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Cellular response of the rat brain to single doses of ^{137}Cs γ rays does not predict its response to prolonged ‘biologically equivalent’ fractionated doses

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Abstract

Purpose—To determine if the brain’s response to single doses predicts its response to ‘biologically equivalent’ fractionated doses.

Methods—Young adult male Fischer 344 rats were whole-brain irradiated with either single 11, 14, or 16.5 Gy doses of ^{137}Cs γ rays or their ‘biologically equivalent’ 20, 30, or 40 Gy fractionated doses (fWBI) delivered in 5 Gy fractions, twice/week for 2, 3, or 4 weeks, respectively. At 2 months post-irradiation, cellular markers of inflammation (total, activated, and newborn microglia) and neurogenesis (newborn neurons) were measured in 40 μm sections of the dentate gyrus (DG).

Results—Although the total number of microglia in the DG/hilus was not significantly different ($p > 0.7$) in unirradiated, single dose, and fWBI rats, single doses produced a significant ($p < 0.003$) increase in the percent-activated microglia; fWBI did not ($p > 0.1$). Additionally, single doses produced a significant ($p < 0.002$) dose-dependent increase in surviving newborn microglia; fWBI did not ($p < 0.8$). Although total proliferation in the DG was reduced equally by single and fWBI doses, single doses produced a significant dose-dependent ($p < 0.02$) decrease in surviving newborn neurons; fWBI did not ($p > 0.6$).

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Declaration of interest

The authors report no conflicts of interest.

Supplementary material available online

Supplementary Figure 1.

Conclusions—These data demonstrate that the rat brain's cellular response to single doses often does not predict its cellular response to 'biologically equivalent' fWBI doses.

Keywords

Brain irradiation; single dose responses; fractionated dose responses; inflammation; neurogenesis

Introduction

Up to 30% (Jemal et al. 2011) of the > 1.6 million individuals diagnosed with cancer in the US during 2012 will develop brain metastases (Patchell 2003); most will receive fractionated partial or whole-brain irradiation (fWBI). Approximately 170,000 of these patients will survive 6 months (Khuntia et al. 2006); 50–90% of these are at risk of developing radiation-induced cognitive impairments that severely impact their quality of life (QOL) (Crossen et al. 1994, Shaw and Robbins 2006). The hallmarks of radiation-induced cognitive impairment include impairments in verbal memory, spatial memory, attention, and novel problem-solving (Twijnstra et al. 1987, Roman and Sperduto 1995); the incidence and severity of these cognitive impairments increase over time (Nieder et al. 1999). Often these cognitive impairments occur without clinical or radiographic evidence of demyelination or white matter necrosis (Dropcho 1991, Shaw and Robbins 2006). To overcome these QOL issues requires research in translatable pre-clinical models to study the mechanisms of fWBI-induced cognitive impairment, and test therapeutics that target these mechanisms. Presently, the majority of radiation-induced brain injury studies have used single doses (Monje et al. 2002, 2003, Mizumatsu et al. 2003, Ramanan et al. 2009, Hua et al. 2012, Son et al. 2014) or a short fractionation regimen (Shinohara et al. 1997), rather than the prolonged fWBI regimen used in the clinic.

A preponderance of evidence supports the hypothesis that late radiation-induced brain injury, including cognitive impairment, is driven by acute and chronic oxidative stress as well as inflammatory responses (Robbins and Zhao 2004, Zhao et al. 2007, Zhao and Robbins 2009). In general, ionizing radiation produces its biological effects either directly or indirectly by generating reactive oxygen species (ROS) that lead to molecular changes such as damage to DNA (Gobbel et al. 1998, Otsuka et al. 2006), lipids (Limoli et al. 2004), and proteins (Stadtman and Berlett 1997, Manda et al. 2007), as well as activation of early response genes (Hong et al. 1995, Moore et al. 2011, Moore et al. 2013) and signal transduction pathways (Schmidt-Ullrich et al. 2000, Dent et al. 2003). Activation of these pathways leads to: (i) Induction of reparative and restorative processes (Mattson et al. 1997, Moyer et al. 1998, Kojima et al. 1998, Heck et al. 1999); (ii) changes in the cytokine milieu (Zhou et al. 2001, Son et al. 2006, Lee et al. 2010), and (iii) activation/influx of inflammatory cells (Moravan et al. 2011).

