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Cerebrovascular Remodeling and Neuroinflammation is a Late Effect of Radiation-Induced Brain Injury in Non-Human Primates

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Abstract

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Fractionated whole-brain irradiation (fWBI) is a mainstay of treatment for patients with intracranial neoplasia; however late-delayed radiation-induced normal tissue injury remains a major adverse consequence of treatment, with deleterious effects on quality of life for affected patients. We hypothesize that cerebrovascular injury and remodeling after fWBI results in ischemic injury to dependent white matter, which contributes to the observed cognitive dysfunction. To evaluate molecular effectors of radiation-induced brain injury (RIBI), real-time quantitative polymerase chain reaction (RT-qPCR) was performed on the dorsolateral prefrontal cortex (DLPFC, Brodmann area 46), hippocampus and temporal white matter of 4 male Rhesus macaques (age 6–11 years), which had received 40 Gray (Gy) fWBI (8 fractions of 5 Gy each, twice per week), and 3 control comparators. All fWBI animals developed neurologic impairment; humane euthanasia was elected at a median of 6 months. Radiation-induced brain injury was confirmed histopathologically in all animals, characterized by white matter degeneration and

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necrosis, and multifocal cerebrovascular injury consisting of perivascular edema, abnormal angiogenesis and perivascular extracellular matrix deposition. Herein we demonstrate that RIBI is associated with white matter-specific up-regulation of hypoxia-associated lactate dehydrogenase A (LDHA) and that increased gene expression of fibronectin 1 (FN1), SERPINE1 and matrix metalloprotease 2 (MMP2) may contribute to cerebrovascular remodeling in late-delayed RIBI. Additionally, vascular stability and maturation associated tumor necrosis super family member 15 (TNFSF15) and vascular endothelial growth factor beta (VEGFB) mRNAs were increased within temporal white matter. We also demonstrate that radiation-induced brain injury is associated with decreases in white matter-specific expression of neurotransmitter receptors SYP, GRIN2A and GRIA4. We additionally provide evidence that macrophage/microglial mediated neuroinflammation may contribute to RIBI through increased gene expression of the macrophage chemoattractant CCL2 and macrophage/ microglia associated CD68. Global patterns in cerebral gene expression varied significantly between regions examined ($P < 0.0001$, Friedman's test), with effects most prominent within cerebral white matter.

INTRODUCTION

Approximately 200,000 people per year receive fractionated whole-brain irradiation (fWBI) for the treatment of primary or metastatic intracranial neoplasia (1), and late-delayed normal tissue injury is a major deleterious consequence. Fifty to 70% of patients that survive long enough to develop late-delayed RIBI (>6 months) develop higher order cognitive dysfunction that negatively affects quality of life (2, 3). Additionally, a subset of patients will develop ataxia, urinary incontinence and profound dementia (4) characterized by memory impairment and behavioral abnormality. Multifocal cerebrovascular and white matter injury is associated with this loss of function (5–7), and extent of white matter injury is correlated with degree of cognitive impairment (8–10). There is currently no effective treatment and the pathogenesis is unclear.

Studies evaluating mechanisms of late-delayed radiation injury in humans are confounded by the concurrent presence of neoplasia, treatment with chemotherapeutics, and comorbid factors such as hypertension and diabetes mellitus (11, 12). Rodents are often used as a model for humans, but differ neuroanatomically [e.g., they possess greater capillary network density and a significantly lower white:gray matter ratio(13, 14)]. Higher order cognition is difficult to evaluate in these species. Furthermore, rodents do not develop white matter necrosis after fWBI (15, 16), as do humans. We and others (7, 17–19) have demonstrated that Rhesus macaques (*Macaca mulatta*) that receive fWBI (total dose of 40–80 Gy, 2–5 Gy per fraction) develop cerebrovascular and white matter lesions which are spatially, temporally and morphologically similar to those observed in humans with late-delayed RIBI (19). Furthermore, these irradiated animals developed progressive cognitive dysfunction (20), particularly at high cognitive workloads, and thus serve as a more human-like alternative to rodent models. Moreover, nonhuman primates (NHPs) possess greater neuroanatomic and genetic similarity to humans, which may increase translatability of findings (21).

In the current study, we hypothesize that radiation-induced cognitive dysfunction is partially mediated by abnormal cerebrovascular remodeling, resulting in subsequent ischemia to dependent brain parenchyma. To identify potential molecular effectors which may contribute late-delayed RIBI, we conducted RT-qPCR on 92 genes associated with neuroinflammation, angiogenesis, cerebrovascular remodeling, blood-brain barrier integrity, neurotransmission and extracellular matrix deposition. We anticipated increases in mRNAs associated with inflammation, angiogenesis and extracellular matrix deposition, and decreases in those associated with blood-brain-barrier integrity, neurotransmission and vascular maturation and stability.

