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Research Paper

Persistent oxidative stress in human neural stem cells exposed to low fluences of charged particles

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ABSTRACT

Exposure to the space radiation environment poses risks for a range of deleterious health effects due to the unique types of radiation encountered. Galactic cosmic rays are comprised of a spectrum of highly energetic nuclei that deposit densely ionizing tracks of damage along the particle trajectory. These tracks are distinct from those generated by the more sparsely ionizing terrestrial radiations, and define the geometric distribution of the complex cellular damage that results when charged particles traverse the tissues of the body. The exquisite radiosensitivity of multipotent neural stem and progenitor cells found within the neurogenic regions of the brain predispose the central nervous system to elevated risks for radiation induced sequelae. Here we show that human neural stem cells (hNSC) exposed to different charged particles at space relevant fluences exhibit significant and persistent oxidative stress. Radiation induced oxidative stress was found to be most dependent on total dose rather than on the linear energy transfer of the incident particle. The use of redox sensitive fluorogenic dyes possessing relative specificity for hydroxyl radicals, peroxynitrite, nitric oxide (NO) and mitochondrial superoxide confirmed that most irradiation paradigms elevated reactive oxygen and nitrogen species (ROS and RNS, respectively) in hNSC over a 1 week interval following exposure. Nitric oxide synthase (NOS) was not the major source of elevated nitric oxides, as the use of NOS inhibitors had little effect on NO dependent fluorescence. Our data provide extensive evidence for the capability of low doses of charged particles to elicit marked changes in the metabolic profile of irradiated hNSC. Radiation induced changes in redox state may render the brain more susceptible to the development of neurocognitive deficits that could affect an astronaut's ability to perform complex tasks during extended missions in deep space.

Introduction

Ionizing radiations come in many types and a critical property that distinguishes sparsely from densely ionizing radiations is stopping power, or the rate at which energy is deposited per unit track length. Linear energy transfer (LET), expressed in keV/μm, is the term used to classify radiation quality in terms of high versus low LET. With the exception of protons, the charged particles found in space fall into the high LET category [1,2]. These fully ionized, highly energetic nuclei are referred to as HZE particles, derived from high (H) atomic number (Z) and energy (E). While radiobiologist have quantified the differences among radiations of varying LET for numerous biological endpoints, characterizing similar responses for central nervous system (CNS) endpoints has proven more challenging [3]. LET dependent trends for changes in cognition, electrophysiology, neurogenesis and related biochemical and structural parameters have been difficult to demonstrate conclusively owing to the complexity of the CNS radiation response [3]. A large fraction of the brain is comprised of radioresistant, post-mitotic cells and at space relevant fluences, radiation induced changes transpire in the relative absence of cell death [4]. As a result, it is necessary to measure neuronal function and to identify biochemical changes that impact cognitive performance.

Despite the general radioresistance of the brain, the neurogenic regions contain crucial radiosensitive populations of neural stem and progenitor cells that can be depleted by exposures to relatively low doses of radiation [5,6]. While the radiation induced depletion of stem cell pools in the brain has been shown to disrupt neurogenesis and cognition at higher, clinically relevant doses of radiation [7,8], it is unclear whether similar effects will be observed following exposure to space radiations at doses expected to be incurred during short term or extended space travel. As NASA contemplates longer term missions beyond the protective

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magnetosphere of Earth, the capability of charged particles to elicit changes in redox metabolism become increasingly important since biochemical changes capable of causing oxidative stress provide a mechanism for altering regional or global neurotransmission [9]. Considerable work from our laboratory has clearly demonstrated that radiations of varied quality (i.e. LET) elicit oxidative stress to differing extents [9–12]. Higher LET radiations were found to elicit more persistent and significant increases in oxidative stress than lower LET radiation modalities [9,12]. While these and other studies have characterized the capability of HZE particle exposure to elicit oxidative stress, they did not undertake a systematic study to determine how changes in LET and energy of different incident particles impact the onset and duration of radiation induced oxidative stress. Here we report our findings using human neural stem cells exposed to four different charged particles and three different energies to elucidate the microdosimetric relationships for the induction and persistence of oxidative stress.

Materials and methods

Cell culture

Low passage EnStem-A human neural stem cells (EMD Millipore, Billerica, MA) were cultured in flasks treated with poly-L-ornithine (20 μg/ml, Sigma-Aldrich, St. Louis, MO) and laminin (5 μg/ml, Sigma-Aldrich). Cells were maintained at 37 °C and 5% CO_2 in EnStem-A neural expansion media (EMD Millipore) containing neurobasal media supplemented with L-glutamine (2 mM, Invitrogen, Life Technologies, Grand Island, NY), basic fibroblast growth factor (20 ng/ml, EMD Millipore) and 1 × MEM non-essential amino acids (Life Technologies). These cells were routinely passaged 1:2 every other day.