Chronic oxidative stress is generally thought to result from an inflammatory response where irradiation activates microglia and causes immune cells to infiltrate into the brain (Chiang et al. 1993, Monje et al. 2002, Yuan et al. 2003, Moravan et al. 2011). These cells then generate ROS which in turn activate more microglia that can maintain or increase the level of oxidative stress (Hanisch and Kettenmann 2007). Evidence for a chronic, radiation-

induced inflammatory response in rodent models includes: (i) Elevation of inflammatory cytokines up to 6 months post-irradiation (Hong et al. 1995, Lee et al. 2010); (ii) a significant increase in the number of activated microglia in the dentate gyrus (DG) (Monje et al. 2002, 2003, Lee et al. 2012, Greene-Schloesser et al. 2014); (iii) increased expression of the CCR2 receptor in the subgranular zone (Rola et al. 2005); and (iv) persistent microglial and astrocyte activation (Chiang et al. 1993, Schindler et al. 2008).

In an uninjured brain, microglia monitor the microenvironment to ensure that homeostasis is maintained (Stoll and Jander 1999). These microglia generate neurotrophins that selectively regulate microglial function and proliferation (Elkabes et al. 1996). After injury, microglia become activated, a process that is characterized by rounding of the cell body, retraction of cell processes, proliferation, and increased production of ROS, cytokines, and chemokines that mediate neuroinflammation (Stoll and Jander 1999, Gebicke-Haerter 2001, Pocock and Liddle 2001, Kim and de Vellis 2005). Although microglial activation plays an important role in phagocytosis of dead cells, sustained activation is thought to contribute to a chronic inflammatory state in the brain (Gebicke-Haerter 2001, Joo et al. 2012). Measurements of a chronic inflammatory brain response to single doses and fWBI in rodent models include: (i) Elevation of TNF α (Hong et al. 1995); (ii) up-regulation of IL-1 β (Lee et al. 2010, Schnegg et al. 2013); (iii) a marked increase in the percentage of activated microglia (Monje et al. 2002, 2003, Lee et al. 2012, Schnegg et al. 2013, Greene-Schloesser et al. 2014); and (iv) persistent microglial activation (Schindler et al. 2008, Ramanan et al. 2009, Conner et al. 2010, Lee et al. 2012, Greene-Schloesser et al. 2014). Previous studies have also suggested that microglial activation may be associated with decreased hippocampal neurogenesis and cognitive function (Monje et al. 2002, 2007, Raber et al. 2004).

The hippocampus has been shown to play a major role in learning, consolidation, and retrieval of information (Eichenbaum 2001, 2004). Thus, the majority of radiation-induced brain injury studies have focused on the hippocampus. The hippocampus consists of three regions: CA1, CA3, and the dentate gyrus (DG) which have been linked to both rodent and human cognition. Additionally, the DG is one of two sites of adult neurogenesis in the mammalian brain. Neuronal stem cells (NSC) in the DG are capable of self-renewal, and generation of new neurons, astrocytes, and oligodendrocytes (Eriksson et al. 1998, Gage et al. 1998). Neurogenesis is dependent on a specific neurogenic microenvironment where endothelial cells and astrocytes promote/regulate the process (Palmer et al. 2000). Irradiating the rodent brain has been shown to result in, a dose-dependent decrease in NSC (Tada et al. 2000), decreased proliferation of surviving NSC (Monje et al. 2002, Mizumatsu et al. 2003), and decreased differentiation of NSC into neurons (Monje et al. 2002, Mizumatsu et al. 2003). Reductions in hippocampal neurogenesis have also been correlated with radiation-induced cognitive impairment (Raber et al. 2004, Rola et al. 2004).

There is a paucity of studies that have compared the cellular and molecular responses of single doses to fWBI doses. In a study that examined apoptosis in the subependyma of young adult rats after single doses and fWBI, the investigators found that single 0.5–2.0 Gy doses resulted in an initial steep increase in apoptosis followed by a plateau from 2–30 Gy, while 1.5 Gy fractions administered over a 7-day period resulted in an initial increase in apoptosis with the first fraction, followed by a steep decrease after the 2nd and 3rd fractions,

