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Antioxidant Dietary Supplementation in Mice Exposed to Proton Radiation Attenuates Expression of Programmed Cell Death-Associated Genes

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Abstract

Dietary antioxidants have radioprotective effects after ionizing radiation exposure that limit hematopoietic cell depletion. We sought to determine the mechanism of proton-induced hematopoietic cell death in animals receiving a moderate dose of whole-body proton radiation. In addition, animals were maintained on diets supplemented with or without dietary antioxidants. In the presence of the dietary antioxidants, total bone marrow mRNA and protein expression of apoptosis-related genes were decreased compared to the expression profiles in the irradiated mice not receiving the antioxidant formulation. These data confirm high-energy proton-induced gene expression of classical apoptosis markers including BAX, caspase-3 and PARP-1. Antioxidant supplementation resulted in decreased expression of these genes in addition to increased protein expression of the anti-apoptosis markers Bcl2 and Bcl-xL. In conclusion, oral supplementation with antioxidants appears to be an effective approach for radioprotection against hematopoietic cell death.

INTRODUCTION

NASA's plans for the future will include exploration class missions in which astronauts will have increased risks of adverse health effects due to exposure to space radiation. Of particular concern for astronaut health and subsequent completion of mission objectives is potential exposure to solar particle event (SPE) radiation. SPE radiation consists primarily of high-energy protons. The estimated maximum deep dose of SPE radiation is 2 Gy (1).

Previously we determined that dietary antioxidants prevent the loss of hematopoietic cells induced by total-body irradiation (TBI) with X rays (2) and reduce the number of proton-induced neoplastic lesions derived from cells of hematopoietic origin (3). Recently, we completed a study using proton TBI and reported that antioxidant supplementation prevents the radiation-induced decrease of (1) circulating white blood cell numbers, (2) bone marrow cellularity, and (3) spleen mass in animals exposed to 1 Gy protons (4). As part of this previously published study, we sought to determine the molecular mechanism(s) involved in proton radiation-induced and antioxidant-mitigated death of bone marrow-derived cells.

It has been reported that apoptosis plays a role in proton-induced tumor cell death; however, only lethal doses of 10–12 Gy were used in these studies (5–7). Besides the morphological appearance of chromatin condensation or DNA ladder formation, biomarkers for the transduction of apoptotic signaling include gene expression and protein expression patterns for initiator caspases, such as caspase-8, and executioner caspases, including caspase-3 (8). We report here that the expression of genes involved in apoptosis is differentially regulated in hematopoietic cells of the bone marrow in animals receiving a moderate dose of total-body radiation (1 Gy) from protons. Thus apoptosis may contribute to the observed proton-induced decrease in hematopoietic cell counts reported previously (4). Further, we show that supplementation with dietary antioxidants mitigates the expression of apoptosis-related genes.

METHODS

Animals

Male ICR mice aged 4–5 weeks were purchased from Taconic Farms Inc. (Germantown, NY). Animals were acclimated for 7 days in the Brookhaven National Laboratory (BNL) Animal Facility. Ten animals were housed per cage (19'' L × 10 ½'' W × 6 ⅛'' H) with *ad libitum* access to water and rodent chow. The animal care and treatment procedures were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and BNL. Rodent chow (AIN-93G) was prepared by Bio-Serve (Frenchtown, NJ). Antioxidant-supplemented chow was prepared with the following combination of dietary agents: vitamins C and E, α-lipoic acid, coenzyme Q10, N-acetylcysteine (NAC) and L-selenomethionine (SeM). The levels of SeM, vitamin E and ascorbic acid used in these studies are equivalent on a weight basis in humans to the established maximum levels of daily nutrient intake that are likely to pose no risk of adverse effects. The antioxidant combination in the animal diets was formulated to provide the equivalent of 2000 mg/day, 1000 mg/day and 400 μg/day, which represent the upper limit of the established Recommended Dietary Allowances (RDAs) for vitamin C, vitamin E succinate and selenium (9). The control diet (AIN-93G rodent diet) contains vitamin E (75 IU/kg) and selenium (0.18 mg/kg) at levels in the animal diets that are comparable on a weight basis to the human RDA levels of these compounds. Although there is no published RDA for NAC or α-lipoic acid, these thiol supplements were formulated according to previously determined effective doses in humans, 2400 mg/day and 1200 mg/day, respectively (10, 11), that did not exhibit chronic toxicity. Supplemented or non-supplemented AIN-93G was apportioned to the animals 1 week prior to the date of irradiation and after irradiation until euthanasia (chow was not available to animals during the radiation exposure).

