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PROGENITOR CELL MOBILIZATION BY GAMMA-TOCOTRIENOL: A PROMISING RADIATION COUNTERMEASURE

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Abstract—**This article reviews studies of progenitor mobilization with gamma-tocotrienol (GT3), a tocol under advanced development as a radiation countermeasure for acute radiation syndrome (ARS). GT3 protects mice against high doses of ionizing radiation and induces high levels of granulocyte colony-stimulating factor (G-CSF). GT3-induced G-CSF in conjunction with AMD3100 (a chemokine receptor antagonist clinically used to improve the yield of mobilized progenitors) mobilizes progenitors; these mobilized progenitors mitigate injury when infused to mice exposed to acute, high-dose ionizing radiation. The administration of a G-CSF antibody to GT3-injected donor mice abrogated the radiomitigative efficacy of blood or peripheral blood mononuclear cells (PBMC) in irradiated recipient mice. The efficacy of GT3-injected donor mice blood or PBMC was comparable to a recently published article involving blood or mononuclear cells obtained from mice injected with G-CSF. The injected progenitors were found to localize in various tissues of irradiated hosts. The authors demonstrate the efficacy of a bridging therapy in a preclinical animal model that allows the lymphohematopoietic system of severely immunocompromised mice to recover. This suggests that GT3 is a highly effective agent for radioprotection and mobilizing progenitors with significant therapeutic potential. Therefore, GT3 may be considered for further translational development and ultimately for use in humans.**

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Key words: blood; bone marrow; mice; radiation, gamma

INTRODUCTION

THE SEARCH for treatments to counter potentially lethal radiation injury has been underway for the past several decades, resulting in multiple classes of radiation countermeasures. However, to date only granulocyte colony-stimulating factor (G-CSF) has been approved by the United States Food and Drug Administration (FDA) for the treatment of acute radiation syndrome (ARS) (USFDA 2015). Several compounds derived from natural products have been investigated for prevention and therapy of human diseases because they are "generally recognized as safe" and considered appropriate for medicinal purposes (Papas 1999; Singh et al. 2013). Vitamin E has been introduced to radiation countermeasure research and is well known for its antioxidant, neuroprotective, and anti-inflammatory properties (Singh et al. 2013, 2014a). Vitamin E is a family of eight compounds that are collectively known as tocols. Tocols exist as four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). All tocols have powerful antioxidant activity that helps regulate peroxidation reactions and control free radical production within the body (Palozza et al. 2008, 2006). These agents also help prevent oxidative damage caused by irradiation-induced free radicals. Though the majority of radioprotection investigations have used tocopherols, recent discoveries demonstrate that the therapeutic targets are distinct between tocopherols and tocotrienols, indicating that the members of the vitamin E family work through different mechanisms with biological functions that do not significantly overlap (Nesaretnam 2008; Packer 1991). Tocotrienols have clearly distinct functions in maintaining health and treating disease, and a number of studies have demonstrated that tocotrienols are superior antioxidants compared to tocopherols (Kamal-Eldin and Appelqvist 1996; Pearce et al. 1994, 1992; Sen et al. 2006; Serbinova et al. 1991). Gamma-tocotrienol (GT3) is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Baliarsingh et al. 2005; Qureshi et al. 1986). In recent years, it has received

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a great deal of attention and appears to be one of the most promising radioprotectors (Singh et al. 2014a, 2015b). In a recently conducted study, GT3 demonstrated radioprotective efficacy in nonhuman primates (unpublished observation).

Here recent studies are summarized demonstrating that GT3-induced G-CSF mobilizes progenitors, and administration of such progenitor-enriched whole blood or peripheral blood mononuclear cells (PBMC) to irradiated recipient mice mitigates radiation injury. The administration of a G-CSF antibody to GT3-injected donor mice abrogated the radiomitigative efficacy of blood or PBMC obtained from such donors. Progenitors obtained from mice were found to localize in various tissues of recipient mice. The authors suggest that the ability of GT3 to mobilize hematopoietic progenitors can be exploited for treating injuries that result from exposure to ionizing radiation.