and then by a plateau after fractions 4–7 (Shinohara et al. 1997). These findings indicate that there may be differences in the cellular response of the brain to single and fWBI doses. However this study was not designed to directly compare the effects of single doses to ‘biologically equivalent’ fWBI doses, rather it was designed to characterize time, dose, and fractionation effects on the apoptotic response within the subependyma. Gaber et al. (2003) reported that the acute inflammatory response in a mouse model occurred earlier and was more robust after large single doses than after fWBI doses. However, in this study, the single 10 and 20 Gy doses were compared to fractionated 10 and 20 Gy doses, so the responses could not be expected to be ‘biologically equivalent’. Finally, a recent study found that the number of newborn-neurons was significantly decreased by both single and fractionated total body irradiation (TBI). However, the decrease after a single TBI dose was ~3.6-fold greater than that observed after fractionated TBI doses (Park et al. 2012). Again, the single 5 Gy dose and the fractionated 5 Gy dose regimen (1 Gy/fraction, 5×/week and 0.5 Gy/fraction, 10×/week) would not be expected to be ‘biologically equivalent’.

In the present study, we compared the cellular response of the rat hippocampus to 20, 30 and 40 Gy fWBI doses delivered in 5 Gy fractions, 2×/week for 2, 3, or 4 to the calculated (Hall and Giaccia 2012) ‘biologically equivalent’ single doses of 11, 14, and 16.5 Gy. Cellular markers of inflammation (total microglia, microglial activation, surviving newborn microglia) and neurogenesis (total proliferation, surviving newborn neurons) commonly employed in rodent radiation-induced brain injury studies were compared at 2 months post-irradiation. The data demonstrate that the cellular response of the rat brain after single doses often does not predict its cellular response after ‘biologically equivalent’ fractionated doses.

Methods

Animals

Young adult (10–12 weeks old) male Fischer 344 rats (Harlan Laboratories; Indianapolis, IN, USA) were housed in pairs on a 12:12 light:dark schedule with free access to food and water. All animal handling and experiments were performed in strict accordance with the NIH Guide for Care and Use of Laboratory Animals as approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee. Rats were randomized to seven experimental groups ($n = 6$ rats/group); three single dose groups (11, 14, or 16.5 Gy), three fractionated dose groups (20, 30, or 40 Gy), and a sham-irradiated group. All rats were weighed weekly to assess their overall health; no adverse effects were measured.

Irradiation procedures

All irradiations were performed in a 267 TBq (7,214 Ci) self-shielded ^{137}Cs irradiator using lead and Cerrobend[®] shielding devices to collimate the beam. The whole brain, including the brain stem, was irradiated, and the eyes were shielded to prevent vision loss. The average dose rate to the midline of the brain was ~4 Gy/min. Each lightly anesthetized (Ketamine [75 mg/kg]/xylazine [7 mg/kg]) rat had the twice-weekly dose delivered to alternate sides of the head on alternate days to ensure that each rat received the same midline brain dose (Brown et al. 2005). Rats received whole-brain irradiation with either single doses of 11, 14, or 16.5 Gy at 20 weeks of age or ‘biologically equivalent’ fractionated 20,

30, or 40 Gy doses starting at 15 weeks of age administered in 5 Gy fractions twice/week for 2, 3, or 4 weeks (Supplementary Figure 1, available online at <http://informahealthcare.com/oi/abs/10.3109/09553002.2014.933915>). The sham-irradiated rats were lightly anesthetized twice/week for 4 weeks starting at 15 weeks of age. The biologically effective dose (BED) for each regimen was calculated using an α/β ratio of 3 for normal brain late effects (Fowler 1989, Lee et al. 1998) and the following equation (Hall and Giaccia 2012):

$$BED = (nd) \times \left(1 + \frac{d}{\alpha/\beta}\right)$$

BrdU labeling

Bromodeoxyuridine (BrdU) was used to measure the number and percentage of surviving newborn (BrdU+) microglia and newborn (BrdU+) neurons in the DG. Rats received daily intraperitoneal (ip) injections of BrdU (50 mg/kg of body weight) for 7 days starting 1 month after the single doses (24 weeks of age) or 1 month after the last fraction of fWBI (20 Gy fWBI = 21 weeks of age, 30 Gy fWBI = 22 weeks of age, 40 Gy fWBI = 23 weeks of age) as previously described by us (Ramanan et al. 2009, Supplementary Figure 1, available online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.933915>). At 2 months post-irradiation (single doses = 28 weeks of age, 20 Gy fWBI = 25 weeks of age, 30 Gy fWBI = 26 weeks of age, 40 Gy fWBI = 27 weeks of age) the rats were euthanized with CO₂, decapitated, and the brains excised rapidly.

