut-1 IHC was also demonstrated (Fig. 3N) in single spinal cord sections.
Radiation Myelopathy in Mice with Altered VEGF. The irradiated spinal cord of adult VEGF-Lac-Z-transgenic mice showed evidence of lac-Z expression, as revealed by staining with the lac-Z substrate Xgal (Fig. 4A), and by IHC for Lac-Z protein (Fig. 4B). Lac-Z-positive cells with glial morphology were seen in white matter. Activated VEGFR-2 (VEGFR-2-bound VEGF) was detected using the antibody GV39M. Cytoplasmic staining was observed in cells with glial morphology 9 weeks after 75 Gy in sections from VEGF-Alo/H11001, VEGF-Ahi/H11001, and wild-type animals (Fig. 4, D–F). The staining was extranuclear, with an appearance similar to that observed for other markers expressed in glial cytoplasmic extensions. Sections from VEGF-Alo/H11001 animals had less intense staining and a narrower margin of staining around positive cells compared with wild-type and VEGF-Ahi/H11001 animals. This may reflect decreased VEGF activity and a decrease in activated VEGFR-2 in VEGF-Alo/H11001 animals. Wild-type animals exhibited intermediate staining intensity, although the immunoreactivity of some cells overlapped that in VEGF-Alo/H11001 animals.

Transgenic mice were followed 74–325 days after a dose of 55 or 75 Gy to the thoracolumbar spine. VEGF-Alo/H11001 mice with reduced functional VEGF showed a longer latency for radiation-induced hindlimb paralysis and weakness compared with wild-type mice. A longer latency was also seen compared with VEGF-Ahi/H11001 mice (Fig. 5, A-D). The VEGF-Alo/H11001 versus wild-type comparisons reached significance by log-rank test for 75 Gy, scores 1, 2, and 3 and for 55 Gy, score 1, with a trend for longer latency for 55 Gy, score 2 (P = 0.07). VEGF-Ahi/H11001 versus wild-type comparisons did not yield significant differences for any dose or score. Log-rank P's and median times to event are shown in Tables 1 and 2.

DISCUSSION
In a previous study, we showed that areas of hypoxia develop in white matter in association with paralysis in the irradiated rat spinal cord (14). This suggested a role for hypoxia in inducing gene expression changes important in injury. In the present study, cells responding to hypoxia were identified by their up-regulation of HIF1α, and a progressive increase in HIF1α expression in the weeks before paralysis was demon-
detection and associated variability, the time of initial expression of is observed (7). Given the small number of positive cells at initial weeks after 22 Gy irradiation, when significant BSCB breakdown the number of cells positive for both HIF1 and VEGF exhibited a steep dose response above 17 Gy. Interestingly, half-maximum value at about 19 Gy, which is the ED 50 for fore -

VEGF-A hi/lo expression was present in the period 16–20 weeks after 22 Gy irradiation, when significant BSCB breakdown is observed (7). Given the small number of positive cells at initial detection and associated variability, the time of initial expression of HIF1α and VEGF can be considered coincident. Both HIF1α and VEGF exhibited a steep dose response above 17 Gy. Interestingly, the number of cells positive for both HIF1α and VEGF reaches a half-maximum value at about 19 Gy, which is the ED50 for fore-limb paralysis caused by white matter necrosis in the rat myelopathy model (3).

VEGF-A hi/lo mice had longer latency to development of weakness and paralysis compared with wild-type animals. Weakness and paralysis (scores 2 and 3) represent objective measures of significant loss of function and are thus the impor-
tant endpoints in the mouse model. Loss of hindlimb reflex (score 1) is a subjective assessment, and its clinical relevance is less clear. Our data suggest that attenuated VEGF activity may be protective in development of CNS radiation injury. The lack of detrimental effect with VEGF activity beyond wild-type levels may be related to saturation of this response. The mechanisms of VEGF-mediated permeability increases are not understood, and we will have to learn more about possible mechanisms before this can be explained in more detail.

Fig. 4 Characterization of vascular endothelial growth factor (VEGF)-lac-Z transgenic mice. Transgenic mice with a modified VEGF allele express the VEGF-lac-Z transcript as adult animals. This is apparent in spinal cord segments stained for lac-Z activity using the lac-Z substrate Xgal, which stains the tissues blue. No staining is seen in wild-type (VEGF-A hi/lo) animals. (A, top to bottom, VEGF-A hi/hi, VEGF-A hi/lo, VEGF-A lo/lo, 15 weeks after 55 Gy; ×20 magnification). Expression of lac-Z is also evident by lac-Z immunohistochemistry (IHC) in spinal cord sections from transgenic animals (B, VEGF-A hi/lo, 75 Gy, 9 weeks) but not in sections from wild-type animals (C, VEGF-A lo/lo, 75 Gy, 9 weeks). Many lac-Z-positive glial cells (reddish-brown-stained cells) are seen in white matter (original magnifications, ×400). IHC with an antibody specific to the VEGF-vascular endothelial growth factor receptor-2 complex (GV39M) reveals staining in cytoplasmic processes of glial cells (brown-staining arrows, D, E, F, VEGF-A hi/lo, VEGF-A hi/hi, VEGF-A lo/lo, respectively). All sections were obtained 9 weeks after 75 Gy. Decreased staining area and intensity was observed in spinal cord sections from VEGF-A hi/lo animals (D) compared with VEGF-A hi/hi (E) and wild-type animals (F; original magnifications, ×1000).