MATERIALS AND METHODS

Mice

Six to eight week-old male, specific pathogen-free CD2F1 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and housed in the Armed Force Radiobiology Research Institute's (AFFRI) facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-International. All animal procedures were performed according to a protocol approved by the AFRRI 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 Research, U.S. National Research Council, U.S. National Academy of Sciences (National Research Council of the National Academy of Sciences 2011).

Drug preparation, progenitor mobilization and G-CSF neutralization

The efficacy of blood or PBMC administration after total-body irradiation was evaluated using donor and recipient mice. All donor mice received administrations of either a previously determined, optimal dose of GT3 (200 mg kg^{-1}; Yasoo Health, Inc., Johnson City, TN, USA) or vehicle 72 h before blood collection. All donor mice also received AMD3100 (commercially known as plerixafor or Mozobil; Sigma-Aldrich, St. Louis, MO, USA) 1 h before blood collection (0.1 mL, sc, 23 G needle) to mobilize progenitors from the bone marrow into peripheral blood (Singh et al. 2014b, 2014d, 2012c). Mobilization of progenitors by GT3 was evaluated by analyzing c-Kit^+ and Sca-1^+ cells in blood samples of GT3- or vehicle-injected mice by flow cytometry.

To abrogate GT3-induced progenitor mobilization, half of the mice that received GT3 administrations received either G-CSF antibody or isotype control (R&D Systems Inc., Minneapolis, MN, USA) intraperitoneally (ip; 1,000 μg per mouse in 0.2 mL) (Kulkarni et al. 2013; Singh et al. 2010a) with a 23 G needle 8 h after GT3 administration.

Blood collection and isolation of PBMC for transfusion

Donor mice were terminally anaesthetized with isoflurane (Abbott Laboratories, Chicago, IL, USA), and blood was drawn from the caudal vena cava into syringes treated with citrate dextrose (BD Diagnostics, Franklin Lakes, NJ, USA) using a 23 G needle. PBMCs were isolated by layering diluted blood (1:1 with phosphate buffer saline) on histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuging as described earlier (Singh et al. 2014d). Recipient mice were administered whole blood or PBMCs 24 h after irradiation via the retro-orbital sinus [intravenously (iv)] and were monitored for survival for 30 d.

Irradiation

Mice were placed in compartmentalized and ventilated Plexiglas boxes and exposed to bilateral gamma-irradiation (0.6 Gy min^{-1}; 9.2 Gy $LD_{90/30}$ dose for CD2F1 mice) in AFRRI's ^{60}Co facility as described earlier (Singh et al. 2012c). After irradiation, mice were returned to their cages and monitored for 30 d. Radiation dosimetry was based primarily on the alanine/EPR (electron paramagnetic resonance) system (ISO-ASTM 2004; Nagy 2000), currently accepted as one of the most accurate methods and used for comparison between national metrology institutions.

In vivo tracking of PKH26-labeled progenitors

Bone marrow cells from healthy donor femurs were collected and labeled with PKH26, a general cell membrane marker, using a PKH26 red fluorescent cell linker kit (Sigma-Aldrich) and lineage antibody cocktail (BD Biosciences Pharmingen, San Diego, CA, USA) as described earlier (Singh et al. 2014c). These labeled cells were then sorted for $PKH26^+$, c-Kit^+ (PerCP-eFluor 710; BD Biosciences Pharmingen), and Lin^- (eFluor 450, BD Biosciences Pharmingen) live cells using FACS (fluorescent-activated cell sorting; BD LSRII Flow Cytometer, BD Biosciences). Recipient mice were irradiated with 11 Gy (0.6 Gy min^{-1}), and 24 h after irradiation, mice were administered 5 X 10^5 $PKH26^+$ sorted cells (iv, retro-orbital sinus). At 48 h after cell administration, the jejunum, sternum, liver, lung, kidney, and heart were collected in a low-light setting, and all samples were stored on ice until processing could begin. To process, collected organs were placed in 4% paraformaldyhde (Sigma-Aldrich) for 2 h. A portion of each organ was then immersed in sucrose solutions (10% and 20%, 10 min each), then kept in 30% sucrose solution until cryosectioning to visualize PKH26 stained cells. Another portion of each organ or tissue was placed in 10% neutral buffered formalin until sectioning for tissue structure visualization. Preserved samples were processed by Histoserv, Inc. (Germantown, MD, USA) for cryotomy and hematoxylin and eosin slides. Frozen sections were counterstained with