MATERIALS AND METHODS

Brain Tissue and Subjects

Archived frozen and formalin fixed paraffin embedded brain tissues from 7 adult male Rhesus macaques (6–11 years old, 5.3–8.7 kg) were used in this study for molecular and histologic analyses, respectively. Whole-brain irradiated animals (n = 4, aged 5.6–10.7 years, 7.5–8.7 kg) received 40 Gy fWBI 4–7 months prior to necropsy as part of a previous experiment. Housing, diet and experimental procedures are detailed in Hanbury *et al.* (19). Control comparators (n = 3, aged 5.5–6.6 years, 5.3–8.3 kg) had been part of an unrelated study in which they had received single dose, 10 Gy thoracic irradiation and subcutaneous injections of vehicle [1 mL SQ, sterile phosphorus-buffered saline(PBS)], twice daily for 4 months, ceasing 4 months prior to necropsy. Housing, diet, euthanasia and tissue collection were identical to that of the irradiated group. Both experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee (IACUC). The Wake Forest University School of Medicine is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and conforms to all state and federal animal welfare laws.

Irradiation

Fractionated whole-brain irradiation procedures are as described by Hanbury *et al.* (19) and Robbins *et al.* (20). Briefly, NHP were sedated with ketamine HCl (15 mg/kg body weight, intramuscularly) and maintained on isoflurane gas (3% induction, 1.5% maintenance) in 100% oxygen during irradiation. A total of 40 Gy mid-plane was given (8 fractions $\times$ 5 Gy/fraction; 2 fractions/week $\times$ 4 weeks; nominal dose rate of 4.9–5.4 Gy/min) using a clinical linear accelerator and opposed lateral fields of 6MV X rays.

Control comparators received 10 Gy, single-dose thoracic irradiation delivered to the anterior-posterior thoracic mid-plane as part of a previous study. X rays (6 MV) were delivered with parallel opposed anterior-posterior fields from a clinical linear accelerator at a nominal dose rate of 4 Gy/min. The field of radiation included the heart, mediastinum and superior, lateral and inferior lung fields extending up to 4 cm caudal to the xyphoid. The maximum dose administered to the brain in thorax-only irradiated animals is estimated to be 0.1 Gy.

Clinical and Cognitive Assessment

Animals were assessed daily by trained technical staff during the study period; any animal developing impairment received veterinary neurologic evaluation, and assessment using a standardized cage-side neurologic checklist. Humane euthanasia was provided for animals with severe neurologic impairment. All whole-brain irradiated animals (n = 4) underwent daily cognitive assessment during the study period, using a delayed-match-to-sample task (DMS), the procedure for which has been described previously (20).

Tissue Collection, Preparation and Histopathology

Four to 7 months after irradiation, the animals were humanely euthanized in accordance with the American Veterinary Medical Association's Guidelines on Euthanasia (22) by deep anesthesia with pentobarbital, followed by exsanguination and perfusion of the vascular system with 2 liters of cold normal saline. The entire brain was removed intact, placed in a stainless-steel brain matrix with cutting guides, and sectioned coronally in 4 mm intervals. Once removed from the matrix, all slices were photographed, and then alternating sections either immediately frozen on dry ice or immersed in 4% cold paraformaldehyde for 24 h. Fixed brain tissues were transferred to cold 70% ethanol solution until embedding.

Fixed brain tissues from 8 standard regions were then selected and trimmed in accordance with Society for Toxicologic Pathology's Recommended Practices for Sampling and Processing the Nervous System (23), with the addition of prefrontal cortex, and embedded in paraffin and sectioned coronally at 4 μ m. Sections were stained with hematoxylin and eosin and examined histologically by a board certified veterinary pathologist (JMC). Lesions were scored as described elsewhere (19); absent (0), minimal (1 = inflammatory or vascular changes without disruption of the neuropil or clear neuronal loss); mild (2 = focal vascular injury and inflammation with loss of neuropil or neurons and microglial activation); moderate (3 = extensive or multifocal vascular injury, hemorrhage, disruption of the neuropil, neuronal loss and microglial activation); or severe (4 = extensive or multifocal vascular injury, hemorrhage, disruption of the neuropil, neuronal loss and microglial activation, with additionally extensive zones of necrosis within neural tissue). The presence of vascular lesions in each region was recorded as follows: a: endothelial hypertrophy; b: perivascular extracellular matrix deposition, c: perivascular edema, d: disorganized vascular morphology. The assessor was blinded to the irradiation status of the animals.