Approximately 43% of HIF1α-positive cells were identified as astrocytes. Only cells with clearly evident GFAP expression were counted as astrocytes, and therefore this may be a conservative estimate of the proportion comprising that subpopulation. Future methods allowing detection of GFAP and RCA-1 with greater sensitivity might identify more cells as astrocytes or microglia, respectively. In our earlier study, 74% of VEGF expressing cells were astrocytes, and 4% were microglia (14). In this study, individual cells expressing both HIF1α and VEGF were present. HIF1α and VEGF expression was present in the period 16–20 weeks after 22 Gy irradiation, when significant BSCB breakdown is observed (7). Given the small number of positive cells at initial detection and associated variability, the time of initial expression of HIF1α and VEGF can be considered coincident. Both HIF1α and VEGF exhibited a steep dose response above 17 Gy. Interestingly, the number of cells positive for both HIF1α and VEGF reaches a half-maximum value at about 19 Gy, which is the ED50 for fore-limb paralysis caused by white matter necrosis in the rat myelopathy model (3).

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VEGF-A hi/lo mice had decreased immunoreactivity for the VEGF-VEGFR-2 complex compared with VEGF-A hi/lo or wild-type mice. This may represent a reduction in activated VEGFR-2 in VEGF-A hi/lo animals that is relevant to the observed response. VEGFR-2 binding is of interest because the vast majority of receptor-mediated activities demonstrated for VEGF have been attributed to VEGFR-2, rather than VEGFR-1 (34). The difference in immunoreactivity observed was in the intensity and area of stained cytoplasm. This is potentially quantifiable with digital image analysis, but this would not add greatly to the information obtained. Glial VEGF-VEGFR localization is not surprising, because glial VEGFR expression has been demonstrated in the CNS in other studies (35, 36) as well as in our own experiments. We did not observe endothelial expression of VEGFR-1 or -2,4 nor endothelial localization of
Fig. 5 Radiation-induced hindlimb weakness and paralysis in vascular endothelial growth factor (VEGF)-lac-Z transgenic mice. Event-free probability curves (5, A and B) show time to onset of hindlimb weakness (score 2) in VEGF-A\textsuperscript{+/+}, VEGF-A\textsuperscript{hi/+}, and VEGF-A\textsuperscript{lo/+} mice after 75 and 55 Gy, respectively. VEGF-A\textsuperscript{lo/+} mice show prolonged latency to development of weakness compared with VEGF-A\textsuperscript{+/+} (P < 0.05 and 0.07 for 75 and 55 Gy, respectively). Latency to injury is also longer compared with VEGF-A\textsuperscript{hi/+} animals. A similar relationship is seen in time-to-onset of paralysis (C and D, score 3 and 5). The onset of paralysis was shifted to later times for VEGF-A\textsuperscript{lo/+} mice compared with VEGF-A\textsuperscript{+/+} at 75 Gy (P = 0.0006). A similar trend was seen for 55 Gy.
VEGF-bound VEGFR-2. This is in agreement with prior findings that endothelial VEGF receptor is usually undetectable or at most weakly expressed in adult brain (35, 36). There is evidence that mechanisms of VEGF-mediated permeability that do not depend on receptor binding may be important (37).

Glut-1 is a blood-brain barrier protein important in glucose transport necessary for physiological energy demands in the CNS. Its expression accompanies maturation of the blood-brain barrier in the developing brain (38). In the rat brain, Glut-1 expression has been localized to astrocytes, as well as endothelial cells (39). Increased Glut-1 expression in areas of BSCB disruption and hypoxia may represent an adaptive response in meeting cellular energy demands. VEGF and Glut-1 showed similar temporal and spatial patterns of expression. This is consistent with their coinduction under hypoxia. Up-regulation of other HIF1-target genes may be observed in future studies. Whereas some hypoxia-induced genes may have negative (VEGF) or homeostatic (Glut-1) effects, others such as erythropoietin and heme oxygenase-1 have shown neuroprotective potential (40).

Increased expression of HIF1-target genes has been shown to parallel up-regulation of HIF1α in other CNS injuries. Expression of Glut-1 and several glycolytic enzymes colocalized with HIF1α in a rat middle cerebral artery occlusion model (23). Coexpression of HIF1α and VEGF mRNA was described in a mouse model of focal ischemia (41). In our study, the parallel dose-response, spatial, and temporal relationships of HIF1α and HIF1-target genes are evidence supporting induction of VEGF and Glut-1 by hypoxia, as opposed to induction through other mechanisms, against a background of hypoxia (and EF5 staining). The observed expression of both HIF1α and VEGF by individual cells in the neuropil suggests that an autocrine regulatory pathway may apply.

