Beta Androstenediol Mitigates the Damage of 1 GeV/n Fe Ion Particle Radiation to the Hematopoietic System

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Abstract

Space exploration is associated with exposure to 1–3 Gy solar particle radiation and galactic cosmic radiation that could increase cancer rates. Effective nontoxic countermeasures to high linear energy transfer (LET) radiation exposure are highly desirable but currently not available. The aim was to determine whether a single subcutaneous injection of androstenediol (Δ^5 androsten-3 β , 17 β -diol [AED]) could mitigate and restore the mouse hematopoietic system from the radiation-mediated injury of 3 Gy whole-body high LET $^{56}\text{Fe}^{26+}$ exposure. The findings show that postradiation AED treatment has an overall positive and significant beneficial effect to restore the levels of hematopoietic elements ($p < 0.001$). Androstenediol treatment significantly increased monocyte levels at days 4, 7, and 14 and, similarly, increased red blood cell, hemoglobin, and platelet counts. Flow cytometry analysis 14 days after radiation and AED treatment demonstrated an increase ($p < 0.05$) in bone marrow cells counts. *Ex vivo* osteoclastogenesis studies show that AED treatment is necessary and advantageous for the development and restoration of osteoclastogenesis after radiation exposure. These findings clearly show that androstenediol functions as a countermeasure to remedy hematopoietic injury mediated by high LET iron ion radiation. Presently, no other agent has been shown to have such properties.

Key words: androstenediol, bone marrow, high LET radiation, hematopoietic, osteoclastogenesis, radioprotector

Introduction

NASA's current Vision for Space Exploration calls for manned missions of longer duration than ever before. By necessity, these forays would require astronauts to spend extended periods beyond low-Earth orbit. However, exposure to charged particle radiation present in space has been identified as a critical risk factor, thereby warranting additional research.¹ Within this type of radiation, energetic iron (Fe) particles are especially important because they are predicted to account for the largest dose-equivalent that astronauts would receive from any single element in galactic cosmic rays.² The potential for increased cancer rates and damage to the central nervous and hematopoietic systems of

astronauts after exposure to these high linear energy transfer (LET) particles is of particular concern.³

Several research groups have recently tested the potential of various compounds to function as possible countermeasures to the negative effects of charged particle, high LET radiation in ground-based studies. Amifostine and other thiols appear to be effective radioprotectors, but they have significant side effects that will likely make them impractical for use in space flights.⁴ Many of the other agents examined thus far are either antioxidants or have antioxidant properties. However, because antioxidants function to scavenge free radicals, they may not be effective against damage by the type of densely ionizing radiation like that present in space, where DNA is ionized directly.⁵ Consequently,

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effective nontoxic countermeasures to high LET radiation exposure are highly desirable but are not presently available. Therefore, this study tested the potential for Δ^5 androsten-3 β , 17 β -diol (AED) to mitigate the negative effects of charged particle radiation exposure to the hematopoietic system of mice. AED has advantages over many of the compounds previously tested for their ability to serve as radioprotectors in that it can be administered at low physiological concentrations, is stable at room temperature for years, and acts by increasing the host immunity. Previous reports demonstrated that a single subcutaneous injection of AED protects the host from death and restores radiation-mediated destruction of the myelopoietic system after exposure to whole-body gamma radiation dose of 8 Gy (LD100 for C57BL/6J; human LD100 6 Gy).⁶⁻⁹ Gridley et al.^{10,11} and Pecaut et al.^{12,13} have reported on the acute effects of whole-body iron particle radiation to C57BL/6J mice. Four days after exposure to a dose of 3 Gy, spleen weight was reduced by 52% and white blood cell (WBC) counts, monocytes, and lymphocytes levels were reduced by about 80%. Exposure to a 2 Gy dose had a similar but less pronounced effects on WBC and bone marrow cell counts.^{11,14,15}

The aim of this study was to demonstrate that a single subcutaneous injection of AED could counteract the depletion of the mouse hematopoietic system resulting from whole-body irradiation with 3 Gy of high LET $^{56}\text{Fe}^{26+}$ ions. The parameters of measurements were counts of WBCs, monocytes, granulocytes, and lymphocytes, bone marrow osteoclastogenic cultures, and fluorescent-activated cell sorter (FACS) analysis of bone marrow cells.