Molecular Assessment of Cerebral Gene Expression

Relative gene expression was evaluated by RT-qPCR within 3 brain regions [dorsolateral prefrontal cortex (Brodmann area 46), hippocampus and temporal white matter] from all animals. Ribonucleic acid (RNA) was isolated by homogenization of 60–120 mg of brain tissue in TRI Reagent (Molecular Research Center, Inc.; Cincinnati, OH), followed by phase separation with 1-bromo-3-chloropropane (BCP). RNA was purified by RNeasy Mini kit (Qiagen; Valencia, CA). RNA content and purity was determined at 260 nm, and with 260/280 ratios between 2.0 and 2.2, as obtained by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE). Absence of genomic DNA contamination was confirmed during RT-qPCR by RT² qPCR Primer Assay for Rhesus Macaque Genomic DNA (gDNA) Contamination, (Qiagen) and run concurrently with all samples. Amplification for all

samples was absent or below the limit of detection (>35 Ct). cDNA was synthesized by RT² First Strand kit (Qiagen; Valencia, CA); reaction efficiency was confirmed by reverse transcriptase control within each plate and run concurrently with all samples.

RT-qPCR was performed on an ABI 7500 Fast system (Applied Biosystems), using a Qiagen Custom RT2 Profiler Array utilizing Rhesus macaque specific primers. Plates were designed to assess 92 target genes, selected for their roles in inflammation, angiogenesis, blood-brain barrier integrity, extracellular matrix deposition, neurotransmission, hypoxia and DNA damage repair (Table 1). Standard nomenclature as designated by the HUGO Gene Nomenclature Committee (HGCN) (25) is utilized.

Calculation of Changes in Gene Expression

For each sample, the threshold cycle (Ct) for the gene of interest and the selected housekeeping genes were determined. The limit of detection was set to 35 Ct. Results for claudin-3 and S1PR4 were excluded from statistical analyses as Ct values were below the threshold of detection for all samples. Target gene expression was normalized to the geometric mean of two control genes (β -actin and hypoxanthine-guanine phosphoribosyltransferase) in each sample; the $Ct \{Ct(\text{target gene}) - Ct[\text{housekeeping gene(s)}]\}$ was calculated to quantitate relative gene expression. Fold change was calculated by 2^{-Ct} as described by Livak (26).

Statistics

Statistical analyses were performed under the guidance of a professional statistician (JAT) and in Statistica 13 (StataCorp., College Station, TX) unless otherwise noted. Ct values were used for all comparisons. The effect of brain region on relative gene expression was evaluated by Friedman's test in GraphPad Prism software [version 6.07 for Windows (www.graphpad.com)] followed by paired *t* tests or Wilcoxon signed rank for parametrically and nonparametrically distributed data, respectively. Gene expression with respect to irradiation status was stratified by region and evaluated as follows: Normality and equality of variances were assessed by Shapiro-Wilk and Levene's tests, respectively. Nonparametric data were compared by Mann-Whitney U test. Normally distributed data were compared by Student's *t* and Welch's tests for data with equal and unequal variances, respectively. Fold changes ± 2 and *P* values <0.01 were considered significant to adjust for the small sample size. As these data were considered preliminary analyses, no adjustments for multiple comparisons were made.

RESULTS

Clinical Parameters

All four whole-brain irradiated animals were euthanized during the study period (4.3–6.6 months postirradiation, mean: 5.6 months) due to the development of severe neurologic signs (Table 2). Concurrent unilateral inner ear infection precluded interpretation of the clinical signs in NHP1, as impaired vestibular function secondary to otitis media/interna could not be clearly distinguished from a central nervous system lesion affecting the vestibular system. Neurologic deficits in the remaining three animals were attributed to late-

delayed radiation-induced brain injury, as no other inciting cause or evidence of local or systemic disease was identified clinically or histopathologically.

As control comparators had received single high-dose thoracic irradiation as part of a previous study, clinical data regarding cardiac and respiratory function were reviewed prior to use of archived tissues. Neurologically these animals were normal. Respiratory rate, heart rate, pulse oximetry and ejection fraction as determined by echocardiography were within normal limits until euthanasia.

Histopathology

All fWBI animals demonstrated histopathologic lesions consistent with late-delayed RIBI, including white matter degeneration, necrosis and cerebrovascular injury consisting of perivascular edema with or without protein deposition, perivascular fibrosis, endothelial hypertrophy and disorganized angiogenesis (Fig 1). Individual animals differed in lesion severity and distribution of vascular morphologic abnormality (Table 3), although forebrain white matter was affected in all animals examined.

Regional Effects of Radiation on Gene Expression

The effect of radiation on relative gene expression differed with respect to region ($P < 0.0001$, Friedman's test); further analyses were stratified by region. No significant change in hippocampal gene expression ($P > 0.01$ for all genes examined) was associated with late-delayed RIBI. Mean fold changes were of the greatest magnitude within temporal white matter (−19-fold to 49-fold).

