Combined Therapy of Pegylated G-CSF and Alxn4100TPO Improves Survival and Mitigates Acute Radiation Syndrome after Whole-Body Ionizing Irradiation Alone and Followed by Wound Trauma

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Exposure to ionizing radiation alone or combined with traumatic tissue injury is a crucial life-threatening factor in nuclear and radiological incidents. Radiation injuries occur at the molecular, cellular, tissue and systemic levels; their mechanisms, however, remain largely unclear. Exposure to radiation combined with skin wounding, bacterial infection or burns results in greater mortality than radiation exposure alone in dogs, pigs, rats, guinea pigs and mice. In the current study we observed that B6D2F1/J female mice exposed to ⁶⁰Co gamma-photon radiation followed by 15% total-body-surface-area skin wounds experienced an increment of 25% higher mortality over a 30-day observation period compared to those subjected to radiation alone. Radiation exposure delayed wound healing by approximately 14 days. On day 30 post-injury, bone marrow and ileum in animals from both groups (radiation alone or combined injury) still displayed low cellularity and structural damage. White blood cell counts, e.g., neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets, still remained very low in surviving irradiated alone animals, whereas only the lymphocyte count was low in surviving combined injury animals. Likewise, in surviving animals from radiation alone and combined injury groups, the RBCs, hemoglobin, hematocrit and platelets remained low. We observed, that animals treated with both pegylated G-CSF (a cytokine for neutrophil maturation and mobilization) and Alxn4100TPO (a thrombopoietin receptor agonist) at 4 h postirradiation, a 95% survival (vehicle: 60%) over the 30-day period, along with mitigated body-weight loss and significantly reduced acute radiation syndrome. In animals that received combined treatment of radiation and injury that received pegylated G-CSF and Alxn4100TPO, survival was increased from 35%

to 55%, but did not accelerate wound healing. Hematopoiesis and ileum showed significant improvement in animals from both groups (irradiation alone and combined injury) when treated with pegylated G-CSF and Alxn4100TPO. Treatment with pegylated G-CSF alone increased survival after irradiation alone and combined injury by 33% and 15%, respectively, and further delayed wound healing, but increased WBC, RBC and platelet counts after irradiation alone, and only RBCs and platelets after combined injury. Treatment with Alxn4100TPO alone increased survival after both irradiation alone and combined injury by 4 and 23%, respectively, and delayed wound healing after combined injury, but increased RBCs, hemoglobin concentrations, hematocrit values and platelets after irradiation alone and only platelets after combined injury. Taken together, the results suggest that combined treatment with pegylated G-CSF and Alxn4100TPO is effective for mitigating effects of both radiation alone and in combination with injury. © 2017

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INTRODUCTION

Injuries induced by ionizing radiation exposure alone or in combination with trauma from blast and thermal energy exposure (“combined injury”) are inevitable after the detonation of radiation dispersal devices or nuclear weapons. Published *in vivo* (1) and *in vitro* (2, 3) studies indicate that combined injury enhances pathophysiological responses to radiation, including DNA double-strand breaks (DSBs), activates signal transduction pathways, elevates cytokine/chemokine concentrations in the peripheral blood, bone marrow injury and small intestinal damage and increases systemic bacterial infection, thereby leading to cell death and multiple organ dysfunction and failure (1, 4–6). Through these means, combined injury therefore increases mortality (1, 5–7). The responses to radiation and combined injury are complex, occurring in a multilayered manner at the molecular, cellular, tissue and systemic

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levels, thereby making it very difficult to identify countermeasures for prophylaxis, mitigation or therapy. Thus, identifying countermeasures for combined injury is needed. G-CSF (Neupogen®) and pegylated G-CSF (Neulasta®) have been approved recently by the U.S. Food and Drug Administration (FDA) under the animal rule [CFR Title 21, Sections 314.600–314.650 and 601.90–601.95 (8, 9)].

