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Mobilization of progenitor cells into peripheral blood by gamma-tocotrienol: A promising radiation countermeasure

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ABSTRACT

Gamma-tocotrienol (GT3), a vitamin E isoform, is shown to induce high levels of granulocyte colony stimulating factor (G-CSF) in mice. G-CSF is a key cytokine used for stimulation of hematopoiesis, and mobilization of hematopoietic stem and progenitor cells into peripheral blood. GT3 is also shown to induce vascular endothelial growth factor (VEGF), another important cytokine necessary for vasculogenesis and endothelial progenitor mobilization. Since GT3 induces both these cytokines, we tested whether GT3 mobilizes hematopoietic and endothelial progenitors in mice. GT3 (200 mg/kg) was injected in 10-week-old CD2F1 mice and mobilization of progenitors in peripheral blood was analyzed at 24, 48, and 72 h post-administration. Circulating hematopoietic progenitor cells (HPCs, Lin^-, $cKit^+$), endothelial progenitor cells (EPCs, Lin^-, $CD34^+$, Flk^+), and stromal progenitor cells (SPCs, Lin^-, $CD29^+$, $CD105^+$) in peripheral blood mononuclear cells (PBMCs) were analyzed simultaneously by flow cytometry. Mobilized HPCs, EPCs and SPCs in PBMC were also measured by colony-forming unit (CFU) assay in progenitor-specific media. Three groups of mice received vehicle, GT3 and GT3 plus AMD3100, a receptor antagonist used to enhance mobilization. GT3 induced significant mobilization of all three progenitor cell types compared to vehicle in peripheral blood; AMD3100 enhanced GT3-induced mobilization even further. Mobilization of progenitor cells in peripheral blood by GT3 indicates that GT3 can be used as an alternative to G-CSF and VGEF to mobilize HPCs and EPCs.

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1. Introduction

Bone marrow is a reservoir of several types of progenitor cells including blood forming (hematopoietic), as well as non-blood forming (endothelial and stromal) progenitor cells [1]. In response to injury or stress stimulus, these progenitors are mobilized from bone marrow into circulation to be recruited at the site of insult. Mobilization of progenitors is a complex process of signal transduction, where progenitors are detached from the stroma in the presence of certain growth factors and chemokines [2]. The detachment of progenitor is also associated with increase in proliferation status of these progenitors. Therefore, mobilized progenitors are more successfully engrafted than bone marrow derived progenitors [2].

Mobilized hematopoietic stem and progenitor ($CD34^+$) cells have been used widely as a source of hematopoietic stem cells (HSCs), replacing bone marrow cells to treat patients who need bone marrow transplantation [3]. G-CSF is known to mobilize progenitor cells into peripheral blood, which facilitates collection of progenitor-enriched cell populations for subsequent transfusion to treat immune-suppressed patients [3–5]. G-CSF is also a Food and Drug Administration (FDA)-approved drug marketed under the names Neupogen® and Neulasta® (a polyethylene glycol derivative of G-CSF) for chemotherapy-induced neutropenia due its ability to stimulate hematopoiesis and proliferation [6–8]. However, use of G-CSF may cause side effects varying from mild headache, nausea, vomiting, nosebleed, and bone or muscular pain to severe anaphylaxis [9]. A more cost-effective alternative to G-CSF for collecting enriched progenitor cells is being investigated.

We demonstrated recently that gamma-tocotrienol (GT3) induces myelopoiesis and increases WBC counts in mice, and protects them against radiation injury [10,11]. We also reported recently that GT3 induces high levels of G-CSF and other key cytokines important in hematopoiesis [12]. The exact mechanisms of the radioprotective effects of GT3 have not been elucidated. G-CSF has been evaluated extensively for its role in radioprotection and mitigation of radiation injuries [7,13,14]. GT3 is also shown to induce vascular endothelial growth factor (VEGF) in an independent study [15]. AMD3100, commercially known as Plerixafor, a CXCR4 antagonist (i.e., a CXC chemokine receptor), is often used to accelerate mobilization of hematopoietic

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progenitor cells from bone marrow into peripheral blood [16,17]. AMD3100 combined with G-CSF in mice and healthy human volunteers in clinic demonstrated a synergistic increase in $CD34^+$ cell mobilization [14,18]. AMD3100 alone or in combination with G-CSF has been approved in clinic for rapid mobilization of $CD34^+$ cells for autologous transplantation in patients with non-Hodgkin's lymphoma [19,20]. In this study we report that GT3 alone or in combination with a CXCR4 antagonist (AMD3100) can induce mobilization of HPCs, EPCs and SPCs at significant levels into blood probably by inducing G-CSF and VEGF. This is the first report to document that the vitamin E analog GT3 mobilizes HPCs, EPCs and SPCs into circulating blood.

2. Materials and methods

2.1. Reagents

Histopaque® 1083 was purchased from Sigma-Aldrich Corporation (Cambridge, MA, USA). Antibodies for flow cytometry, a mouse lineage cocktail antibody conjugated with V450 fluorochrome, CD34 antibody conjugated with Alexa700, CD117 antibody conjugated with phycoerythrin (PE)-Cyanine (Cy)7, Flit-like ligand (Flk)1 antibody conjugated with allophycocyanin (APC), and CD29 antibody conjugated with fluorescein-isothiocyanate (FITC), were purchased from BD Biosciences (Chicago, IL, USA). CD105 conjugated with phycoerythrin (PE) was purchased from Abcam (Cambridge, MA, USA). MethoCult® media (GF M3434) and MesenCult® media were purchased from StemCell Technologies (Vancouver, Canada). EGM-2-MV BulletKit® was purchased from Lonza (Walkersville, MD, USA). AMD3100 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer saline (PBS) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Fibronectin plates used for culturing EPC were purchased from EMD-Millipore (Billerica, MA).