Deposition of Extracellular Matrix

RIBI was associated with increased expression of fibronectin 1 (FN1: DLPFC: 3.2-fold up-regulated, $P < 0.01$; WM: 4.6-fold up-regulated, $P < 0.0001$), Serpin E1 (SERPINE1: WM: 20.8-fold up-regulated, $P < 0.0006$) and matrix metalloproteinase 2 (MMP2: DLPFC: 6.5-fold up-regulated, $P < 0.003$; WM: 34.6-fold up-regulated, $P < 0.001$), factors associated with extracellular matrix deposition and remodeling (Fig. 2). No significant changes were observed in expression of TGFB1, nidogen 1, HSPG2, tissue plasminogen activator, or any of the laminins, tissue inhibitors of matrix metalloproteinases and other matrix metalloproteinases evaluated.

Angiogenesis, Vascular Maturation and Stabilization

The relative expression of factors associated with vascular stability and survival [tumor necrosis factor super family member 15 (TNFSF15: 44.4-fold up-regulated, $P < 0.003$) and vascular endothelial cell growth factor beta (VEGFB: 2.3-fold up-regulated, $P < 0.002$)] was increased within white matter in late-delayed RIBI (Fig. 3). Radiation had no significant effect on other members of the vascular endothelial cell growth factor (VEGF) family (VEGFA, VEGFC, VEGFD or associated receptors KDR and FLT1). No significant changes were seen in other molecules associated with angiogenesis and vascular maturation and stabilization [angiopoietin 1 or 2 (ANGPT1, ANGPT2), DLL4, FIGF, midkine (MDK), NOTCH3, PDGFB, PDGFRB, sphingosine-1-phosphate receptors 1-5, TEK or WNT1].

Neuroinflammation

Relative expression of macrophage/microglia associated CD68 and CCL2 was up-regulated within both dorsolateral prefrontal cortex (CCL2: 30.5-fold up-regulated, $P < 0.002$) and temporal white matter (CCL2: 49.0-fold up-regulated, $P < 0.003$; CD68: 27.8-fold up-regulated, $P < 0.005$) (Fig 4B). Endothelial associated PECAM1 and lymphocyte associated CD3G expression was also increased within temporal white matter (PECAM1: 4.8-fold up-regulated, $P < 0.0005$; CD3G: 23.2-fold up-regulated, $P < 0.0003$) (Fig. 5A and B). fWBI had no significant effect on the relative gene expression of inflammatory mediators IL6, AIF1, IFNG, TNF, COX2, NOS2, IL10, NFKB1, transferrin (TFRC) or B-cell associated MS4A1.

Hypoxia

Expression of LDHA (2.9-fold up-regulated, $P < 0.004$) and FAS (12.7-fold-upregulated, $P < 0.0004$) was increased within white matter in irradiated animals (Fig. 5C and D). No significant changes were observed in the relative expression of hypoxia-inducible factor family members (HIF1a, EPAS1, ARNT), erythropoietin (EPO) or heme oxygenase 1 or 2 (HMOX1, HMOX2).

Neurotransmission

RIBI was associated with decreased expression of neurotransmission associated synaptophysin (SYP: 3.4-fold down-regulated, $P < 0.004$), glutamate receptor, ionotropic, AMPA4 (GRIA4: 6.7-fold down-regulated, $P < 0.007$) and glutamate ionotropic receptor NMDA type subunit 2A (GRIN2A: 6.7-fold down-regulated, $P < 0.01$) in white matter (Fig. 6). There was no significant effect on the expression of GRIA2 and GRIN2B.

Blood-Brain Barrier Structural Integrity

Late-delayed RIBI did not affect the relative gene expression of astrocyte-associated GFAP, AQP4, Connexin 43 or Agrin. No significant effect was observed on relative gene expression of endothelial cell–cell junction associated VE-Cadherin, B-Catenin, zona occludens 1 and 2, claudins 5 and 12, occludin, and junctional adhesion molecules 1, 2 and 3. Expression of claudin 3 was below the level of detection in all samples. fWBI did not affect the relative gene expression of channel associated KCNJ10, KCNJ8, ABCB1 or ABCC9.

DNA Damage

No significant changes were observed in any of the markers examined associated with DNA damage and repair [CHEK1, CHEK2, Nibrin (NBN) and H2AFX].

DISCUSSION

These data reaffirm that non-human primates develop multifocal cerebrovascular and white matter lesions in late-delayed RIBI that are consistent with those observed in human patients. As the primary clinical manifestation of late-delayed RIBI in patients is progressive cognitive decline (2, 3), the parent study for all whole-brain irradiated animals ($n = 4$) included daily cognitive assessment as used previously (20). As neurological decline

necessitated humane euthanasia prior to experimental endpoint, cognitive data were incomplete and full statistical analysis of cognition for the animals in the present cohort was not possible. Task measured cognitive decline preceded ataxia observations; however, as the ataxia developed, animals stopped working the task. Previous data have demonstrated working memory impairment beginning four months postirradiation (20).