4', 6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) to prevent rapid photo-bleaching. Fluorescent cells were scored in a 100X high power field under a Nikon Eclipse Ti-U fluorescent microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a CoolSnap HQ^2 imaging camera (Photometrics, Tucson, AZ, USA).

Statistical analysis

A Fisher's exact test was used to compare survival rates at the end of 30 d, with a Bonferroni correction used to control for type-I error if multiple comparisons were used. For c-Kit^+ and Sca-1^+ data analyses, mean values with standard errors (SE, when applicable) were reported. Analysis of variance (ANOVA) was used to detect whether there were significant differences between experimental groups. When significance was indicated, a Tukey's post-hoc test was used to determine significant differences between particular groups. All statistical tests were two-sided with a 5% significance level and performed using the statistical software SPSS version 19 (IBM, Armonk, NY, USA).

RESULTS

Mobilization of progenitors to peripheral blood by GT3 and AMD3100 administration

Data presented in Fig. 1 demonstrate that blood samples from GT3- and AMD3100-injected mice had significantly higher numbers of c-Kit^+ and double positive cells (c-Kit^+ and Sca-1^+) compared to vehicle- and AMD3100-injected or untreated control. GT3-injected mice had higher numbers of Sca-1^+ cells compared to the untreated control.

Efficacy of infusing whole blood or PBMCs from GT3-injected donor mice on the survival of recipient irradiated mice and effect of G-CSF antibody injection to donor mice on the efficacy of whole blood or PBMC administration

The authors' interest was to evaluate the radiomitigative potential of GT3-mobilized progenitors and the effect of G-CSF antibody on the efficacy of whole blood or PBMC administration. Recipient mice received whole blood or PBMCs from GT3- or vehicle-treated mice 24 h post-irradiation. Recipient mice also received whole blood or PBMC's from GT3 and G-CSF antibody- or isotype-injected donors. A control group of irradiated mice received no blood from donors to serve as irradiated control.

Data presented in Fig. 2a demonstrate that administration of whole blood (100 μL) from GT3-treated donors had significantly higher survivors compared to survivors for the mice receiving vehicle-treated whole blood ($p<0.001$). Similarly, data presented in Fig. 2b demonstrate that mice receiving 5×10^6 GT3-mobilized PBMC provided 100% survival, a significant improvement over survival of the mice receiving vehicle-mobilized PBMC (38%, $p<0.001$).

As demonstrated in Fig. 2c, mice treated with whole blood from the GT3- and isotype-injected donors had 93% survivors compared to the 0% survivors in the group