Different cell types have been found to up-regulate HIF1α in response to hypoxia in various types of CNS injury. In global cerebral ischemia after cerebral artery ligation in the rat, HIF1α immunoreactivity localized to neurons of the hippocampus and cortex. Dual immunostaining showed that some neurons expressed both HIF1α and VEGF (42). In a rat model of chronic hypoxia induced through reduced inspired O2, neurons, astrocytes, ependymal cells, and endothelial cells displayed HIF1α expression, and VEGF and Glut-1 showed a similar induction (43). In focal brain ischemia induced by middle cerebral artery occlusion in the rat, there was up-regulation of HIF1α and HIF1-target genes in all ischemic areas, with a concentration of expression in areas dense in neurons (23). These results and our own data suggest damage-specific patterns of expression.

Expression of HIF1α and VEGF was seen in association with areas of BSCB disruption. Whereas gross vascular lesions after radiation are inconspicuous in the rat and mouse models, increased vascular permeability clearly occurs. This suggests that the mechanisms of radiation-induced permeability do not depend on structural breakdown of the vessel wall (7). Attention may instead be directed at factors that impact permeability and BSCB competence by altering endothelial cell function. These may include stimuli from the microenvironment or substances released by adjacent cells. Growth factors and other molecules such as cytokines released by astrocytes are important in diverse CNS injuries (44). VEGF has been shown to increase permeability in the CNS (12, 45), and our data suggests that it may have a role in permeability changes after radiation. The mechanisms of VEGF-mediated permeability increases are not clear from the literature or our studies. There is evidence that important effects of VEGF may occur through mechanisms that do not depend on receptor binding (37). These may include effects on tight junction proteins (46–48) or on adhesion molecules important in barrier properties such as intercellular adhesion molecule 1 (12, 49).

Endothelial cell density was found to decrease beginning at 90 days after irradiation (7) at a time when focal disruption of BSCB was first observed. Clonogenic death of endothelial cells may well be the initiating event for BSCB disruption. The present data, however, suggest a model where a component of secondary damage contributes to the ultimate injury. This is consistent with current views on CNS injury that development of damage is a dynamic process featuring interaction of different cell types and the microenvironment (50). In the irradiated rat

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Median times to event scores 1, 2, and 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparisons</td>
<td>55 Gy</td>
</tr>
<tr>
<td>Score 1</td>
<td>VEGF-A hi/ vs. VEGF-A lo/</td>
</tr>
<tr>
<td></td>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
</tr>
<tr>
<td>Score 2</td>
<td>VEGF-A hi/ vs. VEGF-A lo/</td>
</tr>
<tr>
<td></td>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
</tr>
<tr>
<td>Score 3</td>
<td>VEGF-A hi/ vs. VEGF-A lo/</td>
</tr>
<tr>
<td></td>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
</tr>
<tr>
<td></td>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
</tr>
</tbody>
</table>

*VFG, vascular endothelial growth factor.

**Table 1** Log-rank Ps for event-free probability estimates

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>55 Gy</th>
<th>75 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 1</td>
<td>0.004</td>
<td>0.0090</td>
</tr>
<tr>
<td>VEGF-A hi/ vs. VEGF-A lo/</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
<td>0.01</td>
<td>0.0009</td>
</tr>
<tr>
<td>Score 2</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VEGF-A hi/ vs. VEGF-A hi/</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Score 3</td>
<td>0.006</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* VEGF, vascular endothelial growth factor; NS, not significant.

1. Score 1, loss of hindlimb spread reflex when animal suspended by tail.
2. Score 2, severe weakness (inability to grasp cage rim with hindlimbs).
3. Score 3, flaccid hindlimb paralysis.
spinal cord, we postulate that once the barrier is disrupted after endothelial cell death or damage, hypoxia and VEGF up-regulation may be involved in a secondary cycle of damage (14). Increased permeability may result in hypoxic areas from decreased perfusion, edema, and increased interstitial fluid pressure. Glial cells, predominantly astrocytes, respond to hypoxia by production of HIF1α. This results in expression of HIF1-target genes, including VEGF. VEGF up-regulation produces a further increase in vascular permeability and exacerbates BSCB disruption. Interrupting this secondary cycle of damage may serve as a neuroprotective strategy against CNS radiation injury.

ACKNOWLEDGMENTS

The suggestions and critical insights of Drs. Richard Hill and Abhijit Guha are gratefully acknowledged. Technical assistance of James Ho in immunohistochemistry was invaluable. The expertise of Dr. Duncan Galbraith in calibration and calculation of radiation doses was essential in performing the mouse myelopathy experiments. The suggestions and critical insights of Drs. Richard Hill and Abhijit Guha are gratefully acknowledged. Technical assistance of James Ho in immunohistochemistry was invaluable. The expertise of Dr. Duncan Galbraith in calibration and calculation of radiation doses was essential in performing the mouse myelopathy experiments.

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