Performance on this DMS task is reliant on processing by both the prefrontal cortex (rule based) and hippocampus (working and/or short-term); thus the impaired cognitive function cannot be localized to either brain region solely on the basis of DMS task outcome. Rather, impaired performance on this task necessitates error analysis of the behavioral task and/or further experimental manipulations to determine the mechanism of impaired performance. In other studies, extra-hippocampal injury (prefrontal cortical lesioning) alone is sufficient to impair performance on the DMS task (27), and electrophysiology demonstrates that inter-laminar processing by the DLPFC is required for successful execution of the task (28). These injuries suggest that NHPs err when following the wrong task rule, as directed by the DLPFC, rather than incorrectly remembering due to hippocampal processing. This, in conjunction with our demonstrated regional patterns in gene expression, challenges the postulation that hippocampal-sparing irradiation alone will be sufficient to spare cognitive function after fWBI (29, 30).

Our gene expression data reveal that whole-brain irradiation does not result in significant changes in hippocampal gene expression for our selected targets. Rather, fWBI results in patterns of gene expression indicating that cerebrovascular remodeling, neuroinflammation and alterations in neurotransmission contribute to late-delayed RIBI, and that these changes most prominently affect white matter.

Our molecular data indicate that fibronectin 1, MMP2 and SERPINE1 may contribute to vascular remodeling in RIBI and influence the deposition of perivascular extracellular matrix. Fibronectin, exuded in soluble form from the vascular compartment as a component of blood plasma or secreted by endothelial cells, is a major component of extracellular matrix following cell-mediated assembly, and its expression is up-regulated in a number of fibrotic diseases (31, 32). Additionally, fibronectin triggers the activation of microglia *in vitro* (33), and thus may stimulate macrophage/microglial activation in RIBI. Fibronectin is also increased in the cortex (34) and plasma (35) of humans with clinical and subclinical Alzheimer's disease. The cortical deposition of fibronectin may play a role in the pathology of cognitive dysfunction.

Both fibronectin and SERPINE1 expression may be influenced by Rho/ROCK signaling (36, 37), suggesting a common pathway of induction. Increased SERPINE1 expression in late-delayed RIBI is known to contribute to perivascular fibrosis through the inhibition of fibrinolysis (38, 39) and stimulation of fibronectin matrix assembly through interaction with $\alpha v\beta 5$ and $\alpha 5\beta 1$ integrins (40). Furthermore, endothelial cell death in radiation-induced intestinal injury is dependent on SERPINE1 expression (41), which serves as a potential explanation for the decreases in microvascular density observed following fWBI in rat studies (16, 42).

Increased expression of the gelatinase MMP2 is associated with blood-brain barrier disruption in rodent models of focal transient cerebral ischemia (43), leukemia (44), and pneumococcal meningitis (45), and in human brain tissue affected by cerebral amyloid angiopathy-associated hemorrhagic stroke (46). Additionally, MMP2 expression and activity is increased in rodents receiving single high-dose whole-brain irradiation (10 Gy) and fWBI (40 Gy, 8 fractions $\times$ 5 Gy/fraction) and associated with the degradation of type IV collagen (47), which may contribute to blood-brain barrier breakdown and perivascular edema in RIBI. In addition to the potential of MMP2 to degrade extracellular matrix, increased expression and activation contributes to cardiac, hepatic and renal fibrosis (48–50). We postulate that MMP2 contributes to perivascular fibrosis in RIBI by remodeling the deposited substrate.

The perivascular deposition of extracellular matrix components, including fibronectin, may create a diffusion barrier and induces ischemic injury to the dependent brain parenchyma. We propose that cerebral white matter is most vulnerable to this ischemic injury, as the cerebrovascular architecture transitions from larger bore penetrating arterioles and venules to a predominately capillary network within white matter (51) and capillary density is decreased relative to gray matter (52). Our hypothesis is supported by the white matter-specific increased relative expression of LDHA, an enzyme which catalyzes the conversion of L-lactate to pyruvate in the final step of anaerobic glycolysis. While there were no significant changes in relative gene expression among members of the hypoxia-inducible factor family (HIF1A, EPAS, ARNT), regulation of these factors most commonly occurs at the protein level (53, 54), which may explain lack of change at the transcriptomic level. Additionally, hypoxia induces the expression of both SERPINE1 (55) and fibronectin (56, 57), and thus hypoxia may potentiate perivascular fibrosis in RIBI. Hypoxia is known to induce the expression of cell-surface death receptor Fas (58), however the white matter-specific up-regulation of Fas is difficult to interpret as it is involved in many physiologic processes necessitating apoptosis.