Irradiation

Total-body irradiation of animals was performed with 1 GeV protons [approximate linear energy transfer (LET) of 0.24 keV/μm] at dose rates ranging from 20–70 cGy/min at the NASA Space Radiation Laboratory (NSRL) at BNL. Proton irradiation was carried out within the nonstopping region (Bragg plateau) of the curve for energy deposition as a function of depth. The animals were restrained in plastic holders during radiation exposures and were returned to their cages afterward. Animals were returned to the animal facility once their radiation levels were determined to be at background. Sham-irradiated animals were restrained similarly.

Bone Marrow Cell Isolation, Quantification, RNA Isolation and cDNA Synthesis

Animals were euthanized by CO₂ asphyxiation 4 h after irradiation or sham irradiation. Tibiae and femurs were removed and the ends of the bones were bluntly cut. The bone marrow cavity was flushed with PBS using a sterile 22-gauge needle. The bone marrow was

carefully resuspended to obtain a single cell suspension, centrifuged at 1000 rpm for 10 min at 4°C, resuspended in RNeasy lysis solution (Qiagen, Valencia, CA), or directly frozen at -80°C. Total RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was synthesized using the Superscript II First Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA) using 2 µg Total RNA, then was diluted, frozen in aliquots, and stored at -20°C until use.

Quantitative Real-Time PCR

Primers were designed using the default parameters of the Primer Express® software v2.1 (PE Applied Biosystems, Foster City, CA). Primer sets were designed based on the relevant murine nucleotide sequences as deposited on GenBank and supplied by IDT (Coralville, IA). The primer sequences were as follows:

BAX (Bcl-2 associated X protein) forward primer 5'-AGACACCTGAGCTGACCTTGGA-3' and reverse primer 5'-GAGACACTCGCTCAGCTTCTTG-3'; Bcl2 forward primer 5'-TGCCACCTGTGGTCCATCT-3' and reverse primer 5'-GTGCAGCTGACTGGACATCTCT-3'; caspase-9 forward primer 5'-TCACGGCTTTGATGGAGATG-3' and reverse primer 5'-GAGGATGACCACCACAAAGCA-3' caspase-8 forward primer 5'-AAGGCAATCTGTCTTTCCTGAAA-3' and reverse primer 5'-TCGGTTGCAGTCTAGGAAGTTG-3'; TGFβ1 (Transforming growth factor beta, one) forward primer 5'-TGGAGCAACATGTGGAATC-3' and reverse primer 5'-GTCAGCAGCCGGTTACCA-3'; NFκB1 forward primer 5'-GACCACTGCTCAGGTCCACT-3' and reverse primer 5'-TCATCTATGTGCTGCCTCGT-3'; GAPDH forward primer 5'-CATGGCCTTCCGTGTTCTTA-3' and reverse primer 5'-GCGGCACGTCAGATCCA-3'.

cDNA was amplified in Sybr Advantage qPCR Premix (Clontech, Mountain View, CA) consisting of a full-length Taq™ polymerase with a hot-start Taq antibody and SYBR Green I by using an ABI 7300 Real Time PCR System (PE Applied Biosystems). A sample volume of 20 µl that contained a 1× final concentration of SYBR Advantage Premix, 250 nM gene specific primers, and 2 µl cDNA template was used for all assays. Assays were carried out using the following protocol: stage 1, 95°C for 20 s, stage 2, 95°C for 3 s, 58–64°C for 34 s (depending on optimal annealing temperature), with stage 2 repeated for 40 cycles followed by the dissociation stage. A cycle threshold (C_t) was assigned at the beginning of the logarithmic phase of PCR amplification, and the differences in the C_t values of the control and experimental samples were used to determine the relative expression of the gene in each sample. Relative expression levels were normalized to the constitutively expressed housekeeping gene GAPDH.

