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Granulocyte colony-stimulating factor antibody abrogates radioprotective efficacy of gamma-tocotrienol, a promising radiation countermeasure

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ABSTRACT

This study aimed to determine the role of granulocyte colony-stimulating factor (G-CSF), induced by a promising radiation countermeasure, gamma tocotrienol (GT3), in protecting mice from lethal doses of ionizing radiation. CD2F1 mice were injected with an optimal dose of GT3 and a G-CSF antibody, and their 30-d survival was monitored. An appropriate antibody isotype was used as a control. Multiplex Luminex was used to analyze GT3-induced cytokines. G-CSF neutralization by exogenous administration of a G-CSF antibody was confirmed by analyzing serum cytokine levels. Our results demonstrate that GT3 significantly protected mice against ionizing radiation, and induced high levels of G-CSF in peripheral blood 24 h after administration. Injection of a G-CSF neutralizing antibody to the GT3-treated mice resulted in complete neutralization of G-CSF and abrogation of its protective efficacy. Administration of a G-CSF antibody did not affect levels of other cytokines induced by GT3. Histopathology of bone marrow from GT3-treated and -irradiated mice demonstrated protection of the hematopoietic tissue, and also that such protection was abrogated by administering a G-CSF antibody. Our results suggest that induction of high levels of G-CSF by GT3 administration is responsible for its protective efficacy against radiation injury.

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1. Introduction

Exposure to ionizing radiation is a serious concern due to the deleterious effects of radiation on biological systems. These effects vary based on the dose absorbed and radiation quality. The harmful effects of ionizing radiation are observed in almost all tissues, leading to multi-organ dysfunction syndrome [1]. Wound inflictions along with radiation exposure are of even more serious concern because the immune system is extremely radiosensitive. Since immunosuppressed individuals are highly susceptible to opportunistic infections [2], protection of hematopoietic tissue from acute radiation injury is an important criterion in developing radiation countermeasures. This need has prompted research among government laboratories, academic institutions and pharmaceutical companies to identify potential radiation countermeasure candidates. This area of research is equally important for protection of cancer patients undergoing radiation therapy because of radiation-induced neutropenia and thrombocytopenia [3,4].

Several classes of radiation countermeasures have been investigated based on their ability to (a) reduce free radicals, (b) inhibit apoptosis, and (c) stimulate the hematopoietic system [5]. These include thiols and antioxidants, small-molecule apoptosis inhibitors, and cytokines and cytokine mimetics [6–11]. Although these approaches have led to several pre-clinical studies, not one of these candidate drugs has been approved by the U.S. Food and Drug Administration (FDA) to treat acute radiation syndrome.

Gamma-tocotrienol (GT3), a vitamin E isoform, has shown promising radioprotective efficacy [12]. At a dose of 200 mg/kg administered subcutaneously (sc) 24 h before radiation exposure, the GT3 dose reduction factor was 1.29. GT3 also accelerated the recovery of total white blood cells, neutrophils, monocytes, platelets, and reticulocytes in irradiated mice, compared to vehicle-injected, irradiated controls. GT3 has been shown to protect hematopoietic stem cells as well as reduce instant and persistent DNA damage [13]. These observations are consistent with GT3's antioxidant nature. The radioprotective efficacy of GT3 was enhanced further by combining it with pentoxifylline, an FDA-approved phosphodiesterase inhibitor [14]. GT3 in combination with pentoxifylline significantly improved survival compared to GT3 alone, and offered full protection against lethality even after exposure to a radiation dose of 12.5 Gy.

Granulocyte colony-stimulating factor (G-CSF) along with other cytokines exhibits protective effects against hematopoietic syndrome and radiation-induced neutropenia [3,15]. Recently, it has been shown that G-CSF induced by radiation countermeasures or irradiation itself plays an important role in protection against the harmful effects of radiation exposure [6,16]. In this study, our objec-

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tive was to investigate the role of G-CSF induction in the protection afforded by GT3 in mice exposed to ^{60}Co γ-radiation. To achieve this, we neutralized G-CSF with an antibody in GT3-treated mice, and monitored post-irradiation (9.2 Gy) survival for 30 days. We also measured G-CSF levels in GT3-treated unirradiated and irradiated mice injected with G-CSF antibody to confirm G-CSF neutralization. Our results demonstrate that administering a G-CSF antibody completely neutralized GT3-induced G-CSF in peripheral blood and was accompanied with complete loss of radioprotection afforded by GT3. These effects also were confirmed by histopathology and immunostaining in the hematopoietic tissue.

2. Materials and methods

2.1. Mice

Male 6–8 week-old specific pathogen free (SPF) CD2F1 mice were purchased (Harlan, Indianapolis, IN, USA) and housed (8 per cage) in an air-conditioned facility at Armed Forces Radiobiology Research Institute (AFRRI) accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Upon arrival, mice were quarantined for 10 days. A microbiological examination of representative samples ensured the absence of *Pseudomonas aeruginosa*. Mice were provided certified rodent rations (Harlan Teklad Rodent Diet, Harlan Teklad, WI, USA), acidified water (HCl, pH = 2.5–2.8) *ad libitum*, sterilized bedding and housed in rooms with a 12-h light/dark cycle as described earlier [17]. The mouse holding room was maintained at 21 ± 2 °C with 10–15 hourly cycles of fresh air and a relative humidity of 50 ± 10%. Research was conducted according to the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, U.S. National Academy of Sciences [18]. Mice were considered moribund when they showed an inability to remain upright, were cold, unresponsive or displayed decreased or labored respiration [19,20]. Mice found moribund were humanely euthanized. Once mice were considered morbid, we monitored them at least twice daily, early morning and late afternoon. In addition to the principal investigator's research staff, mice also were observed twice a day independently by veterinary science department staff. All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee.