As histologic evaluation of tissue revealed disorganized vasculature with reactive nuclei, reminiscent of endothelial proliferation, we anticipated increased expression of markers associated with the induction of angiogenesis. On the contrary, we found no increase in markers associated with new blood vessel formation, but increased expression of VEGFB and TNFSF15, factors involved in vascular quiescence and stability. VEGFB, unlike other members of the vascular endothelial growth factor (VEGF) family, does not induce the formation of new blood vessels, but is critical for the survival and maintenance of newly formed and preexisting vasculature (59). Additional evidence from rodent studies suggests that VEGFB participates in vascular repair after brain injury (60). Circulating TNFSF15 also increased after brain injury, and greater blood levels of TNFSF15 as compared to VEGFA was associated with higher rates of recovery in patients (61). Furthermore, rodent studies demonstrated that administration of TNFSF15 to mice in an experimental model of traumatic brain injury resulted in decreased neuroinflammation, and increased blood-brain barrier integrity with decreased permeability (62). Further characterization of these tissues including assessment of microvascular area fraction is required to meaningfully interpret the alterations in angiogenic gene expression; however, we postulate that increased expression

of VEGFB and TNFSF15 may represent an adaptive response to ongoing cerebrovascular injury.

Neurotransmission-associated synaptophysin, GRIA4 and GRIN2A are most prominently decreased within irradiated white matter. This finding contradicted our initial hypothesis that decreases in markers associated with synapses and neurotransmission would be most prominent within the cortex and hippocampus, where the neuron soma and synapses themselves reside. We propose these decreases in expression reflect a loss of deep white matter synapses, sites in which axons communicate directly to NG2⁺ oligodendrocyte progenitor cells (OPCs) via vesicular release of the neurotransmitter glutamate (63–65). Whether the observed reduction in GRIN2A and GRIA4 mRNAs is a reflection of axonal loss or a reduction in number of NG2⁺ precursor cells requires further investigation, particularly in nonhuman primates. Rodent data are conflicting. Studies completed in rats demonstrate that after initial depletion, NG2⁺ OPC populations in irradiated spinal cord begin proliferating by 2 weeks after single high-dose irradiation (66), with recovery to near normal numbers by 6 weeks (66, 67) to 6 months (68). In contrast, studies in mice demonstrate that OPC populations are less severely affected after irradiation, but lack the capacity to repopulate the irradiated area (69). While both astrocytes and NG2⁺ precursors express GRIA4 (70, 71), the observed decreased relative expression is not presumed to be associated with decreases in total astrocyte number, as GFAP, AQP4 and AGRN were not significantly altered. We additionally infer from these data that disruption of the blood-brain barrier is less likely to be due to transcriptionally mediated alterations of astrocyte associated integrity.

Instead, the increased expression of CCL2 and CD68, in conjunction with the aggregates of macrophages/microglia observed on histopathology and infiltration of parenchyma by IBA-1 positive cells in our previous work (19), indicates that RIBI occurs in an environment of macrophage/ microglial mediated neuroinflammation. Increased expression of CCL2 directly induces the proliferation and activation of microglia (72, 73), and further drives neuroinflammation by stimulating the recruitment of circulating monocytes into the CNS parenchyma (73). CD68 expression, in addition to its historical use as a marker of macrophages and microglia, is also associated with the phagocytic capacity of a cell (74). Accordingly, the white matter specific increase in CD68 expression may correlate to the histologically observed necrosis, a process necessitating phagocytosis and debris removal. Furthermore, enhanced phagocytic capacity is associated with microglial activation(75) that in turn produces a number of pro-inflammatory cytokines (76–78), which may contribute to a sustained pro-inflammatory milieu.

In addition to macrophage/microglial activation and monocyte recruitment, CCL2 increases the expression and surface localization of PECAM1 within endothelial cells (79). Further studies in cell culture and mouse models demonstrate that PECAM1 facilitates the endothelial transmigration of leukocytes, including CD3⁺ T-lymphocytes (80–82). Thus, increased expression of PECAM1 may contribute to the lymphocytic perivascular aggregates observed histologically in RIBI (83, 84), which we believe correspond to the increased expression of CD3G observed in this study. We do not believe that the concurrent otitis media/interna in one animal affected neuroinflammatory gene expression, as the infection

did not extend within the cranial vault and no outliers were detected during statistical analysis. Further characterization of the neuroinflammatory contributors, including localization, quantification and immunophenotyping is pending and will be the focus of our future studies.

