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Pathology of Fractionated Whole-Brain Irradiation in Rhesus Monkeys (*Macaca mulatta*)

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Abstract

Fractionated whole-brain irradiation (fWBI), used to treat brain metastases, often leads to neurologic injury and cognitive impairment. The cognitive effects of irradiation in nonhuman primates (NHP) have been previously published; this report focuses on corresponding neuropathologic changes that could have served as the basis for those effects in the same study. Four rhesus monkeys were exposed to 40 Gy of fWBI [5 Gy×8 fraction (fx), 2 fx/week for four weeks] and received anatomical MRI prior to, and 14 months after fWBI. Neurologic and histologic sequelae were studied posthumously. Three of the NHPs underwent cognitive assessments, and each exhibited radiation-induced impairment associated with various degrees of vascular and inflammatory neuropathology. Two NHPs had severe multifocal necrosis of the forebrain, midbrain and brainstem. Histologic and MRI findings were in agreement, and the severity of cognitive decrement previously reported corresponded to the degree of observed pathology in two of the animals. In response to fWBI, the NHPs showed pathology similar to humans exposed to radiation and show comparable cognitive decline. These results provide a basis

for implementing NHPs to examine and treat adverse cognitive and neurophysiologic sequelae of radiation exposure in humans.

INTRODUCTION

Each year, approximately 200,000 individuals receiving radiotherapy for primary and metastatic brain tumors will survive long enough (>6 months) to suffer from radiation-induced brain injury (1) and 70% will do so within two years of treatment (2). Though injury and cognitive decrements are commonly observed results of brain irradiation, both the extent of damage and how quickly it presents depend largely on total dose, dose per fraction (fx), number of fractions, dose rate, host factors and radiation quality (3). It is difficult to study the cognitive and physiologic effects of ionizing radiation in the human brain, as damage caused by the neoplasms being treated is often confounding (4). Moreover, individuals receiving radiotherapy often either have been, or are currently being treated with chemotherapeutic drugs (1). Thus, rodents are frequently employed in studying radiation effects. One disadvantage to this model is that rodents lack anatomic and physiological similarity to humans (5), which limits the translation of the findings [e.g., rodents have a different white matter vascular architecture than primates (6)]. Thus, NHPs are a logical alternative for studying the effects of radiation. Early studies characterized the pathologic and histologic response of rhesus macaques to fWBI at varying dose levels (7, 8), however cognition has not been evaluated in detail in the same NHPs.

Previously, Robbins *et al.* (9) reported the cognitive responses for a group of three NHPs that received 40 Gy fWBI in 8 fx of 5 Gy, given twice per week, a dose-fractionation regimen shown in rats to produce both cognitive (10) and histopathologic effects (11) similar to that which is seen in humans. Fourteen months after irradiation, the NHPs were euthanized and studied histopathologically. We report here the corresponding histologic and neurobiologic sequelae of fWBI in these same three animals. A fourth NHP is also reported here that was irradiated and cognitively assessed with the previous three but was euthanized for humane reasons prior to the end of the study.

MATERIALS AND METHODS

Subjects

Four adult, male rhesus monkeys (*Macaca mulatta*, age 6–9 years, weight 8–12 kg) housed at the Wake Forest School of Medicine (WFSM) were used in this study. The NHPs were individually housed in stainless-steel cages in temperature and humidity-controlled rooms with a 12 h light/dark cycle. They were fed commercially available monkey chow (Purina, St. Louis, MO) supplemented daily with fresh produce. Because these NHPs were administered cognitive tasks in which the reward for a correct response was a sip of juice, they were water regulated for the duration of testing in accordance with the WFSM Institutional Animal Care and Use Committee (IACUC) Policy on Food and Water Regulated Animals. Experimental procedures were approved by the WFSM IACUC, and animals were under veterinary care throughout the study. WFSM 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

The NHPs were sedated with ketamine HCl (15 mg/kg body weight, IM), transported by handcart to the linear accelerator, and administered isoflurane gas (3% induction, 1.5% maintenance) in 100% oxygen was used during irradiation. Animals were placed in a supine position with the chin extended and secured in a curved head and neck support. They received 40 Gy of 6 MV X rays in eight 5 Gy fx (2 fx per week for four weeks) from a clinical linear accelerator at a nominal dose rate of 4 Gy/min. Dose was specified at the sagittal midplane of the brain (nominal depth = 4.5 cm, nominal midplane diameter = 9 cm), in the center of each radiation field. Cylindrical alloy (lead, tin, bismuth) eye-blocks were used to shield both eyes for each fraction delivered (9). The central axis of each beam was placed at the outer canthi for zero divergence of each beam into the contralateral eye (see Fig. 1). The olfactory region of the brain, which is prominent in NHPs and extends anteriorly between the eyes, was also shielded by the eye-blocks. Each field was imaged immediately prior to irradiation with an electronic portal imaging device to confirm correct radiation field geometry. Irradiation planning, dose computation procedures, and routine linear accelerator quality assurance procedures were the same as the methods used for human whole-brain radiation treatments and according to national quality assurance recommendations (12).