2.2. Irradiation

Mice were placed in ventilated Plexiglas boxes compartmentalized to accommodate 8 mice per box and exposed to bilateral irradiation in the AFRRI cobalt-60 facility at a dose rate of 0.6 Gy/min to total midline doses indicated in experiments as described earlier [21]. After irradiation, mice were returned to their cages and monitored. Sham-irradiated mice were treated in the same manner as irradiated animals except that the cobalt-60 rods were not raised from the pool of shielding water. Radiation dosimetry was based on the alanine/EPR (electron paramagnetic resonance) system [22,23], currently accepted as one of the most accurate methods and used for intercomparison among national metrology institutions. The calibration curves used in dose measurements at the Armed Forces Radiobiology Research Institute (spectrometer e-Scan, Burker Biospin, Inc., Madison, WI, USA) are based on standard alanine calibration sets purchased from the United States National Institute of Standards and Technology, Gaithersburg, MD, USA.

2.3. Drug preparation and administration

Gamma-tocotrienol (2,7,8-trimethyl-2-(4,8,12-trimethyltrideca-3,7,11-trienyl) chroman-6-ol) formulation in 5% Tween-80 in saline was purchased from Yasoo Health, Inc. (Johnson City, TN, USA). Olive oil was used as vehicle control (equivalent to the quantity of GT3) in 5% Tween-80. The final GT3 concentration (200 mg/kg) was adjusted to administer 0.1 ml. Control mice received 0.1 ml of vehicle. The sc injections of the drug and vehicle were done at the nape of the neck with a 23 G needle 24 h before irradiation. No infections or local reactions were noted at the site of injection.

2.4. G-CSF neutralization

Mice were administered GT3 (200 mg/kg), sc, 24 h before blood harvest or irradiation. The GT3-treated mice then received either the G-CSF antibody (0.2 ml, 600 μg/mouse) or the isotype control (0.2 ml, 600 μg/mouse) intraperitoneally (ip), 8 h after GT3 administration as described earlier [24]. A monoclonal anti-mouse G-CSF antibody (8.21 mg/ml in phosphate buffered saline: PBS) and rat IgG1 isotype control (8.90 mg/ml in PBS) were purchased from R&D Systems Inc., MN, USA. The antibody/isotype was diluted to 3,000 μg/ml in PBS and administered in a volume of 0.2 ml using a 23 G needle. Before injection, G-CSF antibody and isotype were tested for 12 viral agents by BioReliance (Rockville, MD, USA) by MAP-IT (Molecular antigen PCR-identification test for mice) assay (Cat # 104253), and found negative for all agents tested. Blood samples were collected 16 h after G-CSF antibody injection (24 h after GT3 administration) to analyze cytokine induction.

2.5. Blood collection and Luminex analysis of cytokines

Blood was collected from anesthetized (Isoflurane, Abbott Laboratories, Chicago, IL, USA) mice via the inferior vena cava using a 23 G needle. After collection, blood was transferred to Capiject serum separator tubes (3T-MG; Terumo Medical Corp., Elkton, MD, USA), allowed to clot for 30 min, and centrifuged at 400g for 10 min. The serum was collected and stored at −70 °C until used.

Luminex 200 (Luminex Corp., Austin, TX, USA) was used to simultaneously detect different cytokines. Mouse serum samples were analyzed as described elsewhere [24] for interleukin-1β (IL-1β), IL-6, IL-10, IL-12(p70), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), keratinocyte-derived chemokine (KC), and tumor necrosis factor-α (TNF-α) using a custom ordered kit (M200003JZX, Bio-Rad Inc., Hercules, CA, USA). Cytokine quantification was performed using Bio-Plex Manager software, version 5.0 (Bio-Rad Inc.).

2.6. Histopathology of sternum of irradiated mice

Bone marrow from the sternum was used for immunohistochemistry. Sternums collected from mice at 6 h after 9.2-Gy irradiation, were immersion-fixed in formalin for 1–7 days, using a 20:1 volume of fixative. Fixed sternums were then decalcified for 3 h, and routinely processed with progressive dehydration in ethanol, clearing in xylene, paraffin vacuum-infiltration, and paraffin embedding. Longitudinal sections of sternum, with ideally 5–6 sternebrae, were cut with a manual rotary microtome at 4 μm and stained with hematoxylin and eosin (H&E) [25]. Bone marrow cellularity was evaluated by subjective analysis as described earlier [26,27].

2.7. Evaluation of phospho–histone H3 (pH3) protein expression as a mitotic marker

Phosphorylated H3 protein levels in bone marrow were analyzed as a mitotic marker. Sternum sections were deparaffinized briefly in Histo-Clear (National Diagnostics, Atlanta, GA USA),