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Maintenance of white matter integrity in a rat model of radiation-induced cognitive impairment

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ABSTRACT

Radiation therapy is used widely to treat primary and metastatic brain tumors, but also can lead to delayed neurological complications. Since maintenance of myelin integrity is important for cognitive function, the present study used a rat model that demonstrates spatial learning and memory impairment 12 months following fractionated whole-brain irradiation (WBI) at middle age to investigate WBI-induced myelin changes. In this model, 12-month Fischer 344×Brown Norway rats received 9 fractions of 5 Gy delivered over 4.5 weeks (WBI rats); Sham-IR rats received anesthesia only. Twelve months later, the brains were collected and measures of white matter integrity were quantified. Qualitative observation did not reveal white matter necrosis one year post-WBI. In addition, the size of major forebrain commissures, the number of oligodendrocytes, the size and number of myelinated axons, and the thickness of myelin sheaths did not differ between the two groups. In summary, both the gross morphology and the structural integrity of myelin were preserved one year following fractionated WBI in a rodent model of radiation-induced cognitive impairment. Imaging studies with advanced techniques including diffusion tensor imaging may be required to elucidate the neurobiological changes associated with the cognitive impairment in this model.

1. Introduction

Radiation therapy is a cornerstone of modern cancer management and approximately half of all newly diagnosed cancer patients will receive radiotherapy at some point during the treatment of their disease [1]. Whole-brain irradiation (WBI) following tumor resection or radiosurgery can effectively reduce tumor recurrence and is used prophylactically to kill metastatic cells that would otherwise seed the brain [2]. Along with the therapeutic benefits, however, WBI can lead to delayed, dose-dependent neurological complications including progressive cognitive impairment [3,4]. Importantly, the physical and social burden of these neurological side effects can be more debilitating and detrimental than the primary disease [3,5]. With the marked improvements in long-term cancer survival, radiation-induced neurotoxicity is an issue of increasing concern [6].

The pathophysiological changes characterizing late-delayed WBI-induced neurotoxicity are dynamic and complex, and involve both the brain vasculature [7,8] and parenchyma [9]. These late-delayed effects manifest more than 6 months following WBI and are progressive and irreversible [10]. Earlier studies of long-term WBI survivors [11] have revealed white matter changes such as periventricular white matter abnormalities, ventricular dilation, leukoencephalopathy, and diffuse demyelination [9,12,13]. It is important to note that published clinical data are derived from brain tumor patients. Not only are the brains exposed to the tumor microenvironment, but many patients also receive multimodality therapy, including surgery, radiosurgery, and/or chemotherapy. Use of such multimodality therapy provides significant biological challenges to normal brain tissue and can lead to severe neurotoxicty [2,3,14], confounding the interpretation of the effects of WBI [15]. Rodent models provide valuable tools to directly and thoroughly examine the late-delayed effects of WBI on brain white matter in the absence of the complicating effects of brain tumors or other treatment modalities. Indeed, recent studies have reported radiation-induced changes including apoptosis of oligodendrocytes and decreased expression of myelin-associated proteins in rodent spinal cord [16] as well as white matter necrosis in rat optic nerve neuropathy [17].

A recent study reported spatial learning and memory impairment 12 months following 45 Gy of WBI delivered as 9 fractions of 5 Gy whole-brain irradiation over 4.5 weeks to middle-aged Fischer 344×Brown Norway (F344×BN) rats [18]. These cognitive impairments were

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associated with changes in glutamate receptors in the hippocampus, but not with a decrease in hippocampal neuron number [19]. This rat model of fractionated WBI at middle age is of clinical importance because the incidence of cancer with a propensity to metastasize to the brain such as lung cancer, breast cancer, and malignant melanoma, increases significantly by middle age [20]. The present study investigated potential late-delayed changes in brain white matter in this rat model of fractionated WBI.

Maintenance of white mater integrity is critically important for cognitive performance [21,22] and a positive correlation has been established between cognitive performance and white matter integrity [23]. Moreover, in the aged population, a region-specific correlation between measures of cognitive performance and white matter integrity has been reported [24]. Accordingly, the present study quantified measures of white matter integrity 12 months following 45 Gy of fractionated WBI at middle age. By using the same experimental design that resulted in WBI-induced cognitive impairment [18], the rats in the previous study served as a behavioral index for the parallel cohorts of rats studied here. Using morphometric analysis of light and electron microscopic material, we quantified the size of major forebrain commissures, the number of oligodendrocytes, the size and number of myelinated axons, and the thickness of myelin sheaths and found no WBI-induced change on any of the measures. These findings suggest that the cognitive impairment present in this model one year after fractionated WBI at middle age occurs in the absence of structural changes on these measures of myelin integrity.

