Progress Report (Final – cycle 3; w/NCE): Report period: Dec 2010 to June 2014

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Scope of Work - Overview

We have now tested these ideas by irradiating multipotent neural cells derived from humans and rodents and by undertaking an extensive in vivo analysis of irradiated mice to assess the impact of low dose exposures on neurogenesis. The following summarizes our progress over the funding period.

Progress to date: In vitro studies

We have continued our characterization of radiation effects in multipotent neural cells and have used a number of different irradiation paradigms that include low dose and low dose rate experiments using a ^{57}Co flood source, low dose and high dose rate studies using a ^{137}Cs irradiator and low dose and multiple dose rate studies using protons at Loma Linda University.

Flood source studies: We have subjected a range of neural stem cells to flood source exposures (~1-2 cGy/day) and found that under these conditions, oxidative stress was induced. Cells routinely exhibited higher levels of ROS/RNS and superoxide, increased proliferation, and enhanced DNA repair. Oxidative stress increased by ~ 1.5 fold over sham irradiated controls, while flood source doses of 10 cGy led to a rise (~4%) in proliferation when assayed 4 and 5 days later. When flood source exposed cells were subjected to a 5 Gy challenge dose, survival increased from 5-7% when compared to cell exposed acutely. Cells subjected to similar dosing paradigms and assessed for DNA DSB repair by following the formation and removal of g-H2AX foci showed enhanced rates of DSB repair, suggesting that flood source exposure primed the DNA damage response, possibly through redox sensitive signaling, to facilitate the repair of cytotoxic DNA DSBs.

Low dose studies with multipotent neural cells: Neural stem cells subjected to low dose exposure were also analyzed by 2-photon microscopy to measure their metabolic index (MI), quantified as the individual fluorescence signals of flavoproteins and NAD(P)H. We have found that low dose irradiation elicits a drop in MI, suggesting that cells were preparing to bolster their proliferative state by shunting energy reserves away from ATP production in favor of the synthesis of macromolecular precursors. Dose-dependent drops in MI were not found at higher doses and point to the differences between low and high dose radiobiology, where lower doses tend to stimulate multipotent neural cell division while higher doses impede this process.

Low dose exposure alters gene expression profiles analyzed by qRT-PCR. Neural stem cells exposed to low dose γ-rays were evaluated for specific neurotrophic factors, along with multipotent and cell fate specific markers over a 2-week period of differentiation. Compared to unirradiated controls, exposure to relatively low dose gamma irradiation increased brain-derived (BDNF) and glial cell-line derived (GDNF) neurotrophic factors from 10-100 fold at various post-irradiation times. Further analyses of irradiated cultures (0.5 – 2 Gy) revealed that the immature neuronal marker beta-tubulin (tuj1) showed a dose-dependent enhancement (from 2 to

5-fold) compared to unirradiated controls. Ongoing work will extend these studies to other multipotent neural stem cells (see below) and additional antioxidant enzymes (SOD, catalase, GPx and TRdx).

To more fully characterize the response of pluripotent and multipotent stem cells to irradiation. Human embryonic stem (ES) cells, human induced pluripotent stem (iPS) cells, and iPS-derived human neural stem cells (iPS-hNSCs) cells were irradiated and analyzed for cell survival parameters, differentiation, DNA damage and repair and for oxidative stress at various times after exposure. All cells exhibited dose-dependent reductions in cell number and a dose-dependent increase in the metabolic activity in surviving cells. Irradiation did not preclude germ layer commitment of ES cells, but did promote the neuronal differentiation of hNSCs from either source, as found above. ES cells subjected to irradiation exhibited early apoptosis, inhibition of cell cycle progression, but otherwise normal repair of DNA double-strand breaks. Cells surviving irradiation also exhibited ~~a~~ significantly higher levels of reactive oxygen and nitrogen species, superoxide and nitric oxide. The enhanced levels of these reactive species underscores the capability of stem cells to alter their redox homeostasis to adapt to noxious stimuli, and suggests that radiation-induced oxidative stress plays a role in regulating the function of stem cells within tissues compromised by radiation injury.