Materials and Methods

Fifty-six male C57BL/6J (6–8 weeks old) mice from Jackson Bar Harbor Laboratory were acclimated at the Brookhaven National Laboratory Animal Facility for 5 days before exposure of 48 animals to whole-body radiation. Eight untreated animals (i.e., no radiation, vehicle, or β AED administration) were used as an additional control. Each unanesthetized mouse was removed from its cage and placed into a rectangular polyethylene holder designed for beam line exposure immediately before irradiation. The holder permits restricted movement of the animal and is inert to the radiation beam. After exposure (3 minutes), the mice were promptly returned to their cages. Eight mice were exposed at a time to a beam of 1 GeV/nucleon $^{56}\text{Fe}^{26+}$ particles delivered to the NASA Space Radiation Laboratory at Brookhaven National Lab. The ion beam was extracted at 1005 MeV/nucleon and had energy at the target surface of 969 MeV/nucleon and an LET of 151.4 keV/m. The dose rate was 1 Gy/min for all irradiations for a total exposure of 3 Gy dose. Thirty minutes after whole-body exposure to a dose of 3 Gy, animals were injected with a single subcutaneous dose of 320 mg/kg of Δ^5 androsten-3 β , 17 β -diol (AED; Sigma Chemical Company), or the vehicle DMSO:EtOH at a 1:1 ratio. The total injection volume was limited to 0.2 mL, and the animals were sacrificed on days 4, 7, and 14 using an overdose of anesthesia.

Hematopoietic procedures

Total differential blood cell counts (i.e., neutrophils, monocytes, granulocytes, lymphocytes, red blood cell [RBC],

and platelet counts) were obtained using a veterinary hematology analyzer (VetScan HMII—Product of Abaxis). Duplicate counts were obtained on 16 vehicle-treated and 17 AED-treated irradiated animals.

Bone marrow cell isolation. Specimens of whole bone marrow were isolated from mouse femurs as previously described.¹⁶⁻¹⁸ Briefly, bone marrow cells were flushed out of the femurs into alpha minimal essential medium (α -MEM) using a 1 mL syringe fitted with a 28-gauge needle and transferred to a sterile 15 mL tube. A portion of the cells were used in osteoclastogenesis analysis. The remainder cells were passed through a 40 μM filter. Cell counts were obtained using a hemocytometer and stained for FACS procedure.

Flow cytometry

For flow cytometry procedures, 0.5 to 3.0×10^6 bone marrow cells were used. Fc receptors were blocked with 0.5 μg of anti-CD16/CD32 per 1.0×10^6 cells and kept on ice for 10 minutes. The following antibodies were used: FITC-B220, FITC-CD90.2, FITC-GR-1, FITC-CD11b, FITC-Ter119, PE/Cy7-Ter119, PE-Gr-1, PE-c-kit, APC-CD11b, and APC-Sca-1 (Biollegend, Inc.). A total of 0.25 μg of each antibody per 1.0×10^6 cells bone marrow cells was added and incubated for 30 minutes at 4°C in the dark. The cells were washed with 5 mL of phosphate-buffered saline (PBS) and centrifuged, and the supernatant was discarded. The cells were then fixed in 1 mL of 4% paraformaldehyde and stored at 4°C in the dark. Before analysis the suspension was washed with PBS, spun down, and resuspended in 0.5 mL of PBS. The cells were run in the Beckton Dickinson FACSCanto machine, and 10,000 events were quantified for each sample. The events were observed in forward scatter (FSC) versus side scatter (SSC) dot plot and the cells were gated, separated from the debris, and analyzed using FCS express software (De Novo Software) in a dual-antibody plot where quadrants were set to separate the negative, positive, or double-positive events. The percentages of the subpopulations under study were obtained and multiplied by the total of cells counts in the suspension.

Osteoclastogenesis

Data were collected from the 7- and 14-day postirradiation mice for this study. Bone marrow cells were added to α -MEM containing 10% FBS and 1% penicillin/streptomycin complete α -MEM and cultured overnight. Nonadherent cells representing a heterogeneous mixture of cells that included monocyte progenitors were collected and transferred to a clean flask and cultured for 7 days in complete α -MEM containing macrophage colony stimulating factor (m-CSF) (50 ng/mL). The medium was changed at mid-week. The resulting adherent cells from this step were bone marrow macrophages (BMM). The monolayers of BMM cells were collected by trypsinization and 2.0×10^4 BMM per well were seeded into 12-well plates for use in osteoclast differentiation. Osteoclastogenesis was initiated by addition of RANKL (100 ng/mL) to complete α -MEM containing m-CSF (25 ng/mL) and culturing for an additional 10 days with medium changes again at mid-week and after 7 days. The progression of osteoclastogenesis was observed by light microscopy and analyzed by tartrate resistant acid phosphatase (TRAP)

staining.^{19,20} On day 10, the cells were fixed with a sodium citrate solution (provided in the acid phosphatase TRAP staining kit; Sigma-Aldrich) acetone (1:4) fixative and washed with PBS, followed by TRAP staining and counterstaining with hematoxylin. Osteoclasts were distinguished as enlarged cells with positive TRAP stain and containing three or more nuclei.