Western Blot Analysis

Frozen bone marrow cell pellets were homogenized in homogenizing buffer: 0.1% Tween-20 in phosphate-buffered saline containing phosphatase inhibitors (Calbiochem, San Diego, CA) and protease inhibitors (Sigma, St. Louis, MO). Equal concentrations of protein lysates were separated by SDS-PAGE (4–12%) under reducing conditions followed by electrotransfer to a nitrocellulose membrane (Invitrogen, Carlsbad, CA). Membranes were blocked in 5% nonfat dry milk and incubated with anti-Bcl2, anti-Bcl-xL, anti-PARP1, anti-caspase-3 (Cell Signaling), or β-actin (Abcam) for 1 h at room temperature. Blots were washed three times with 0.1% in Tris buffered saline and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were

visualized using the enhanced chemiluminescence method (GE Healthcare Life Sciences, Piscataway, NJ).

Data Analysis

Data were analyzed using SigmaPlot 10.0 software. To determine whether there were statistically significant differences between the treatment groups, the Student's *t* test was used to compare multiple group means. Differences were considered significant when $P < 0.05$.

RESULTS

mRNA Expression of Apoptosis-Related Genes are Upregulated 4 h after Proton TBI

Four hours after 1 Gy proton TBI, total bone marrow cells were analyzed for mRNA expression of the following genes: Bax, caspase-9, caspase-8, Bcl2, NF κ B1 and TGF β 1. For all genes except Bcl2, a significant increase in mRNA expression was observed in irradiated mice compared to the sham-irradiated controls (Fig. 1A–E). No statistically significant changes were observed in mRNA levels in the sham-irradiated mice fed the antioxidant-supplemented diet (compared to the sham-irradiated mice fed the non-supplemented diet). Bcl2 expression appeared to increase slightly in the sham-irradiated, antioxidant-supplemented mice; however, no statistically significant differences were observed between the treatment groups (Fig. 1F).

Antioxidant Supplementation in the Diet of Proton-Irradiated Mice Attenuates Radiation-Induced Expression of Programmed Cell Death-Related Genes

A significant decrease in expression of genes that were induced by protons was observed in the proton-irradiated mice fed the antioxidant-supplemented diet (Fig. 1B–E). Bax mRNA expression was decreased in the antioxidant-supplemented mice exposed to radiation compared to the nonsupplemented mice exposed to radiation; however, this decrease did not reach statistical significance (Fig. 1A). Caspase-9, caspase-8, NF κ B1 and TGF β 1 mRNA expression in the irradiated antioxidant-supplemented mice was comparable to that in the nonirradiated antioxidant-supplemented mice, suggesting that the dietary antioxidant supplementation may prevent radiation-induced apoptotic gene expression. There was a statistically significant reduction in the expression levels of these genes in the irradiated mice maintained on the antioxidant-supplemented diet compared to the irradiated mice maintained without the antioxidant-supplemented diet. Bcl2 mRNA expression was upregulated in the irradiated antioxidant-supplemented mice compared to the nonsupplemented mice (Fig. 1F); however, the differences between the treatment groups did not reach statistical significance. Bcl2 is an anti-apoptosis regulator (12).

Proton Radiation-Induced Protein Expression of Bcl-Related Proteins is Augmented in the Presence of Antioxidant Supplementation

Bcl2 and Bcl-xL protein expression were detected in total bone marrow cells of mice after proton TBI. In the sham-irradiated control mice, antioxidant supplementation resulted in increased levels of the apoptosis antagonist protein Bcl2 (Fig. 2A). In the presence of radiation, antioxidant supplementation augmented the expression of both Bcl proteins compared to sham-irradiated controls and irradiated mice fed the non-supplemented diet. These data suggest that the anti-apoptosis Bcl-related proteins respond to antioxidant supplementation in both the absence and presence of radiation.

TGFβ1 Expression is Upregulated after Proton TBI

Protein expression of the multifunctional cytokine TGFβ1 was assessed 4 h after proton TBI. Radiation exposure significantly increased the mRNA (Fig. 1D) and protein (Fig. 2A) expression of TGFβ1 in the bone marrow of mice exposed to 1 Gy protons. Radiation-induced mRNA (Fig. 1D) and protein (Fig. 2A) expression were reduced in the presence of antioxidants.