2. Materials and methods

2.1. Animals and irradiation procedure

Male F1 Fischer 344×Brown Norway (F344×BN) rats (Harlan Industries, Indianapolis, IN) were divided randomly into sham-irradiated (Sham-IR; $n=15$) and WBI ($n=19$) groups at 12 months of age. WBI rats were lightly anesthetized and irradiated twice per week for 4.5 weeks in a 444-TBq self-shielded ^{137}Cs irradiator using lead and Cerrobend shielding devices to collimate the beam so that the whole brain was irradiated [18,19]. A total dose of 45 Gy was delivered to the brain as 9 fractions of 5 Gy. The biologically effective dose (BED, 23) calculated for this regimen was 120 Gy_3, assuming an α/β ratio of 3 for late radiation-induced damage [25] and the single dose equivalence was 17.6 Gy [48]. To ensure that each rat received the same midline brain dose, the dose was delivered to alternate sides of the head on alternate days. Sham-IR rats were anesthetized, but not irradiated. All rats were euthanized by an overdose of sodium pentobarbital at 24 months of age. All subsequent experimental procedures were carried out by investigators blinded to irradiation status. Following irradiation, Sham-IR and WBI rats were randomly allocated for evaluation by either light microscopy (LM) or electron microscopy (EM). The animal protocol for this study conforms to NIH guidelines and was reviewed and approved by the Animal Care and Use Committee of Wake Forest University Health Sciences to ensure the ethics of the research and the welfare of the animals. Rats were housed singly in a climate-controlled environment with a 12-hour light/dark cycle and provided food and water *ad libitum*. Steps have been taken to eliminate pain and suffering of animals.

2.2. Tissue preparation for LM

For LM analysis ($n=7$ Sham-IR; $n=11$ WBI), rats were perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer. The brains were dissected from the cranial vault, postfixed overnight, cryoprotected in a graded sucrose, and blocked stereotaxically at Bregma 5.5 and Bregma −15 mm [26]. The brains were frozen in Tissue-Tek OCT (Optimal Cutting Temperature Compound; Sakura Finetek) on dry ice, and stored at −80 °C. Tissue was cryostat-sectioned coronally (40 μm) and the sections were stored in antifreeze at −20 °C until staining.

2.2.1. Heidenhain staining

Sections stained with the Heidenhain myelin stain were used to, i] assess white matter integrity, ii] determine neocortex and forebrain volumes, and iii] measure the decussation area of the major brain commissures at midline. Briefly, every 24th section through the cerebral cortex was washed in phosphate buffered saline (PBS), immersed in iron alum, washed in water, stained with hematoxylin, washed in water, and placed overnight in PBS [27]. Sections then were mounted onto slides, air-dried, dehydrated, cleared, and cover-slipped. Every-other section through the anterior commissure (AC) also was stained and processed as described.

2.2.2. Nissl staining

Nissl is a basophilic stain that provides clear visualization of both neurons and glia. Oligodendrocytes, the glia comprising the myelin sheaths, were quantified in Nissl-stained sections in both corpus callosum (CC) and AC. Previous studies have indicated that oligodendrocytes can be identified morphologically by their size, shape, and cytoplasmic density, as well as by their orientation parallel to axon bundles in white matter [28,29]. Oligodendrocytes identified using these criteria were quantified in order to count the entire population of myelinating glia. The available antibodies to oligodendrocytes only label subsets of oligodendrocytes. Consequently, the number of oligodendrocytes immunolabeled by the available antibodies, either individually or in combination, may not reflect the total oligodendrocyte population. Briefly, every 12th section through the rostral–caudal extent of the CC and every-other section through AC were washed in PBS, mounted on slides, air-dried, washed in PBS and in water, stained in cresyl violet, washed in water, dehydrated, and cover-slipped.

2.3. LM morphometric analysis

2.3.1. Volumetric determinations

Neocortex and forebrain volumes were measured in Heidenhain-stained sections using the *Cavalieri* method [31] and StereoInvestigator software. For these measurements, 16 sections through the rostral–caudal extent of the forebrain (Bregma 5.5 to −9.5 mm) were digitized (Duoscan T2500 scanner, AGFA). A grid (100×100 μm) was superimposed over the section (illustrated schematically for the right hemisphere in Fig. 1A). The areas of the neocortex (cortex dorsal to the rhinal fissure, shown as the stippled area of the left hemisphere in Fig. 1A) and the non-neocortex forebrain were calculated by the software from the grid size and the number of intersections lying over the tissue. The neocortex and total forebrain (neocortex + non-neocortex forebrain) areas were multiplied by the section thickness (40 μm) and separation between measured sections (960 μm) to determine the volumes.

2.3.2. Decussation areas of commissures

The decussation areas at midline of 3 major forebrain commissures were determined in Heidenhain-stained sections: CC (Fig. 2A–D, Bregma 1.0 to −5.6 mm), AC (Fig. 2A and B, Bregma −0.2 to −0.7 mm), and dorsal hippocampal commissure (DHC, Fig. 2C and D, Bregma −1.8 mm to −5.2 mm). All sections were of equal thickness. In every 24th section through the CC and DHC and in every-other section through the AC, the height of each commissure was measured at midline, multiplied by the section thickness (40 μm) and separation, and summed across the rostrocaudal extent of the commissure to derive the midline decussation area for that commissure. This method permitted derivation of commissure areas at midline from coronal sections.