Statistics analysis

The effects of whole-body irradiation on the reduction in hematopoietic cell counts are presented as mean $\pm$ SE, and were analyzed using Excel Student's *t*-test. A three-way analysis of variance (ANOVA) was used to determine the effect of AED over the 14-day experiment.

Results

Effects of high LET radiation

Four days after irradiation with 3 Gy of 1 GeV/nucleon iron ions, a profound effect in the blood cell counts of the mice was measured in accordance with the findings of Gridley and Pecaut.^{10–14} When comparing the exposed animals to the unirradiated controls, total WBC counts were reduced by 86.26% ($p < 0.001$), lymphocyte levels were reduced by 74.72% ($p < 0.01$), monocytes by 77.78% ($p < 0.05$), and granulocytes by 91.79% ($p < 0.01$) (see Figure 1). Further RBC counts were reduced from $8.31 \pm 0.38 \times 10^{12}/L$ in non-irradiated to $7.30 \pm 1.18 \times 10^{12}/L$ in irradiated animals a 12% reduction ($p < 0.01$). Platelets counts were reduced by 34% ($p < 0.01$), from 690.60 ± 134.18 to $457.33 \pm 49.55 \times 10^9/L$, and hemoglobin (HGB) levels were reduced by 25% ($p < 0.01$), from 13.4 ± 0.75 to 10.07 ± 1.47 g/dL (data not shown). Clearly, whole-body exposure to a nonlethal dose of 3 Gy of 1 GeV/nucleon iron ions causes a substantial and significant reduction of peripheral blood elements.

Effects of β AED treatment of irradiated animals

Having verified that exposure to 3 Gy of $^{56}\text{Fe}^{26+}$ ions had a dramatic effect on the hematopoietic system of mice, we

FIG. 1. The effects of irradiation with 1 GeV/nucleon $^{56}\text{Fe}^{26+}$ particles on peripheral WBC counts are illustrated. Data are average value $\pm$ SE. Radiation reduced WBC levels by 86.26% ($p < 0.001$), lymphocyte levels by 74.72% ($p < 0.01$), monocytes by 77.78% ($p < 0.05$), and granulocytes by 91.79% ($p < 0.01$). l, liter; WBC, white blood cell.

sought to determine if AED could mitigate these effects and function as a radioprotector. The effects of a single subcutaneous injection of AED after whole-body exposure to 3 Gy of high LET iron ion radiation on peripheral blood counts are presented in Figure 2. The results show that AED treatment over the 14-day test period is beneficial as compared to treatment with the vehicle. Figure 2 shows an increase in the levels of WBC, granulocytes, and lymphocytes over the 14-day test period. This effect was statistically significant at $p < 0.001$ by three-way ANOVA. Additional evidence to the effects of AED treatment is illustrated in Figure 3, showing that in contrast to its vehicle, AED increased monocyte levels at all time points tested, that is, days 4, 7, and 14, and the

FIG. 2. The effects of a single subcutaneous injection of AED (8.0 mg/25 g mouse) after whole-body radiation exposure to a dose of 3 Gy of high LET iron ions on total WBC, granulocyte, and lymphocyte counts. During the test period a positive effect of AED treatment on peripheral blood cell counts as compared to vehicle is evident ($p < 0.001$). AED, Δ^5 androsten-3 β , 17 β -diol; l, liter; LET, linear energy transfer.

FIG. 3. The effects of a single subcutaneous dose of AED (8.0 mg/25 g mouse) after whole-body radiation exposure to a dose of 3 Gy of high LET iron ions on monocytes levels. Monocyte average $\pm$ SE levels of AED-treated animals as compared to vehicle-treated animals are significantly higher at days 4, 7, and 14, $p < 0.05$ and 0.01, respectively. l, liter.

increase was statistically significant: $p < 0.05$ for days 4 and 7, and $p < 0.01$ for day 14, respectively. Similarly, the result shown in Figure 4 demonstrates a significant effect on the levels of RBC ($p < 0.01$) and HGB concentration ($p < 0.01$) at 7 days after irradiation. By 14 days postradiation, AED treatment of irradiated animals also mediated a significant increase in platelet counts with $p < 0.01$, as illustrated in Figure 5.