While these data provide insight into transcriptional changes in radiation-induced brain injury, further characterization of these factors at both the transcriptional and protein level is required to determine cell of origin and tissue compartment distribution. Additionally, we acknowledge that these analyses are correlative and do not necessarily indicate causation, but indicate potential molecular effectors and provide insight on possible pathogenic mechanism. Further study is required to elucidate the complex role of these molecules in radiation-induced brain injury; however, the factors presented may represent potential targets for therapeutic intervention.

We also acknowledge that the tissues in the current study were acquired from young adult NHP and do not fully recapitulate the aged patient population (approximate age: 60 years) (85–87) receiving fWBI. Retrospective analysis of patient populations indicate that aged patients receiving fWBI have poorer survival than younger patient subpopulations (88, 89), although these results are confounded by tumor progression, treatment regimen, concurrent medical illness, and neurologic comorbidity. Data regarding the neuroinflammatory effects of fWBI on the aging brain are conflicting (15, 90–92) and warrant additional study.

Finally, although gene targets were categorized according to function, we recognize that the selected targets do not behave independently and may contribute to several physiologic processes. Nonetheless, these are the first reported cerebral gene expression changes from NHP receiving a clinically relevant course of fractionated whole-brain irradiation, and set the groundwork for future studies into the molecular mechanisms underlying RIBI and associated cognitive impairment.

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FIG. 1.

Cerebrovascular pathology in late-delayed radiation-induced brain injury. Histopathology showing the spectrum of vascular injury in animals with late-delayed radiation-induced brain injury. Panel A: Normal arteriole from a nonirradiated animal. Panel B: Perivascular accumulation of brightly eosinophilic protein and fibrin. Panel C: Disorganized angiogenesis characterized by haphazardly arranged, tortuous small caliber vessels, often lacking distinct lumina. Endothelial nuclei are enlarged with prominent nucleoli. Panel D: Accumulation of fibrous extracellular matrix surrounding arterioles. Reactive (gemistocytic) astrocytes are numerous within the surrounding parenchyma. Panel E: Pericapillary fibrous extracellular matrix, similar to that in seen in panel D.

FIG. 2.

Changes in gene expression associated with extracellular matrix deposition. Late-delayed radiation-induced brain injury was associated with up-regulated expression of MMP2 (35-fold), SERPINE1 (21-fold) and fibronectin1 (threefold) within white matter and MMP2 (sixfold) and fibronectin1 (threefold) within dorsolateral prefrontal cortex. Bars denote mean fold change, individual points represent gene expression values for each animal. Dashed line indicates twofold-expression change. DLPFC: Dorsolateral prefrontal cortex; HC: Hippocampus; WM: Temporal white matter. * P value indicates with the effect of radiation on relative-gene expression and # P value signifies differences in regional gene expression with respect to irradiation status

FIG. 3.

Up-regulated relative expression of factors associated with vascular maturation and stability. Expression of TNFSF15 and VEGFB mRNAs was up-regulated within temporal white matter in late-delayed radiation-induced brain injury, 44-fold and twofold, respectively. Bars denote mean fold change, individual points represent gene expression values for each animal. Dashed line indicates twofold-expression change. DLPFC: Dorsolateral prefrontal cortex, HC: Hippocampus, WM: Temporal white matter. **P* value indicates with the effect of radiation on relative-gene expression and #*P* value signifies differences in regional gene expression with respect to irradiation status

FIG. 4.

Evidence of macrophage/microglial mediated neuroinflammation. Panel A: High-magnification image of focal white matter necrosis with axonal degeneration, mineralization and infiltration by macrophages/microglia. Panel B: Late-delayed radiation-induced brain injury was associated with increased gene expression of CD68 (28-fold) and CCL2 (49-fold) within temporal white matter. Expression of CCL2 was also increased within dorsolateral prefrontal cortex CCL2 (31-fold up-regulated). Bars denote mean fold change, individual points represent gene expression values for each animal. Dashed line indicates twofold-expression change. DLPFC: Dorsolateral prefrontal cortex, HC: Hippocampus, WM: Temporal white matter. **P* value indicates with the effect of radiation on relative-gene expression and #*P* value signifies differences in regional gene expression with respect to irradiation status

FIG. 5.

Additional changes in gene expression. Panel A: Relative gene expression of endothelial inflammation associated PECAM1 was 4.8-fold increased within temporal white matter. Panel B: Expression of T-lymphocyte associated CD3G was 23.2 increased within white matter. Panel C: Hypoxia associated LDHA expression was 2.9-fold increased within temporal white matter. Panel D: Late-delayed radiation-induced brain injury was associated with a 12.7-fold increase in relative gene expression of cell surface death receptor FAS in temporal white matter. Bars denote mean fold change, individual points represent gene expression values for each animal. Dashed line indicates twofold-expression change. DLPFC: Dorsolateral prefrontal cortex, HC: Hippocampus, WM: Temporal white matter. **P* value indicates with the effect of radiation on relative-gene expression and #*P* value signifies differences in regional gene expression with respect to irradiation status

FIG. 6.