Tissue preparation

Each excised brain was hemisected at the midline. The right hemisphere was flash frozen in liquid nitrogen, and the left hemisphere was immersion fixed in phosphate-buffered 4% paraformaldehyde for 24 h. The left hemisphere was then cryoprotected for 24 h in 10, 20 and 30% sucrose, frozen in embedding medium, coronally cryosectioned at a thickness of 40 μ m, and placed in an antifreeze solution (1:1:2 ethylene glycol, glycerol and 0.1 M of phosphate-buffered saline) until processed for immunohistochemical analyses.

Immunohistochemistry

Immunohistochemistry was performed on the left hemispheres of four brains randomly selected from each experimental group (Ramanan et al. 2009). After sectioning the entire DG (bregma — 1.8 to bregma — 6.8), an initial section was randomly selected from the first 12 sections of the DG. The initial section and every 12th section thereafter through the entire DG was washed in tris-buffered saline (TBS: pH 7.4) to remove the cryoprotectant.

For the inflammatory response, we assessed total microglia, surviving newborn microglia, and activated microglia using the primary antibodies: Rabbit polyclonal anti-ionized calcium-binding adaptor molecule (Iba1) that labels all microglia (Wako, Richmond, VA; 0.083 μ g/ml), anti-BrdU which labels cells in S-phase (MCA2060, AbD serotec, Raleigh, NC; 1:250), and mouse monoclonal anti-rat CD68 (ED1 clone) that labels activated microglia (MCA341R, AbD Serotec, Raleigh, NC; 1:400). Prior to staining, endogenous

peroxidase activity was reduced by a 30 min incubation in 0.6% hydrogen peroxide in TBS. To assess proliferation and surviving newborn microglia, sections were treated with 2 mol/l hydrochloric acid at 37°C to denature the DNA and washed in 1×TBS, pH 8.5 to neutralize the acid. Sections were then incubated in blocking solution (5% normal serum and 0.2% Triton X-100 in TBS) and finally overnight at 48°C with the primary antibodies diluted in blocking solution.

Primary antibodies were detected using biotinylated secondary antibodies (Iba1: goat anti-rabbit; BrdU: rabbit anti-rat; ED1: horse anti-mouse) and visualized using peroxidase conjugated avidin-biotin complex (ABC Elite kit) with nickel enhanced diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) as the substrate for both Iba1 and ED1 and/or SG chromagen (Vector Laboratories, Burlingame, CA, USA) as the substrate for BrdU. Sections were counterstained with the nuclear binding dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Aldrich, St. Louis, MO, USA) to facilitate recognition of anatomical landmarks.

Neurogenesis was assessed using immunofluorescence staining for surviving newborn neurons. Prior to staining, sections were treated to denature the DNA as described above. The sections were then incubated for 2 h in blocking solution (5% normal serum and 0.3% Triton X-100 in 1×TBS) and finally overnight at 4°C with rat mAB-BrdU (MCA2060, AbD Serotec, Raleigh, NC; 1:100) and mouse a-NeuN (MAB377, Chemicon, Billerica, MA; 1:2000). The BrdU and NeuN labeling was detected by use of Cy3-conjugated and Alexa Fluor 488-conjugated secondary antibodies, respectively (Jackson ImmunoResearch, West Grove, PA; 1:200).

Stereological analyses of tissue sections

Stereologic quantification is superior to traditional, single-section estimation techniques because the result is not biased by the shape, size, and orientation of the counting objects or by volumetric variation (West et al. 1991). All analyses were performed using coded sections with a Zeiss AxioImager M2, (Göttingen, Germany) equipped with a semi-automatic stereology system (StereoInvestigator software, Microbrightfield, Inc.) as previously described by us (Lee et al. 2012, Greene-Schloesser et al. 2014). Briefly, the optical fractionator method (Gundersen et al. 1988) was used for quantification of immunolabeled cells in the granular cell layer (GCL), subgranular zone (SGZ) and hilus. The counting parameters for Iba1+, BrdU+ and ED1 + cells in the dentate gyrus/hilus were: Sampling grid size (175×175 µm), counting frame size (75×75 µm), disector height (20 µm), and guard zone thickness (5 µm). BrdU+/Iba1 + cells were identified by the presence of BrdU+ nuclei surrounded by Iba1 + cytoplasm.