Imaging

Structural brain images (T1-weighted) were acquired using a 3.0 Tesla MRI scanner (GE Medical Systems, Milwaukee, WI) one day prior to the first fWBI fraction, and within two weeks prior to euthanasia. For one animal requiring additional diagnostic imaging, a multi-slice computed tomography (CT) scanner (GE Healthcare, Milwaukee, WI) was used to provide axial, noncontrast anatomic images with 5 mm slice thickness. Animals also received PET scans [data reported previously (9)]. Anesthesia for imaging procedures was as for irradiation.

Cognitive and Clinical Assessments

The task (delayed match-to-sample; DMS) providing cognitive assessment was described in detail previously (9). Animals were tested daily by trained technical staff for over a year prior to and for one year after fWBI, with neurologic examinations performed by veterinarians during this time for any animal showing signs of impairment. Euthanasia was provided for animals showing clinical impairment unresponsive to supportive care over this time period.

Preparation of Brain Tissue and Histopathology

Fourteen months after fWBI, the NHPs were humanely euthanized following the American Veterinary Medical Association's Guidelines on Euthanasia (13), by deep anesthesia with pentobarbital (to effect, IV), followed by exsanguination and perfusion of the vascular system with 2 L of cold normal saline. The entire brain was removed, and sectioned

coronally at 4 mm intervals using a Plexiglas brain matrix with cutting guides. All slices were photographed, and alternating slices were either quick frozen on dry ice or fixed in 4% cold paraformaldehyde. Each fixed coronal slice was then embedded in paraffin, sectioned coronally at 4 μ m, stained with hematoxylin and eosin, and examined histologically by a board-certified veterinary anatomic pathologist (JMC). Histologic lesions were scored as 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). Abnormalities noted on MRI were mapped to the corresponding histology. Sections from each subject containing frontal cortex, basal ganglia/striatum, hippocampus, thalamus, midbrain, cerebellum and brainstem were selected for further immunohistochemistry. Tissues were stained for neuron-specific enolase (NSE) (mouse monoclonal, 1:200; Leica Biosystems, New Castle, UK), Factor VIII (FVIII) (rabbit polyclonal, 1:50; Zymed Laboratories, Burlingame, CA), glial fibrillary acidic protein (GFAP) (rabbit polyclonal, 1:2,000; Millipore, Billerica, MA) and ionizing calcium binding adapter molecule-1 (IBA-1) (mouse monoclonal, 1:2,000; Millipore). A streptavidin-linked alkaline phosphatase or peroxidase secondary enzyme-linked reagent was used, and stained cells were visualized using Vector Red alkaline phosphatase substrate kit (Vector Labs, Burlingame, CA) or diaminobenzidine (Fisher Scientific, Pittsburgh, PA). Selected sections were also stained for intratissue hemosiderin (Prussian blue stain) and fibrin (Phosphotungstic acid-hematoxylin).

RESULTS

Clinical Findings

One of the four NHPs (herein referred to as NHP-A) displayed clinical signs in the fourteen months after fWBI, ataxia being the most prominent, but also difficulty swallowing and maintaining a standing posture. A diagnostic CT scan showed fluid filling the inter-trabecular spaces of the mastoid process (interpreted as mastoiditis) and the inner ear (interpreted as unilateral otitis interna). Ceftriaxone antibiotic therapy (500 mg, SID) and pain medication (ketoprofen 5 mg/kg, SID) were largely ineffective, and humane euthanasia was elected (five months post fWBI). Otitis media/interna was confirmed by histology. Although vestibular signs were not observed, this animal's data were excluded from prior cognitive analysis due to uncertainty regarding the effect of the ear infection on cognitive performance. No other NHPs displayed significant outward clinical symptoms, and the remaining animals survived to the end of the study at 14 months post fWBI.