Proton TBI Induces Cleavage of Pro-apoptosis Proteins

Protein expression of two pro-apoptosis genes was evaluated in the bone marrow of mice after proton TBI. PARP-1 cleavage was detected by Western blot analysis using an antibody specific to the cleaved (or activated) form. As a marker for apoptosis, PARP-1 is cleaved by caspase-3 (13). Figure 2B shows significant amounts of cleaved PARP-1 in the bone marrow lysates of mice after 1 Gy proton TBI. Caspase-3 activation is shown in Fig. 2B. Cleaved or activated caspase-3 protein expression is pronounced in the bone marrow of mice after 1 Gy proton TBI compared to the sham-irradiated controls. Unexpectedly, cleaved caspase-3 was detected in the sham-irradiated controls, as shown by Western blot analysis (Fig. 2B), in the absence and presence of antioxidant supplementation, suggesting that the apoptotic program is activated without radiation exposure. However, the levels of cleaved caspase-3 in the irradiated lysates (without antioxidants) are significantly higher than those in the sham-irradiated control lysates (without and with antioxidants), and in the lysates of irradiated mice fed the diet with the antioxidant supplementation.

DISCUSSION

The objective of this study was to characterize the acute molecular response to whole-body proton irradiation in the absence and presence of dietary antioxidants in mice *in vivo*. These data confirm the high-energy proton-induced gene expression of classical markers of apoptosis, including the activation of the downstream effectors caspase-3 and PARP-1 (Fig. 2B), in the bone marrow of mice. In the presence of the dietary antioxidants, total bone marrow mRNA and protein expression of apoptosis-related genes were decreased compared to the expression profiles in the irradiated mice not receiving the antioxidant formulation. It is speculated that the antioxidants are altering mRNA and protein levels by reducing the generation of reactive oxygen species and total cellular antioxidant status (or cellular oxidative stress). A general scheme of the apoptosis-related genes and their potential roles in the mitochondria after ionizing radiation exposure and in the presence of antioxidants is illustrated in Fig. 3.

Significant increases in BAX, Bcl2 and Bcl-xL expression in proton-irradiated mice suggest mitochondrial dysfunction and activation of the cell-intrinsic apoptotic pathway as opposed to the cell-extrinsic pathway involving death receptors (14). BAX, Bcl2 and Bcl-xL, all members of the Bcl superfamily, are downstream target genes of the tumor suppressor gene p53. BAX is a pro-apoptosis gene. In response to cellular stress BAX relocates to the surface of the mitochondria where the anti-apoptosis proteins, including Bcl2 and Bcl-xL, are located. The interaction between the anti- and pro-apoptosis proteins forms pores in the mitochondria, causing the release of cytochrome c, which in turn activates the caspase cascade (Fig. 3). A decrease in mRNA levels of BAX is observed in mice fed the antioxidant diet and exposed to proton radiation (Fig. 1A). The proton radiation-induced increases in BAX mRNA levels are consistent with previous findings of γ-radiation-induced increases of BAX gene expression in hematopoietic tissues (15, 16). Our findings show increased expression of both Bcl2 and Bcl-xL proteins, which prevent the release of cytochrome c (17, 18), in the presence of dietary antioxidants (Figs. 2A and 3).

Caspase-9 mRNA expression is significantly upregulated after proton irradiation and downregulated in the presence of antioxidants after proton irradiation (Fig. 1C). Caspase-9 is activated upon cytochrome c release from the mitochondria, and the activated complex cleaves or activates effector caspase, caspase-3. Caspase-3 cleavage is significantly induced after proton irradiation (Fig. 2B), and protein detection of the activated form of caspase-3 is decreased in the presence of dietary antioxidants (Fig. 2B).

Caspase 8 is known as an initiator caspase of the extrinsic pathway involving transmembrane death receptors. Upon ligation, the death receptors activate caspase-8, initiating effector caspase signaling (19). caspase-8 mRNA expression was increased after radiation exposure (Fig. 1E); however, the active form of caspase-8 was not measured in this study. Expression levels of pro-caspase 8 were significantly decreased after radiation exposure in the presence of dietary antioxidants, suggesting an abrogating effect on this signaling pathway. NF κ B suppresses death receptor signaling (19), and elevated NF κ B mRNA levels were observed after radiation exposure (Fig. 1B), contributing to the extrinsic pathway.

By definition, antioxidants scavenge free radicals. The antioxidants used in this study vary widely in their chemical structure, and this may contribute to other biochemical functions and cellular processes that anti-oxidant treatments have been shown to hinder, including gene expression of apoptosis-related genes (20, 21), as reported here. It is generally accepted that ionizing radiation results in oxidative stress (22) within the biological system and the generation of reactive oxygen species (ROS) (23). ROS are generated in the mitochondria by the respiratory chain, at the level of coenzyme Q10 (one component of the antioxidant mix used in this study). Oxidative stress and ROS are considered to induce apoptotic cell death through direct interaction with DNA or the initiation of the intrinsic pathway for cell death. The latter begins in the mitochondria where ROS cause mitochondrial outer membrane permeabilization (23) and subsequent release of cytochrome c and other proteins. Regulators of the permeabilization include members of the Bcl2 family Bax and Bak, while anti-apoptotic Bcl2 and Bcl-xL inhibit protein release. Cytochrome c release from the mitochondria into the cytoplasm initiates the caspase cascade of events, as mentioned above.