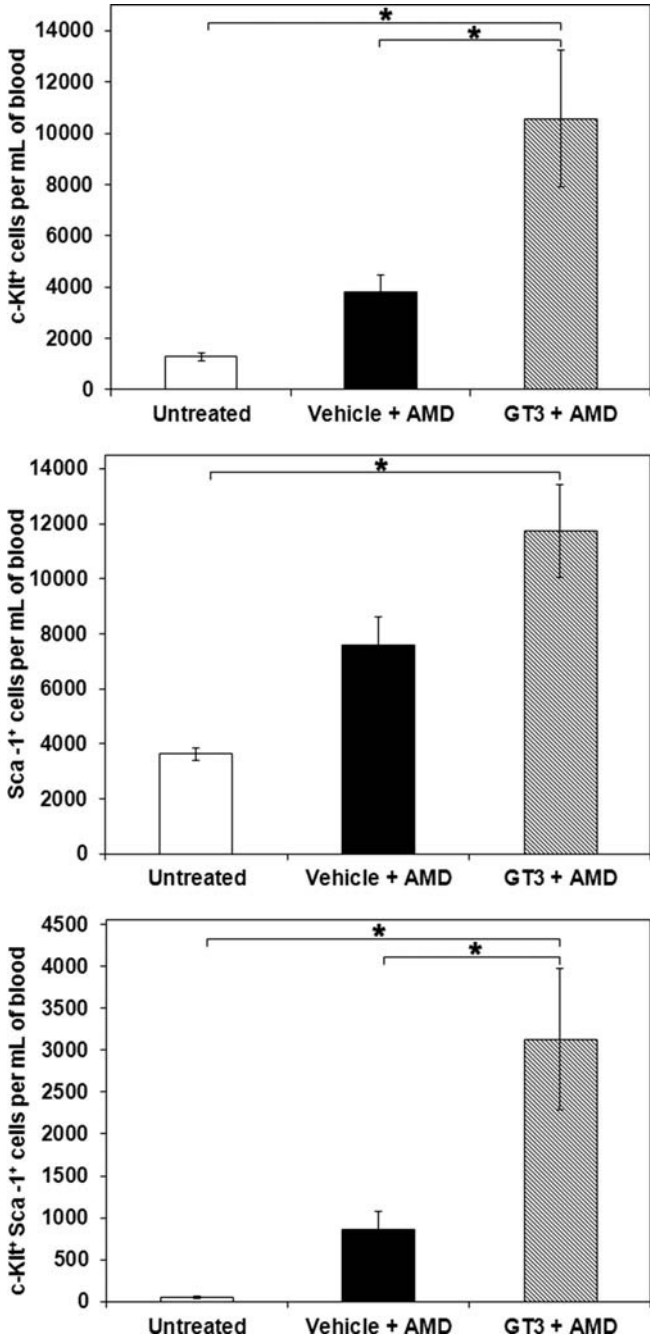

Fig. 1. Mobilization of hematopoietic progenitors by GT3 treatment. Mice were treated (sc) with GT3 (200 mg kg^{-1}) or vehicle. Blood samples were collected from mice 72 h after GT3 injection. AMD3100 was injected 1 h prior to blood collection. Mononuclear blood cells were stained with lineage markers (c-Kit^+ and Sca-1^+) conjugated with fluorochromes and analyzed by fluorescence-activated cell sorting (FACS). Sca-1^+ and/or c-Kit^+ cells were analyzed in lineage negative live population. The data are presented as averages with error bars indicating the standard error of the mean (SEM). *Denotes statistically significant differences between GT3- and vehicle-mobilized or untreated progenitors ($p<0.05$).