Irradiation

One day prior to irradiation, cells were passaged into pre-coated T-25 flasks. These exponentially growing hNSC were either sham irradiated or exposed to 5–100 cGy doses of ^{16}O, ^{28}S, ^{48}Ti or ^{56}Fe particles (600 MeV/n; 10–50 cGy/min). For ^{28}Si and ^{56}Fe particles, energy escalations were also evaluated using 300, 600, 1000 MeV/n beams. Cells were irradiated at the NASA Space Radiation Laboratory (NSRL) at the Brookhaven National Laboratory, Upton, NY. All necessary dosimetry was provided by the Physics Dosimetry Group at the NSRL as previously described [9].

Cell survival analysis

Cell survival was determined using SYBR green fluorescence to quantify DNA using previously described protocols [9]. Briefly, following irradiation the hNSC were seeded into 24 well plates at a density of 50,000 cells per well, maintained for 5 days post-irradiation, and then frozen without media at −80 °C. Cells were then lysed using Mammalian Protein Extraction Reagent (Thermo Fischer Scientific, Waltham, MA) containing 2.5 × SYBR Green I (Invitrogen). SYBR Green I fluorescence was detected using a Synergy MX microplate reader and settings of 497 nm excitation and 520 nm emission (BioTek, Winooski, VT). Cell counts were determined by comparison to standard curves generated for the EnStem hNSC line. The data are presented as mean ± SD of at least 3 replicates after each had been normalized to its respective unirradiated control.

ATP assay

Following irradiation, hNSC were counted and seeded at 500,000 or 200,000 cells per well into coated 24 well plates for analysis at 3 or 7 days post-irradiation, respectively. The ATP assays were preformed as recommended in the manufacturer protocol (Perkin Elmer, Waltham, MA). Briefly, at the appropriate time point cells were lysed in their respective well using the ATPlite lysis buffer, shaken for 5 min and then frozen at −80 °C. Subsequently, luciferase assays were performed as described in the manufacture's protocol. The data are presented as mean ± SD of at least 3 replicates after each had been normalized to its respective unirradiated control.

Oxidative stress

The detection of intracellular ROS and RNS was based on the ability of live cells to oxidize fluorogenic dyes to their corresponding analog. Exponentially growing cells were treated with the ROS/RNS sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate (CM-H_2DCFDA or CM; 5 μM; Invitrogen), the nitric oxide sensitive dye 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF; 5 μM; Invitrogen), or the superoxide sensitive dye Mito-SOX (MS; 0.5 μM; Invitrogen). Immediately after incubation with dye, cells were harvested and analyzed by flow cytometry. For each post-irradiation time, measurements of ROS/RNS (CM), NO (DAF), or superoxide (MS) in irradiated and sham irradiated control cultures were performed in parallel. All measurements were performed in triplicate from separate, independently irradiated flasks of cultured hNSC and the data presented as mean ± SD after each sample had been normalized to its respective unirradiated control.

Inhibition of nitric oxide synthases (NOS)

In order to determine whether nitric oxide synthases (NOS) contributed to radiation induced oxidative stress, hNSC were treated with either 100 μM of the NOS inhibitors L-N^5-(1-iminoethyl)ornithine hydrochloride (L-NIO, Sigma-Aldrich) or N^G-methyl-L-arginine acetate salt (L-NMMA, Sigma-Aldrich). Immediately following irradiation, NOS inhibitor drugs were prepared in culture medium and added to the cells. The cells subsequently received a media change containing freshly prepared inhibitor every 2–3 days and were maintained at 50% confluence until FACS analysis was performed. FACS analysis was performed on post-irradiation day 3 and on day 7 to measure oxidative stress.

Statistical analysis

For all assays, data for irradiated cells were normalized to that assay's sham irradiated concurrent control level for each time point. Data are presented as the mean of at least 3 replicate experiments ± SD. The data were assessed for significance (*i.e.* $P \leq 0.05$) by analysis of variance (ANOVA) with comparisons between groups performed using Bonferroni's post-hoc test using Prism3 software.

Results

Dose dependent survival in human neural stem cells

Human neural stem cells were irradiated with 600 MeV/n ^{28}Si or ^{56}Fe particles using a range of doses (0–100 cGy) to investigate the effects on cell proliferation. As compared to unirradiated controls, cell counts were not decreased by the 5 cGy dose of ^{28}Si particles, but were reduced significantly following the 25 and