2.2. Mice

Six- to 8-week-old male CD2F1 mice were purchased from Harlan Laboratories (Indiana, USA) and were housed (8 per cage) in an air-conditioned facility at the Armed Forces Radiobiology Research Institute (AFRRI) which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Mice were held in quarantine for 2 weeks and were used after microbiology, serology, and histopathology examination of representative samples ensured the absence of *Pseudomonas aeruginosa* and common murine diseases. Mice were provided with certified rodent rations (Harlan Teklad Rodent Diet #8604, Harlan Teklad, WI, USA) and acidified water (with HCl, pH 2.5–3.0) *ad libitum*. All mice were kept in rooms with a 12-h light/dark cycle with lights on from 0600 to 1800 h. All animal procedures were performed in accordance with a protocol approved by the AFRRI's Institutional Animal Care and Use Committee. Research was conducted according to the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, the National Research Council, U.S. National Academy of Sciences.

2.3. Drug formulation and administration

GT3 formulated in 5% Tween-80 was purchased from Yasoo Health Inc. (Johnson City, TN). Tween 80 was used as vehicle control (equivalent to the amount of GT3). Previous studies [12] had shown that 200 mg/kg of GT3 induced high levels of G-CSF, so this dose was used throughout the study. Mice were randomly distributed in four groups; naive, vehicle, GT3, and GT3 plus AMD3100. GT3 (200 mg/kg) was injected sc at the nape of the neck with a 23-G needle. For the control group, Tween 80 was injected sc 24 h before the first blood collection. AMD 3100 (a CXCR4 antagonist) was injected sc at 5 mg/kg (i.e., 125 μg/mouse in 0.1 ml PBS), 1 h before collection. No infections or local reactions were noted at the site of GT3 injection.

2.4. Whole blood collection

Blood (0.6–1.0 ml) was collected at 24, 48 and 72 h after GT3 administration from the posterior vena cava using a 23-gauge needle from mice terminally anesthetized with isoflurane (Hospira Inc., Lake Forest, IL, USA). The animals were deeply anesthetized in a rodent anesthesia machine; the tails were pinched for checking reflexive movement, indicative of insufficient anesthesia. If there was no response, the animals were moved to a station with an individual nose cone for continued anesthesia. An incision was made on the right side of the animals, closest to the IVC, the vein exposed, and blood was drawn with a 23-G needle. Blood was transferred immediately into blood-collection tubes treated with ethylenediaminetetraacetic acid – (EDTA, Sigma-Aldrich, St. Louis, MO, USA), and mixed gently on a rotary shaker until ready for peripheral blood mononuclear cells (PBMCs) isolation. PBMCs were isolated from whole blood by use of Histopaque 1083 solution. Cells were counted by use of the trypan blue exclusion method.

2.5. Flow cytometry

PBMCs were treated with Fc Block™ for 20 min on ice and then a cocktail of antibodies (Lin-V450, CD117 PE-Cy7, CD34 Alexa700, Flk1 APC, CD29 FITC and CD105 PE) was added. After incubation on ice for 20 more minutes, cells were centrifuged at 400 g for 6 min at 4° and resuspended in PBS/FBS. This step was repeated once more. Cells then were fixed with BD Cytofix (BD Biosciences, Chicago, IL, USA) on ice for 15 min and analyzed by flow cytometry. Lin^-, $cKit^+$ (HPCs) [1], Lin^-, $CD34^+$, $FLk1^+$ (EPCs), and Lin^-, $CD29^+$ and $CD105^+$ (SPCs) [21] were monitored, analyzed and reported as percentages of total lineage population. Isotype controls and single-stain controls were used to adjust voltages. Cells were processed on an LSR II HTS flow machine (BD Biosciences, MD, USA) using DIVA™-HTS software. The percentage of cell population of interest was calculated by DIVA™ analysis software.

2.6. Colony forming unit (CFU) assay

2.6.1. HPCs

MethoCult® complete media was used from Stem Cell Technology, Inc. (Vancouver, Canada). 5×10^4 PBMCs were suspended in 100 μl of IMDM (Iscove's Modified Dulbecco's Media) and added to 1.1 ml of MethoCult® complete media (GF M3434) per 35-mm plate and were incubated at 37 °C in CO2 incubator for 11 days before quantification. Colonies from 3 plates for each sample and 9 plates for each treatment group were counted. The total number of colonies was counted and the average value was plotted as colonies per million of cells.

2.6.2. EPCs

5×10^5 cells were added to EPC colony media (EGM-2, endothelial cell growth medium-2 basal media and supplements; and 16% FBS) on 6-well fibronectin-coated plates. Petri dishes were incubated at 37 °C in CO2 incubator for 14 days. Media was changed at day 7 and EPC colonies (EPC-CFU) were counted at day 14. Colonies were photographed after staining with Giemsa stain and counted under a microscope. The total number of colonies was counted and the average value was plotted as colonies per million of cells for each treatment group.

2.6.3. SPCs

5×10^5 cells were added to MesenCult® media, including supplements (Stem-Cell Technologies, Inc.) and stored in 6-well plates. They were incubated at 37 °C in CO2 incubator for 14 days. Media were