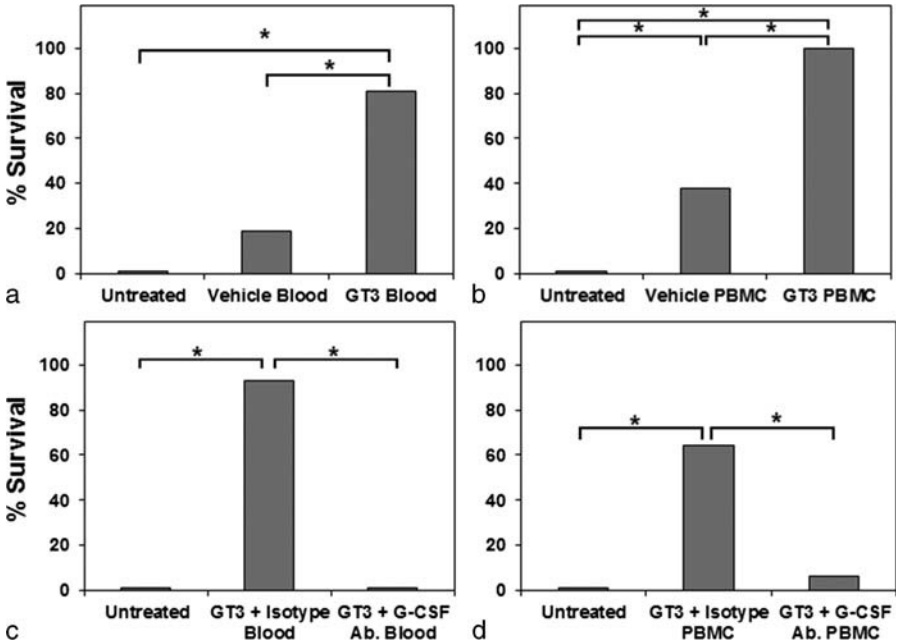

Fig. 2. Evaluation of GT3-mobilized whole blood (a) or PBMCs (b) as a radiomitigator when administered 24 h after irradiation (11 Gy, 0.6 Gy min^{-1}) and the evaluation of such treatments when donor mice were injected with G-CSF-specific antibody (c and d). Donor mice were administered 200 mg kg^{-1} GT3 (sc) or vehicle (olive oil). Half the mice that received GT3 also received either G-CSF antibody or isotype (1,000 μg per mouse, ip) 8 h after GT3 injection. Blood was collected 72 h after GT3 administration. Recipient mice were transfused (iv) 24 h after 11 Gy radiation exposure with GT3-treated whole blood (100 μL) (a), 5×10^6 PBMCs (in 100 μL of PBS containing 1% FBS) from GT3-injected mice (b), whole blood (100 μL) from GT3 and G-CSF antibody-injected mice (c) or 2×10^6 PBMC (in 100 μL of PBS containing 1% FBS) from GT3 and G-CSF injected mice (d). Mice were monitored for survival for 30 d. All treatment groups for survival studies consisted of 16 mice. *Denotes significant differences between treatment groups ($p<0.05$).

of mice (n = 16) that received blood from GT3- and G-CSF antibody-injected mice ($p<0.01$). Mice transfused with 5×10^6 GT3-mobilized PBMC with isotype control had a significantly higher rate of survivors (64%) compared to untreated mice (0%) ($p < 0.001$). Mice transfused with PBMC obtained from GT3- and G-CSF antibody-injected mice had 40% survivors, which was not significantly different than mice receiving PBMCs obtained from the GT3-isotype-injected mice (data not presented). This result indicates that 5×10^6 PBMC from mice (irrespective of treatment) has radiomitigative potential, though to a lesser extent than cells obtained from GT3-treated animals. This experiment was repeated with transfusions containing 2×10^6 PBMCs. Fig. 2d shows that mice injected with 2×10^6 GT3-mobilized PBMCs with isotype control had a significantly higher survival rate (64%) compared to both the untreated mice (0%) and mice administered GT3-mobilized PBMCs obtained from GT3- and G-CSF antibody-injected mice (6%).

Localization of GT3-mobilized transfused donor cells in the recipient mice

The authors investigated the localization of GT3-mobilized transfused cells by tracking PKH26-labeled progenitors in various organs of irradiated host mice. The recipient mice were irradiated at 11 Gy ^{60}Co γ-radiation (0.6 Gy min^{-1}) and injected with 0.5×10^6 PKH26-labeled c-Kit^+ $lineage^-$ cells 24 h after irradiation. The heart, lung, sternum, jejunum, thymus, and spleen of mice were harvested 48 h after cell transfusion. Frozen cross sections of the above organs were processed for the presence of PKH26-labeled cells. PKH26-positive cells were enumerated by microscopic examination. These slides were counterstained with DAPI and observed under a fluorescence microscope. PKH26 labeled cells in spleen, thymus, jejunum, and lung of recipient mice are shown in Fig. 3. Though PKH26 was observed in various organs, the organs with the relatively largest number of fluorescent cells per high powered field were thymus and spleen compared to the other organs harvested. It is important to note that mobilized cells also localized in tissues other than the bone marrow and gut (jejunum), such as lung. These observations suggest that mobilized progenitors migrate from the peripheral circulation to various tissues of the body.