Flow cytometric analysis

Additional confirmation of the beneficial effects of AED was obtained from the flow cytometric analysis of bone marrow cells of AED-treated animals versus vehicle-treated animals 14 days postirradiation. AED treatment, as compared to its vehicle, produced a statistically significant increase ($p < 0.05$) in the levels of three different types of bone marrow cells as shown in Table 1. The increase in the levels of these three types of cells indicated a role of AED treatment in reconstitution of myelopoiesis after radiation exposure.

FIG. 4. The effects of a single subcutaneous injection of AED (8.0 mg/25 g mouse) after whole-body radiation exposure to a dose of 3 Gy of high LET iron ions on RBC levels and hemoglobin (HGB) levels 7 days after treatment. The increase in RBC and HGB levels is statistically significant ($p < 0.01$ and 0.01, respectively). l, liter; RBC, red blood cell.

FIG. 5. The effects of a single subcutaneous injection of AED (8.0 mg/25 g mouse) after whole-body radiation exposure to a dose of 3 Gy of high LET iron ions on platelets levels. Platelet levels (average $\pm$ SE) of AED-treated animals as compared to vehicle-treated animals are significantly higher at day 14. l, liter.

Osteoclastogenesis

Osteoclastogenesis was assessed in both the 7- and 14-day postirradiation mice and in nonirradiated control mice. In general, vehicle-treated mice and their AED-treated counterparts were both capable of generating BMM in culture in numbers similar to age-matched control mice that were not irradiated (data not shown). This observation was true in the day 7 and 14 mice. However, the situation for osteoclastogenesis was different. Osteoclastogenesis data from mice 7 days postradiation are represented in Figure 6. As depicted in Figure 6A, irradiated vehicle control mice yielded fragile osteoclasts that formed initially, but underwent rapid cell death by the day of analysis (10 days in the presence of RANKL). Thin arrows point to red TRAP stain, which was present throughout the culture; larger arrows draw attention to remnants of osteoclasts. AED treatment, on the other hand (Fig. 6B), appeared to rescue osteoclastogenesis and promote large multinucleated osteoclasts. Figure 6C and D depicts the grayscale negative versions, and highlights vigorous healthy osteoclasts from the AED-treated mice (Fig. 6D), as opposed to the random, impaired, and incomplete appearance of the vehicle-treated osteoclasts represented in Figure 6C. The data suggest that beta AED treatment is advantageous for the development and restoration of osteoclastogenesis after high LET iron ion irradiation. Follow-up studies will be necessary to quantify these initial findings.

Discussion

Because of the critical need to identify practical countermeasures to the effects of high LET radiation exposure, the goal of this investigation was to initially determine if AED could function in this capacity. A single subcutaneous injection at a concentration of 8 mg/25 g mouse (320 mg/kg) after exposure to 3 Gy of 1 GeV/nucleon $^{56}\text{Fe}^{26+}$ particles resulted in a highly significant elevation in the numbers of WBC, granulocyte, and lymphocytes over the 14 days of testing ($p < 0.001$). Similarly, circulating monocytes, RBC, HGB, platelets, bone marrow CD11b⁺/Gr-1 progenitors,

TABLE 1. FLUORESCENT-ACTIVATED CELL SORTER ANALYSIS OF HEMATOPOIETIC CELLS FROM Δ⁵ ANDROSTEN-3β, 17β-DIOL, OR VEHICLE-TREATED IRRADIATED ANIMALS

	Mean cell count × 10 ⁶ , AED ± SD	Mean cell count × 10 ⁶ , Vehicle ± SD	Ratio AED/Vehicle
Monocytes, granulocytes: CD11b + /Gr-1 +	4.301 1.58	2.446 1.22	1.75
Hematopoietic stem cells (HSC +): lin-, SCA-1hi, c-kit hi,	0.0191 0.012	0.0072 0.005	2.65
Common myeloid progenitor(CMP+) lin-, SCA-1-, c-kit-hi	0.0704 0.041	0.0273 0.024	2.57

The differences in the mean AED and vehicle values are statistically significant at $p < 0.05$. Ratio of monocytes/granulocytes (CD11b/Gr-1), hematopoietic stem cells (HSCs), and common myeloid progenitor (CMPs) obtained from flow cytometric analysis is significantly increased in AED versus vehicle-treated irradiated animals with $p < 0.05$.

AED, Δ⁵ androsten-3β, 17β-diol.