Cognition

Changes in performance of the cognitive task have been previously reported (9). Briefly, cognitive function declined over time with increasing cognitive load (increasing number of distractors). This was accompanied by diminished FDG-uptake in forebrain, thalamic and

basal ganglia regions normally activated by DMS. In NHP-A, assessment was discontinued at four months post fWBI because physiological impairment prevented task performance.

MR Imaging and Pathologic Findings

All animals had one or more T1-enhancing lesions consistent with focal vascular injury (Table 1); two animals had multifocal lesions consistent with vascular injury in the midbrain and forebrain. In NHP-A and NHP-C, multifocal hemorrhages were grossly visible, corresponding, in NHP-A, to lesions detected by MRI (Fig. 2); smaller lesions were also apparent at the gross level that were not visible by MRI or CT. NHP-C had further multifocal hemorrhages not seen by MRI, but apparent grossly at necropsy. NHP-A was euthanized at five months post fWBI; the other animal was clinically normal until the scheduled study end 14 months after irradiation. Pathologic examination revealed abnormalities in the brains of all four fWBI-treated NHPs (Table 1 and Figs. 2–5). The brain regions most severely affected were the forebrain white matter, midbrain and brainstem, with sparing of the hippocampus. Lesions were multifocal, consisting of acute and chronic vascular degenerative changes, primarily affecting small-bore arterioles (in both gray and white matter) throughout the forebrain, midbrain and brainstem. The distribution of lesions across animals is summarized in Table 1.

Histologic findings included both acute and chronic changes in the brain, consisting of the following: arteriolar degeneration with perivascular edema and PTAH-positive fibrin deposition (Fig. 5C); acute hemorrhage effacing the neuropil (Fig. 5H); chronic hemorrhage evidenced by accumulation of hemosiderin in activated macrophages (brown pigment, Fig. 5A–C); abnormal vascular thickening and reduplication demonstrated as clustered Factor VIII-related antigen-positive channels (Fig. 5G) with perivascular deposition of fibrous connective tissue; infarction with cavitation and mineralization of the parenchyma; white matter vacuolation; focal to extensive microglial activation with formation of IBA1-positive activated microglia (Fig. 5F); and reactive gemistocytic changes in astrocytes adjacent to chronic lesions, demonstrated by expression of glial fibrillary acidic protein (GFAP) (Fig. 5E). One animal (NHP-C) had extensive regional necrosis of the brain. Minor degrees of secondary demyelination (Wallerian degeneration) were evident in white matter tracts throughout the brain. Major histologic findings are shown in Figs. 3–5. NHP-A and -C showed the greatest severity and showed corresponding cognitive decrement (Table 1).

DISCUSSION

The current study revealed that significant injury and neurodegeneration occurred in fWBI-treated NHPs five to 14 months after exposure. The pattern and character of the lesions suggest a spatially stochastic, ongoing, vascular pathogenesis, with predominant forebrain white matter and gray matter damage (Table 1). The damage observed was mirrored by the time course of cognitive decline, the details of which were previously reported (9). Forebrain damage often results in a decline in cognitive performance (14), and the DMS task used in this study is a sensitive indicator of such injury to frontal cortical areas including white and gray matter as shown here (15, 16). Histologic and imaging data revealed that forebrain white matter and cortical gray matter suffered the greatest injury relative to other

brain regions. Structures in other areas such as the hippocampus showed little or no gross or histologic damage, in contrast to fWBI studies in rodents (e.g. 17, 18), which may reflect the dissimilarity between rodent and primate brains; alternatively, future quantitative cellular analysis of the hippocampal formation may reveal injury that was not detected by our histologic examination. Compared to the complex primate brain, the rat brain is lissencephalic and the hippocampus occupies a larger percentage of the brain area [8.64% (19)] than in macaque NHPs [0.41–0.43% (20)] or humans [0.40% (21)].

Previous research has shown that the effects of 40 Gy of fWBI (2 Gy fx, 5 days per week for 4 weeks; BED = 66.7) were minimal in NHPs, with progressive multifocal brain necrosis seen at 60 and 80 Gy, 6–12 months after radiation exposure (7). In the current work, a model with a larger dose per fraction and smaller dose frequency (5 Gy fx, twice per week for 4 weeks; BED = 106.7 Gy) was employed that had previously demonstrated success in rats inducing cognitive (10) and histologic (11) abnormalities. In NHP studies where neural damage has been reported at 40 Gy fWBI or less, defects typically appeared more than two years postirradiation (22). Although the pathology observed in two NHPs in our study was quite severe given the total dose of radiation, it is unlikely that the abnormalities preceded irradiation since FDG-PET scans and MRIs acquired prior to irradiation showed no brain abnormalities in the NHPs. The lesions we observed were morphologically identical to those described by Caveness (7). The occurrence of brain lesions at lower dose and shorter time intervals in our study may relate to differences in fraction size or frequency as described above, or dose rate (2 Gy/min by Caveness (7) vs. 4 Gy/min in this study). We believe potential confounding factors were not relevant; although one animal had an ear infection that may have confounded cognitive assessment, the vascular/degenerative changes in the brain were spatially and pathologically distinct from otitis.