DISCUSSION

The objective of using GT3 to mobilize progenitors is to replace G-CSF in the clinical setting with a cheap product that can be stored at ambient temperature. AMD3100 is always used in conjunction with primary mobilizing agent; hence, AMD3100 is used in this study, though additional

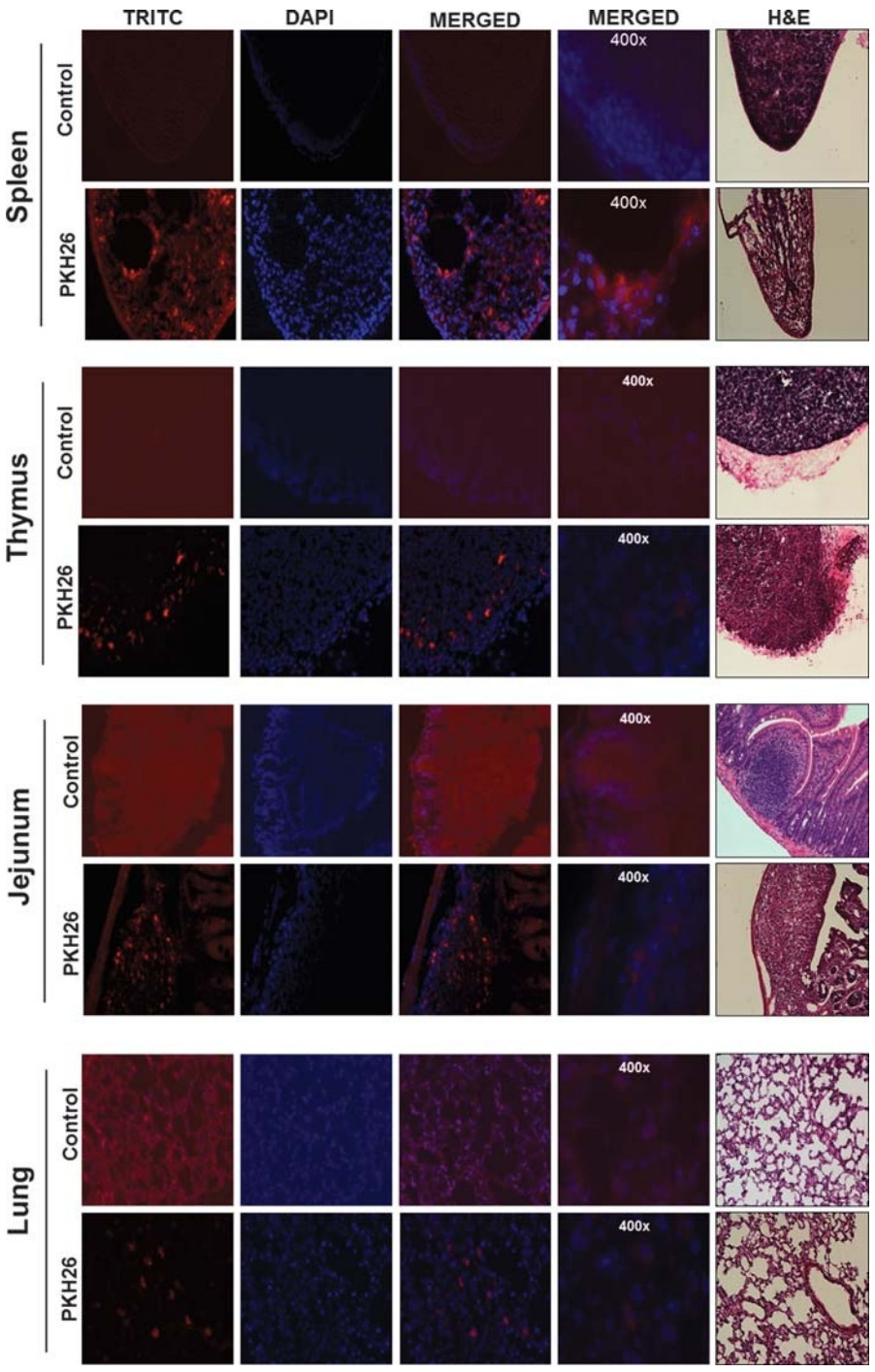

Fig. 3. Tracking of PKH26-labeled donor progenitors in recipient mice after irradiation with 11 Gy. Mice were irradiated (11 Gy, 0.6 Gy min^{-1}) and 24 h after irradiation received iv injection of 0.5×10^6 live PKH26-positive, c-Kit^+ $lineage^-$ cells. The spleen, thymus, jejunum, and lung of recipient mice were harvested 48 h after cell transfusion. Immunohistochemistry of tissues was performed to visualize PKH26-positive cells. All panels are shown at 200X magnification unless otherwise labeled. The top row is control tissue from a mouse with no PKH26 labeled cells administered. The bottom row is from a recipient mouse transfused with PKH26 labeled progenitors. $PKH26^+$ labeled cells are red. Cell nuclei are dyed with DAPI (blue). Images from other tissues have not been included due to space constriction. H&E of representative tissues are provided to compare integrity of the tissues.

studies are needed to confirm the safety profile of this combination for human use. Currently, G-CSF is used to mobilize $CD34^+$ cells into the blood to collect progenitor-enriched cell fractions for subsequent transfusions to treat severely immunocompromised patients as a viable, allogeneic stem-cell transplantation for those who do not respond

to cytokine therapies (Weaver et al. 2001). However, G-CSF requires continuous cold storage, making its availability extremely limited during any disaster scenario. It is important to note that this approach is specifically meant for a small group of victims when mobilized progenitors can be used as primary modality of treatment.