BrdU+/NeuN+ double labeled cells were assessed from image stacks. The counting parameters for BrdU+/NeuN+ cells in the GCL/SGZ were: Sampling grid size (100×100 µm), counting frame size (100×100 µm), disector height (30 µm), and guard zone thickness (5 µm). BrdU/NeuN double-labeling was confirmed using confocal microscopy (Leica TCS SP2, Leica Microsystems, Bannockburn, IL, USA). The precision of the stereological counts was determined using Gundersen-Jensen coefficient of error estimator estimates (Gundersen

et al. 1999, Slomianka and West 2005). All estimates in the current study were less than the required 0.5 criterion.

Statistical analysis

Data are presented as the mean $\pm$ SEM. The generalized linear model (GLM) was used to detect effects of radiation, group (single vs. fractionated), and dose. Linear regression analyses were performed on the fractionated and single dose groups to determine if the effects were dose-dependent. All analyses were performed using SAS (Cary, NC, USA) software.

Results

Effect of single and fractionated doses on the brain's cellular inflammatory response

The total number of microglia in the DG/hilus of both the single dose and fWBI rats was not significantly different ($p > 0.2$ and $p > 0.6$, respectively) from the sham-irradiated rats at 2 months after irradiation (Figures 1A, 2A–C). However, the total number of microglia in the DG/hilus of the single dose rats was significantly ($p < 0.04$) decreased compared to the number of microglia in the fWBI rats (Figure 1A) suggesting that the inflammatory response of the brain to single doses may not predict the inflammatory response to fractionated doses.

The percentage of BrdU+ microglia in the DG/hilus of fWBI rats was not significantly different ($p > 0.80$) from the percentage in sham-irradiated rats (Figures 1B, 2A–C). However, the percentage of BrdU+ microglia in the DG/hilus of the single dose rats was significantly different ($p < 0.05$) from the percentage of BrdU+ microglia in sham-irradiated rats (Figures 1B, 2A–C). The percentage of BrdU+ microglia in the DG/hilus of the single dose rats was also significantly different ($p < 0.0001$) from the percentage of BrdU+ microglia in fWBI rats (Figures 1B, 2A–C). Moreover, this increase in BrdU+ microglia in the single dose rats was marginally dose-dependent ($p < 0.058$; $R^2 = 0.31$). Thus, the BrdU+ microglia response after single doses did not predict the BrdU+ microglia response after fractionated doses.

At 2 months after irradiation, the increase in the percentage of activated microglia measured in the fWBI rats was not significantly different ($p > 0.1$) from the percentage of activated microglia in the sham-irradiated rats (Figures 1C, 2D–F). However, the increase in the percentage of activated microglia in the single dose rats was significantly different from the percentage of activated microglia in both sham-irradiated ($p < 0.003$) and fWBI ($p < 0.004$) rats (Figures 1C, 2D–F). Thus, the activated microglia response after single doses did not predict the activated microglia response after fractionated doses.

Effect of single and fractionated doses on proliferation and neurogenesis

The total number of proliferating cells labeled with BrdU at 1 month post-irradiation that survived until 2 months post-irradiation (i.e., surviving newborn neurons, microglia and neuroglia) after single doses and fractionated doses was significantly ($p < 0.0001$) decreased from that in sham-irradiated rats (Figure 3A). However, the decreased proliferation after single doses was not significantly different ($p > 0.23$) from the decreased proliferation after

fractionated doses (Figure 3A). Thus, the total proliferation measured after single doses predicted the total proliferation measured after fractionated doses.

The percentage of surviving newborn neurons significantly ($p < 0.02$) decreased after both single and fractionated doses (Figures 3B, 4A–C). However, this decrease in surviving newborn neurons was dose-dependent ($p < 0.02$; $R^2 = 0.54$) after single doses, but dose-independent after fractionated doses ($p > 0.68$; $R^2 = 0.02$) (Figure 3B). Consequently, the dose-dependent decrease in neurogenesis after single doses did not predict the dose-independent decrease in neurogenesis after fractionated doses.

Discussion

We and others have used single doses of WBI to predict the outcomes of prolonged fractionated schemes used in the clinic (Monje et al. 2002, Mizumatsu et al. 2003, Raber et al. 2004, Ramanan et al. 2009, Hua et al. 2012, Schnegg et al. 2013). However, results from the present study indicate that single doses responses using commonly employed cellular endpoints to define radiation-induced brain injury often do not predict the outcomes of prolonged fractionation schemes (Figures 1A–C, 2A–F, 3B, 4A–C). In young adult male rats, significant differences in the neuroinflammatory response to single doses and fWBI were measured (Figures 1A–C, 2A–F). Additionally, neurogenesis was differentially affected with a dose-dependent decrease in surviving newborn neurons after single doses and a dose-independent decrease after fractionated doses (Figure 3B). These data demonstrate that extrapolating the results from single doses to the results from prolonged fractionated doses is fraught with difficulties.