The response of the CNS to irradiation depends on multiple factors (e.g., total dose, dose per fx size, host factors) (3). With the exception of host factors, these influences were held constant in this study. Thus, it appears that, at the molecular and cellular level, differences exist in NHPs, which affect radiation response. Radiation-induced brain injury in humans varies similarly, however the contribution of radiation is confounded by the presence of a tumor and other treatments (1, 4). An NHP model allows investigation of radiation effects in normal brain tissue free from these potentially confounding factors in humans. We have refined the *in vivo* model from previous descriptions of gross brain dysfunction to quantitative cognitive assessments (9), and here present the corresponding pathology in the same animals. These data validate cognitive testing as an early measure of radiation-induced neurodegeneration in this NHP model.

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FIG. 1.

Shows the electronic portal imaging device (EPID) images for the right and left lateral fields obtained immediately prior to irradiation for each radiation fraction to confirm correct positioning. The blue field-outline (which is automatically detected and drawn) and eye shield are shown for each field, and the silhouette of the facemask for gas anesthesia delivery is readily visible.

FIG. 2.

Panel A: Coronal T1-weighted MRI, demonstrating focal lesions (arrows). Panel B: Corresponding gross lesions seen at necropsy.

FIG. 3.

Panel A: Normal arteriole, parietal cortex. Panel B: Acutely necrotic arteriole with leakage of serum and fibrin into the parenchyma. Panel C: Chronically degenerate arterioles with reactive astrocytosis (arrow 1); perivascular hemosiderin (arrow 2) indicating past hemorrhage; and marked fibrous thickening of the vascular wall (arrow 3). Panel D: Subacute cerebrocortical hemorrhage, corresponding to the medial MRI lesion shown in Fig. 2.

FIG. 4.

Panel A: Multifocal white matter demyelination and necrosis (arrows). Panel B: Higher magnification of panel A showing white matter loss, microglial infiltration, and vascular wall thickening. Panel C: Higher magnification of panel B, showing infiltration by activated microglial phagocytic cells and abnormal vascular thickening. Panel D: Focal white matter degeneration with vacuolation, microglial activation, and mineralization.

FIG. 5.

Histochemistry and immunohistochemistry of focal subacute cerebral hemorrhage. Panels A and B: Low and high magnification images, respectively, of the same lesion [Hematoxylin and Eosin (H&E)]. Panel C: High magnification image of phosphotungstic acid hematoxylin stain (PTAH); the dark purple color indicates the presence of fibrin. The brown stain in this image is hemosiderin derived from old hemorrhage. Panel D: High magnification image of prussian blue (PB) stain; blue stain in this image indicates iron (hemosiderin). Panel E: High magnification image of glial fibrillary acidic protein (GFAP) staining for astrocytes. Panel F: High magnification image of IBA-1 staining for macrophages. Panel G: High magnification image of factor 8 related antigen (FVIII) indicating the location of blood vessel endothelium. Panel H: High magnification image of neuron specific enolase (NSE), staining the parenchymal meshwork of neuronal processes.

TABLE 1

MRI and Histologic Detection of Focal Necrosis in the Brain and Spinal Cord

NHP Method of detection	A		B		C		D	
	MRI	Histologic	MRI	Histologic	MRI	Histologic	MRI	Histologic
Region/structure								
Forebrain: White matter	7	+++	-	+	17	+++	-	++
Forebrain: Cortical gray matter	4	+	-	-	2	+++	-	+
Basal ganglia/striatum	-	+	-	-	4	+++	-	-
Hippocampus	-	-	-	-	-	-	-	-
Thalamus	-	++	-	-	-	++	-	-
Midbrain	2	+++	-	-	-	++	1	-
Cerebellum	-	+	-	-	-	+	-	-
Brainstem	2	+++	-	-	-	++	-	-
Spinal cord	-	na	1	-	-	++	-	na