Decreases in gene expression associated with neurotransmission. Late-delayed radiation-induced brain injury was associated with decreased expression of neurotransmission associated synaptophysin (−3.4-fold), and glutamatergic neurotransmitter receptor mRNAs for GRIA4 (−6.7-fold) and GRIN2A (−6.7-fold) within white matter. Bars denote mean fold change, individual points represent gene expression values for each animal. Dashed line indicates twofold-expression change. DLPFC: Dorsolateral prefrontal cortex, HC: Hippocampus, WM: Temporal white matter. **P* value indicates with the effect of radiation on relative-gene expression and #*P* value signifies differences in regional gene expression with respect to irradiation status

TABLE 1

Classification of Selected Markers in RT-qPCR Profiler Gene Expression Array

HGNC symbol	Blood-brain barrier structural integrity and transporters	Angiogenesis, vascular maturation and stability	Neuroinflammation	Hypoxia	DNA damage	Neurons and neurotransmission	Extracellular matrix deposition and remodeling
ABCB1		ANGPT1	AIF1	ADM	CHEK1	DCX	FN1
ABCC9		ANGPT2	CCL2	ARNT	CHEK2	GRIA2	HSPG2
AGRN		DLL4	CD34	EPAS1	H2AFX	GRIA4	LAMA4
AQP4		FIGF	CD3G	EPO	NBN	GRIN2A	LAMA5
CDH5		FLT1	CD68	FAS		GRIN2B	LAMB2
CLDN12		KDR	IFNG	HIF1A		SYP	LAMC1
CLDN3		MDK	IL10	HMOX1			MMP10
CLDN5		NOTCH3	IL1B	HMOX2			MMP12
CTNNB1		PDGFB	IL6	LDHA			MMP2
F11R		PDGFRB	MS4A1				MMP3
GFAP		S1PR1	NFKB1				NID1
GIA1		S1PR2	NOS2				PLAT
JAMB		S1PR3	PECAM1				SERPINE1
JAMC		S1PR4	PTGS2				TGFB1
KCNJ10		S1PR5	TFR3				TIMP2
KCNJ8		TEK	TNF				TIMP3
OCLN		TNFSF15					
SLC2A1		VEGFA					
TJP1		VEGFB					
ZO-2		VEGFC					
		WNT1					

TABLE 2

Summary of Clinical Neurological Deficits in Irradiated NHP

Subject ID	Age (years)	Onset (months postirradiation)	Neurologic signs	Survival interval (months postirradiation)
NHP1	10.7	4.4	Ataxia, dysphagia	5.3
			Concurrent mastoiditis and otitis media/interna	
NHP2	5.7	4.0	Visual impairment (incomplete menace response and impaired object tracking)	4.3
			Progressive ataxia and lethargy	
NHP3	6.8	5.0	Ataxia, weakness and intermittent head-pressing	6.0
NHP4	5.6	5.5	Decreased dexterity, progressive ataxia	6.6

TABLE 3
Assessment of White Matter Necrosis and Cerebrovascular Remodeling in Late-Delayed Radiation-Induced Brain Injury

Animal ID	NHP1		NHP2		NHP3		NHP4	
	Necrosis	Vascular	Necrosis	Vascular	Necrosis	Vascular	Necrosis	Vascular
Region/structure								
Forebrain: white matter	+++	<i>a,b,d</i>	++	<i>a,b</i>	++++	<i>a,b,d</i>	++	<i>b,c</i>
Forebrain: cortical gray matter	++	<i>a,d</i>	+	<i>a,b,d</i>	++	<i>a,d</i>	-	-
Prefrontal cortex	-	-	-	-	++	<i>a,d</i>	-	-
Basal ganglia/striatum	+	-	-	-	-	-	-	-
Hippocampus	-	-	+	<i>a,b,d</i>	-	-	-	-
Thalamus	na	na	++++	<i>a,b,c,d</i>	++++	<i>a,b,d</i>	-	-
Midbrain	++++	<i>b</i>	++	<i>a,d</i>	++	<i>a,b,d</i>	-	-
Cerebellum	+	-	-	-	+	-	-	-
Brainstem	+++	-	++	<i>a,b</i>	+++	<i>b</i>	na	na
Spinal cord	na	na	+	<i>b</i>	+	-	-	-

Note: na = not assessed, tissue unavailable for analysis.

^aEndothelial hypertrophy.

^bPerivascular extracellular matrix deposition.

^cPerivascular edema.

^dDisorganized vascular morphology.