Microglia actively monitor the microenvironment to ensure maintenance of brain homeostasis, including the regulation of cell death, neuronal surveillance, neurogenesis, and synapse function (Wake et al. 2013). Previous studies of radiation-induced brain injury in adult rodent models have reported no change in the total number of microglia in the DG/hilus of the hippocampus at 2–3 months after a single doses of WBI (Monje et al. 2002, Hua et al. 2012) or 6 months after fWBI (Lee et al. 2012, Greene-Schloesser et al. 2014). However there are studies that have shown decreases in the total microglia population in the young adult rodent after single doses (Son et al. 2014) or fWBI (Conner et al. 2010). In the present study, there was no significant decrease in the total number of microglia at 2 months after irradiation. Although neither the single nor fWBI groups showed a significant difference in the total number of microglia compared to the sham-irradiation, the total number of microglia in the single dose group was significantly different from the fWBI group (Figures 1A, 2A–C). Single doses resulted in significantly fewer microglia when compared to the fWBI doses, suggesting that microglia may respond differentially to these two treatment regimens.

It has been previously shown that brain injury and chronic diseases of the CNS can lead to an increase in microglia proliferation (Ladeby et al. 2005, Davoust et al. 2008, Kettenmann et al. 2011). Previously, it has been reported that microglial proliferation was increased after single doses of WBI (Monje et al. 2002, 2003, Hua et al. 2012). In the present study, there was a dose-dependent increase in the number and percentage of surviving BrdU+ microglia

2 months after single doses (Figure 1B), while the number of BrdU+ microglia decreased in a marginally dose-dependent ($p < 0.058$, $R^2 = 0.31$) manner after single doses (data not shown). In contrast to single doses of irradiation, fractionated doses did not result in significant changes in surviving newborn microglia as compared to sham-irradiated rats (Figures 1B, 2A–C). Taken together, these data suggest that single doses cause a proliferation, activation, and rapid turn-over of microglia, consistent with what has been observed in other CNS injury models (Vela et al. 2002), that does not occur after fractionated doses of irradiation.

It has been well documented that both single and fractionated doses of irradiation lead to an increase in the activation of microglia in the rodent brain (Monje et al. 2002, Rola et al. 2004, Rosi 2008, Ramanan et al. 2009, Schindler et al. 2008, Conner et al. 2010, Hua et al. 2012, Lee et al. 2012, Schnegg et al. 2013, Greene-Schloesser et al. 2014). In this study, the percentage of activated microglia was greater after single dose rats than after fractionated doses (Figures 1C, 2D–F). These findings suggest that single doses may result in a more pro-inflammatory microenvironment than fractionated doses. This is in agreement with a previous study which found that single doses of WBI resulted in higher expression of the pro-inflammatory cytokines/mediators, TNF- α and ICAM-1, than after fWBI (Gaber et al. 2003). Taken together, these data suggest that the higher inflammatory state induced in the brain by single doses may be responsible for the reported reactive microgliosis characterized by increases in both proliferating and activated microglia.

Previous studies have shown a correlation between increases in the number of activated microglia and reductions in neurogenesis in the rodent brain similar to what we observed after single doses. For example, single doses have been shown to result in a dose-dependent decrease in NSCs (Tada et al. 2000), decreased proliferation of surviving NSCs (Monje et al. 2002, Mizumatsu et al. 2003), and decreased differentiation of NSCs into neurons (Monje et al. 2002, Mizumatsu et al. 2003). Neurogenesis is dependent upon a specific neurogenic microenvironment where endothelial cells, astrocytes, and microglia promote/regulate neurogenesis (Palmer et al. 2000, Kokaia et al. 2012). As expected, proliferation and neurogenesis were significantly reduced in the GCL/SGZ of the DG after both single and fractionated doses (Figures 3A, 3B, 4A–C). Also, there was no significant difference in the total number (data not shown) or percentage of surviving new neurons after single and fractionated doses (Figure 3B). However, single doses resulted in a dose-dependent decrease in neurogenesis that was not observed after fractionated doses (Figure 3B).