There are several other cellular approaches that are being tested (Rosen et al. 2015; Singh et al. 2015a). Cellerant Therapeutics (San Carlos, CA, USA) is developing myeloid progenitors consisting of lineage$^{-/low}$c-Kit^{+} cells expressing varying levels of CD11b^{+} and Gr1^{+}, which indicates a commitment to the myeloid lineage (Singh et al. 2012b). In contrast to whole bone marrow grafts, T and B cells are not present in detectable levels among myeloid progenitors, so the risk of graft-versus-host disease is negligible. In vivo studies demonstrate that allogeneic myeloid progenitors give rise to myeloid and erythroid cells that transiently engraft and functionally protect mice from fungal infection (Arber et al. 2005; BitMansour et al. 2002). This product has recently received FDA investigational new drug (IND) status (Cellerant Therapeutics, Inc., San Carlos, CA, USA—personal communication; 19 May 2015).

The authors are developing a novel strategy to treat individuals who are at high risk for exposure to acute, high doses of ionizing radiation. They demonstrate that GT3 mobilizes high-quality hematopoietic progenitors following its administration. This strategy involves the mobilization of progenitors by GT3 and the subsequent collection of whole blood or progenitor-enriched blood cell fractions well before an ionizing radiation exposure occurs. The efficacy of blood or PBMC transfusion has been tested against a supra-lethal dose of radiation (11 Gy) in CD2F1 mice, which is known to cause hematopoietic as well as gastrointestinal injury (Berbee et al. 2009). GT3-mobilized PBMC or whole blood transfusion mitigated radiation injury in mice against 11 Gy of ^{60}Co γ-radiation. Such progenitor mobilization has been reported for another tocol derivative, α-tocopherol succinate (Singh et al. 2012c). Unlike tocopherol succinate, GT3 is soluble in a FDA-approved excipient (5% Tween-80 in saline), making it more user-friendly for possible clinical use. In rodents, mobilization of progenitors by tocols is as efficient as G-CSF (Singh et al. 2010b). However, pharmaceutical grade GT3 for G-CSF induction and progenitor mobilization has not been tested, and therefore the authors cannot attest to such characteristics. One may need to further purify PBMC samples to remove T and B cells, as these may be contained in the samples. As stated above, myeloid progenitors are devoid of T and B cells.