Loma Linda Studies: Rodent neural stem and precursor cells grown as neurospheres and human neural stem cells grown as monolayers were subjected to acute and multi-dosing paradigms of 250 MeV protons and γ-rays at differing dose rates and analyzed for changes in reactive oxygen species (ROS), reactive nitrogen species (RNS), nitric oxide and superoxide for 2 days after irradiation. While acute exposures led to significant changes in both cell types, hNSCs in particular, exhibited marked and significant elevations in radiation-induced oxidative stress. Elevated oxidative stress was more significant in hNSCs as opposed to their rodent counterparts, and hNSCs were significantly more sensitive to low dose exposures in terms of survival. Combinations of protons and γ-rays delivered as lower priming or higher challenge doses elicited radioadaptive changes that were associated with improved survival, but in general, only under conditions where the levels of reactive species were suppressed compared to cells irradiated acutely. Data corroborates much of our past work and shows that low dose and dose rate exposures elicit significant changes in oxidative stress that have functional consequences on survival.

Progress to date: In vivo studies: To analyze the consequences of low dose irradiation on neurogenesis and if/how activated microglia might impact that process, wild type mice and mice deficient in chemokine receptor 2 (CCR2), the receptor for monocyte chemoattractant protein-1 (MCP-1) were analyzed. The inability of CCR2 knock out mice to activate microglia after irradiation provides the capability to directly test whether radioadaptation caused by prior exposure depends (or not) on neuroinflammation mediated by microglia.

Animals were irradiated as 4 separate cohorts, (0 Gy, 10 cGy, 2 Gy, 10 cGy→2 Gy) with the latter radioadpative cohort involving a 24h time interval between priming and challenge doses involving whole body irradiation with a ^{137}Cs source. 5 days post-irradiation, all mice received daily BrdU injections that continued for 6 days (BrdU at 50mg/kg; 12.5mg/mL). Following the administration of BrdU (25 days) mice were perfused (4% PFA) and prepared for coronal sectioning at 30μm per section and preserved in cryobuffer (ethylene glycol + glycerol + PBS) the assessment of inflammatory and oxidative endpoints by immunohistochemistry.

In vivo immunostaining for neurogenic markers: For WT mice, analysis of BrdU/NeuN doubly labeled cells indicates that either dose (*i.e.* 10cGy or 2Gy) leads to a reduction of mature neurons. A similar response was also found in the radioadpative cohort, indicating that prior exposure was unable to rescue neurogenesis following the higher challenge dose. However, analysis of these same parameters in CCR2 KO mice shows a potential benefit of the priming dose, as the number of proliferating cells in the dentate gyrus of the hippocampus was elevated. The 10cGy priming dose tends to increase the number of proliferating cells and neurogenesis in the hippocampus of CCR2 KO mice, and was significantly different from WT mice. While further work needs to be completed to establish whether such effects are significant, data does suggest that the inability to activate microglia is beneficial, and leads to enhanced survival and neuronal development in the irradiated brain.

Interestingly, the CCR2 KO mice have an inherently higher number of IBA-1 positive resident microglial cells in each of the cohorts analyzed compared to the WT mice. Radioadapative dosing paradigms had little impact on the number of cells expressing IBA-1. A similar pattern was observed in the WT mice. Neither priming nor challenge doses led to any drastic changes in of expression in IBA-1 in WT or CCR2 KO mice. Based on forgoing data we know that the number of resident microglial cells is relatively higher in the CCR2 KO mice as compared to the WT groups.

We are also analyzing the expression of activated microglial cells following irradiation by RT-PCR by immunohistochemistry (ED-1, CD11c work ongoing) and by qRT-PCR. For the qRT-PCR analyses another cohort of WT mice (20 mice total; 4 per dosing group) were ordered for the purpose of determining gene expression levels of the markers IBA-1, ED-1 (activated microglial cells), and CD11c (transmembrane protein involved in the release of reactive oxygen species). Mice (2 months of age) were irradiated as before and sacrificed one month afterwards. Mice were not perfused for these studies, but sacrificed directly, then dissected to isolate the spleen, hippocampus, cortex, and cerebellum. Once dissected, tissues were placed in RNAlater solution (Ambion) and stored at -20°C. RNA extractions are currently underway on each individual sample using an RNA isolation reagent, Trizol (Ambion). Taqman probes will be used for the qRT-PCR.

Current status

We have requested and received No Cost Extensions for completing data collection and analyses and are now completing manuscript submissions. Manuscript/s detailing our DOE studies will be finalized by years end.