A previous study by Monje et al. (2003) found a negative correlation ($R = -0.93$) between activated microglia and neurogenesis which led them to conclude that the extent of inflammation may have a direct titrating role on neurogenesis within the adult DG. We measured a similar negative correlation ($R = -0.99$) when the percentage of activated microglia was plotted as a function of the percentage surviving newborn neurons after single doses (data not shown). The dose-dependent decrease in neurogenesis that we measured after single doses may be due, at least in part, to this proposed ‘direct titrating role’ of inflammation on neurogenesis. No correlation was detected between the percentage of activated microglia and the percentage of newborn neurons after fWBI. All of these results

suggest that the inflammatory microenvironment in the brain after single doses is likely much different than the inflammatory microenvironment after fractionated doses.

In conclusion, our results indicate that the brain's response after single doses often does not predict its response after fractionated doses. Consequently, the selection of a biologically relevant irradiation scheme will depend on the nature of the radiation event being modeled. The biological response to single doses is clearly relevant to understanding the biological effects after catastrophic military, terrorism and accidental exposures. Single doses are also likely relevant to understanding the biological response after Gamma Knife/stereotactic radiosurgery. However, it is likely that only prolonged 'biologically equivalent' fractionation schedules will provide relevant information to understanding the biological response to the 3–6 week fractionated exposures used routinely in the clinic to treat cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to dedicate this article to the memory of the senior author, Mike Robbins, PhD (1954–2012).

The authors alone are responsible for the content and writing of the paper.

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Figure 1.

The microglia response after single and 'biologically equivalent' fractionated doses at 2 months post-irradiation. (A) The total number of microglia were not significantly different in rats after single (■) and fractionated (▨) doses ($p > 0.2$ and $p > 0.6$, respectively) compared to the total number of microglia in sham-irradiated rats. However, the total number of microglia was significantly ($p < 0.04$) decreased in rats after single doses when compared to the number of microglia in rats after fractionated doses. (B) Rats irradiated with single doses had a significant increase in surviving newborn (BrdU+) microglia when

compared to sham-irradiated or fractionated fWBI rats ($p < 0.05$ and $p < 0.0001$, respectively). (C) The percentage of activated microglia was significantly ($p < 0.003$) increased after single doses, but not after fractionated doses. Rats irradiated with single doses had an increase in the percentage of activated microglia when compared to rats irradiated with fractionated doses ($p < 0.004$). Data are presented as Mean $\pm$ SEM; $n = 4$ per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2.

Response of microglia to single or fractionated doses at 2 months after whole-brain irradiation. Panels A–C: Representative regions of the dentate gyrus/hilus showing the total number and BrdU+ microglia in rats that were sham-irradiated (A) irradiated with a single dose of 16.5 Gy (B), or irradiated with fractionated total dose of 40 Gy (C). The total microglia (★) after both single (B) and fractionated (C) doses was not significantly different from that in sham-irradiated (A) rats. Rats irradiated with a single 16.5 Gy dose (B) had a significant increase in the total number of BrdU+ microglia (●) compared to the rats that were sham-irradiated (A) or irradiated with a 40 Gy fractionated dose (C). The total number of activated microglia (★) was significantly increased after both single 16.5 Gy (E) and 40 Gy fractionated (F) doses when compared to that in sham-irradiated (D) rats. A single 16.5 Gy dose (E) resulted in a higher percentage of activated microglia than after a fractionated 40 Gy dose (F).

Figure 3.

The proliferation and neurogenic response of the brain to single doses and 'biologically equivalent' fractionated doses at 2 months after whole-brain irradiation. (A) The total number of proliferating cells decreased to the same extent after single (■) and fractionated (▤) doses. (B) The percentage of surviving newborn neurons was significantly ($p < 0.02$) decreased to the same extent after single and fractionated doses. However single dose decrease was dose-dependent ($p < 0.02$); the fractionated dose decrease was not. Data are presented as the Mean $\pm$ SEM; $n = 4$ per group.

Figure 4. Neurogenic response of the brain after a single 16.5 Gy dose and a ‘biologically equivalent’ fractionated 40 Gy dose at 2 months post-irradiation. Both the single (B) and fractionated (C) doses produced a significant ($p < 0.02$) decrease in surviving newborn neurons (●) when compared to the surviving newborn neurons in sham-irradiated (A) rats.