An additional study was conducted to determine the role of G-CSF antibody administration on the efficacy of GT3-induced progenitors in donor mice. As shown in Fig. 2c and d, the mice that received whole blood or PBMC collected from isotype-injected mice had significant survival benefits compared to mice receiving blood or PBMC from G-CSF antibody-injected animals, suggesting that G-CSF antibody neutralized G-CSF that was induced by GT3. When 5×10^6 PBMC was administered from donor isotype-injected mice to irradiated recipient mice, the transfused PBMC improved the survival of those recipients; PBMC obtained from G-CSF antibody-injected animals also benefitted recipients, although to a lower degree. This observation suggests that 5×10^6 PBMC from healthy donors are capable of mitigating radiation injury to some extent. Though G-CSF induction by GT3 has been confirmed, the exact mechanism of G-CSF induction by GT3 remains unclear; further work to elucidate this mechanism is currently underway (Kulkarni et al. 2013; Singh et al. 2014d).

PKH26-labeled bone marrow progenitors were detected by fluorescence microscopy in various organs of recipient mice. Jejunum histopathology demonstrated that transfusion of GT3-mobilized progenitors into irradiated mice mitigates radiation-induced gastrointestinal injury (data not presented). There was significant recovery in GT3-mobilized progenitor recipient mice compared to untreated control. Similar jejunal recovery of irradiated mice was observed earlier with tocopherol succinate-mobilized progenitors (Singh et al. 2012a). PKH26-labeled bone marrow progenitors were also found to localize in the spleen, thymus and lung. The authors demonstrate that GT3-mobilized cells localize in hematopoietic as well as non-hematopoietic organs (spleen, thymus, jejunum, and lung).

Several characteristics of progenitors make them an attractive option to treat patients with ARS, particularly as a bridging agent for those acute radiation victims that are in the field with minimal infrastructure. GT3 may be used as a substitute for G-CSF to mobilize progenitors since it is stable at room temperature and a cheaper option than G-CSF. GT3 can be administered in an FDA-approved vehicle as shown previously (Singh et al. 2014d). GT3 mobilized progenitors have a long storage life, are essentially non-toxic, and have shown efficacy over a broad range of radiation exposures, including supralethal doses. However, both pre-clinical and clinical trials will be needed to produce a protocol for the clinical management of acute radiation exposure victims.

CONCLUSION

GT3 mobilizes progenitors to peripheral circulation; the infusion of GT3-mobilized, progenitor-containing whole blood or PBMC acts as a bridging therapy and mitigates radiation injury in irradiated mice. Efficacy of such cells can be abrogated by administering a G-CSF antibody to donors, suggesting that mobilization of progenitors by GT3 is a G-CSF-dependent phenomenon. The transfused cells localize in various organs of the host. There are a

number of major advantages that make GT3-mobilized progenitors ideal for treating patients/casualties with ARS: GT3 is non-toxic, an inexpensive product compared to growth factors, stable at room temperature, and GT3-mobilized progenitor therapy clearly allows for a broader treatment range (radiation exposure). This treatment option appears attractive based on studies in the mouse model. With additional studies in large animals, one may be able to provide an appropriate protocol for the clinical management of individuals suffering from exposure to lethal doses of ionizing radiation.

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