FIG. 6. (A, B) Trap staining of multinucleated osteoclasts. Bone marrow macrophages (BMM) were harvested and cultured from mouse femurs at 7 days postirradiation. Conversion of BMM within the osteoclast lineage occurred in the presence of continuous incubation of m-CSF (25 ng/mL) and recombinant human RANKL (100 ng/mL) for 10 days. Thin arrows indicate red tartrate resistant acid phosphatase (TRAP) staining of multinucleated osteoclasts. The large arrow heads reveal remnant osteoclasts, where previously formed osteoclasts underwent a process of cell death. A representative image from irradiated, vehicle-treated animals (A) and from irradiated, AED-treated animals (B) is shown. (C, D) Osteoclastogenesis was random, impaired, and incomplete using BMM from vehicle-treated whole-body irradiated mice. (C) Osteoclastogenesis of BMM harvested from mice treated with AED after whole-body irradiation was characterized by enlarged but mostly stable multinucleated cell structures, (D). Magnification for each panel is 200 ×.

hematopoietic stem cells (HSCs), and common myeloid progenitors were increased after AED treatment of irradiated animals versus their vehicle-treated counterparts.

In the bone marrow environment, as well as on the surface of bone, HSCs are the progenitors of osteoclasts. An osteoclast precursor population is enriched for cells expressing the monocyte-macrophage lineage markers CD14, CD11b, and CD34.²¹ The bone marrow culture data (Fig. 6) illustrate that AED may have a specific role in stimulating and maintaining the development of HSCs into osteoclasts. Previously, it was found that whole-body ionizing radiation caused osteoclast senescence *in vitro*,²² but *in vivo*, ionizing radiation increased osteoclast numbers and surface area in histological long bone preparations.²³ These contradictory lines of evidence are explained by an increased radiosensitivity of proliferating progenitor cells compared to BMs. The present study adds to these findings. Seven days postirradiation, mice were found to possess a full capacity for differentiation of HSC into osteoclasts. However, most of the newly formed osteoclasts died rapidly as a result of senescent-like fragility. In contrast, AED treatment lessened the appearance of senescence features and entirely prevented cell death. This suggests that AED has a positive effect on hematopoietic recovery to radiation damage and in particular may promote stable differentiation of osteoclast precursors. It also provides a partial explanation for the remarkable recovery of hematopoietic elements after whole-body radiation exposure. Prior studies by our group and others indicate that androgenic hormones and AED play a role in promoting bone remodeling as reported in osteoblast cells.^{24,25}

In this first study, the myelopoeitic recovery was incomplete 14 days postirradiation, but the restorative effect of AED is clearly evident. It may be beneficial for future experiments with AED to be carried out for longer durations.

Previously, Loria et al.^{6-8,26,27} reported that AED protected C57BL/6J mice from death after lethal infection challenge by *Pseudomonas*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, Coxsackievirus, Influenza, and different viruses causing encephalitis. Further, a single subcutaneous injection of AED restored the hosts' myelopoeitic system to normal by 21 days after whole-body radiation exposure to a lethal dose of 8-Gy gamma radiation. The effects of AED are mediated by an increase in host resistance and not by a direct antibacterial or antiviral effect, and a restoration of myelopoeisis after irradiation.^{6-9,26,27}

The data presented in this initial report indicate that in addition to restoration from gamma radiation injury, AED also mitigates the damages from high LET charged particle radiation exposure. Since bacteria such as *Pseudomonas aeruginosa* and *Cepacia burkholderi*, as well as opportunistic species such as *Streptococcus pneumoniae*, have been shown to be infectious to individuals with weakened immune systems, they may be potential pathogens for crew members in space.^{28,29} Thus, AED may also be a candidate for the prevention of infections during prolonged space flight.

In summary, the findings of this report demonstrate that AED functions to mitigate the effects of 1 GeV/nucleon ⁵⁶Fe²⁶⁺ particles on the mouse hematopoietic system. We know of no other study that shows that any member of this class of compounds can serve in this capacity. At this stage, our findings are somewhat preliminary due to our limited access to charged particle radiation beams. However, our

data make the initial identification of AED as a counter-measure to the hematopoietic effects of exposure to high LET charged particle radiation. This will enable more comprehensive studies to be performed on this compound in the future.

The results reported here show that AED can reduce the detrimental effects on the hematopoietic system when administered after the radiation exposure has occurred. This could be especially beneficial to future space explorers in the event of an unanticipated exposure to radiation in addition to the dose expected to occur during deep space travels.

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Disclosure Statement

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