Antioxidant supplementation has been shown to prevent radiation-induced oxidative stress (24). We speculate that antioxidant supplementation protects against radiation-induced oxidative stress by either blocking ROS production or neutralizing mitochondrial ROS in animals exposed to 1 Gy proton radiation. The prevention or scavenging of radiation-induced ROS may reduce mitochondrial membrane permeabilization and the levels of expression of pro-apoptosis-related genes that are intimately involved in the mitochondria-associated intrinsic death pathway. These results using small molecule antioxidants are consistent with those of Bai *et al.* (25, 26), who reported that antioxidant enzymes target mitochondrial ROS, affecting apoptotic stimuli.

It is expected that the prevention of radiation-induced apoptosis will result in enhanced survival of the irradiated cells. Because these cells have evaded apoptosis or cell death, the genomic stability of these cells might be considered questionable. The long-term consequences associated with their survival, however, are unknown. It has been hypothesized that radioprotectors may lead to the rescue of damaged cells that could play a role in tumor initiation or progression and ultimately result in increased carcinogenesis (27). While this is a reasonable hypothesis, the evidence thus far does not suggest that the use of radioprotective agents increases radiation-induced carcinogenesis. Most research on radioprotectors and carcinogenesis has been performed with WR2721/WR1065 (also known as amifostine). Amifostine has been shown to have radioprotective (28), antimutagenic (28) and anticarcinogenic (29) properties in numerous *in vivo* and *in vitro* systems, as has been

reviewed (30) by Grdina et al. Because the abilities of the active compound are quite different for cytoprotection and anticarcinogenic activities, this has suggested that the mechanisms of action and pathways that are involved in rescuing cells from radiation-induced cytotoxic effects are quite different from those involved in protection against radiation carcinogenesis (30). Other radioprotectors that inhibit both radiation-induced cell killing and carcinogenesis include the soybean-derived Bowman-Birk inhibitor and the antioxidant combination used in the studies reported here (2–4, 31–34). These results suggest that for these agents, effects on the genes/pathways leading to the rescue of cells destined to die from the cell killing effects of radiation do not have enhancing effects, and may have suppressing effects, on the genes/pathways involved in radiation-induced carcinogenesis. It can be speculated that abrogation of radiation-induced expression of proapoptosis genes could increase or decrease carcinogenic potential, but the long-term effects of radiation induced modification of gene expression are unknown and warrant further research.

The data presented here suggest that proton radiation induces varying molecular mechanisms that simultaneously promote and suppress programmed cell death. Antioxidant supplementation during proton irradiation significantly decreases expression levels of pro-apoptosis genes conferring protection against apoptosis in cells of hematopoietic origin.

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FIG. 1.

Proton radiation induces mRNA expression of the apoptosis-related genes BAX (panel A), NFκB1 (panel B), caspase-9 (panel C), TGFβ1 (panel D), caspase-8 (panel E), and BCL2 (panel F). Total bone marrow cells were collected 4 h after 1 Gy proton TBI and analyzed by qRT-PCR. Data are expressed as means ± SD from three independent experiments, representing three different mice. GAPDH was used as the loading control. Overhanging lines indicate statistically significant differences between the sham-irradiated and irradiated groups with no antioxidant supplementation (-AOX, black bars) or the irradiated group and the irradiated and antioxidant-supplemented (+AOX, white bars) groups.

FIG. 2.

Proton radiation induces protein expression of the apoptosis-related genes Bcl2, Bcl-xL, TGFβ1 (panel A), cleaved PARP-1 and cleaved caspase-3 (panel B). Total bone marrow cell lysates were prepared for Western Blot analysis 4 h after 1 Gy proton TBI. β-Actin was used as the loading control in these experiments. Each blot is representative of three separate experiments. AOX = antioxidant supplementation.

FIG. 3.
Potential role of antioxidants in the mitochondria in response to proton radiation exposure.