Radiation induces a remarkable increase in granulocyte colony stimulating factor (G-CSF) in mouse blood, and combined injury further augments G-CSF increase in mouse blood (7). The increase is initially believed to be a self-defensive response, though its appearance is too late to participate in the repair of bone marrow damage, which usually occurs within hours after exposure (1, 2). G-CSF and pegylated G-CSF (peg-G-CSF) have been used clinically to treat radiation-injured patients (10). It has been reported that this growth factor decreases the period of neutropenia or aplasia in the limited number of radiation accident victims studied and also enhances neutrophil recovery after anti-cancer therapy (10). The cytokine activates or primes neutrophils to enhance their function (11). The peg-G-CSF formulation has a much longer biological half-life than G-CSF (12), thus avoiding the need for daily injections, which would be deleterious for irradiated mice. The drug has no toxic or adverse effects in mice at the dose used. Akin to natural G-CSF peptides, peg-G-CSF initiates the proliferation and differentiation of myeloid progenitors into mature granulocytes and induces hematopoietic stem cell mobilization from the bone marrow into the bloodstream. It is involved in infection recovery (13, 14) and wound healing (15). Peg-G-CSF, when combined with stem cell factors (SCF) and erythropoietin (EPO), was successfully used in the rescue of a hospital technician who had accidentally entered a ^{60}Co irradiation therapy room and received a 4.5 Gy dose (16). Other known radiological accidents where victims received G-CSF treatments have been reported as well (17).

In our B6D2F1/J mouse model (18), subcutaneous (s.c.) administration of G-CSF alone beginning 24 h (10 $\mu\text{g/kg}$, s.c.; day 1–14, once daily) after combined injury, along with topical application of gentamicin cream to the wound (day 1–10, once daily) and *per os* (p.o.) administration of levofloxacin (day 3–21, once daily) significantly increased mouse survival after irradiation alone and combined injury, by 25% and 20%, respectively. Peg-G-CSF injected into B6D2F1 mice (25 $\mu\text{g/mouse}$, s.c., once at 24 h, at day 8, at day 15) after 9.5 Gy irradiation ($\text{LD}_{70/30}$) resulted in 100% survival for 30 days after exposure. However, it was previously reported that peg-G-CSF administration did not improve survival after combined injury with burns (18), demonstrating that peg-G-CSF is an effective radiomitigator for radiation-induced injury only. We have also observed significant recovery of neutrophils, lymphocytes and monocytes in mice that received radiation only, but not in those subjected to combined injury (18). Farese *et al.* (19) reported

that in a NHP model, peg-G-CSF significantly improved neutrophil recovery after 6 Gy. Similarly, MacVittie *et al.* (20) reported that G-CSF significantly improved neutrophil and platelet recovery after 2 Gy in canines.

Hematopoietic tissue is highly susceptible to cytotoxic effects of ionizing radiation, resulting in thrombocytopenia and/or neutropenia, followed by mortality (7). Thrombopoietin (TPO) has been used to prevent thrombocytopenia, accelerate platelet and RBC reconstitution, alleviate neutropenia and promote recovery of immune bone marrow cells (21, 22). Small molecular weight TPO mimetics such as Alxn4100TPO can enhance platelet numbers. Alxn4100TPO is a short chain peptide incorporated in both light and heavy chains of human IgG. In CD2F1 mice, the drug at 2 mg/kg s.c. was effective 12 h postirradiation with 70% survival after exposure to 9 Gy (^{60}Co gamma radiation at 0.6 Gy/min) (23). In our B6D2F1/J mouse model, at 24 h after time of combined injury, s.c. injection of Alxn4100TPO along with topical application of gentamicin cream to the wound (day 1–10) and p.o. administration of levofloxacin (day 3–21) significantly increased mouse survival after combined injury but not after irradiation alone. The treatment also improved body weight, numbers of platelets, neutrophils, eosinophils, spleen weights and numbers of splenocytes only after combined injury (24). The efficacy was independent of gentamicin and levofloxacin (25). Unlike other effective therapeutics for combined injury, including mesenchymal stem cells (26, 27), ghrelin (28) and ciprofloxacin (29–31), which are capable of accelerating wound healing, Alxn4100TPO lacks this capability (24).

It is evident that TPO significantly improves the performance of G-CSF in alleviating severe neutropenia (21), and a combination of TPO and G-CSF stimulates recovery of thrombocytes and erythrocytes in addition to neutrophil recovery (21, 22, 32). We have shown that in B6D2F1 mice, Alxn4100TPO alone improved survival after combined injury but not after irradiation alone (24). We hypothesized that combined treatment with TPO and G-CSF would demonstrate either additive or synergistic therapeutic effects a mouse model subjected to radiation alone or combined with injury. Herein, we examined combined treatment with peg-G-CSF and Alxn4100TPO, after irradiation alone and after combined injury, in combination with gentamicin and levofloxacin to prove our hypothesis. Because the combination of radiation and injury enhances injury to bone marrow and ileum (4, 7), we examined their histopathology as well.

MATERIALS AND METHODS

Animals and Experimental Design

B6D2F1/J female mice (Jackson Laboratory, Bar Harbor, ME) were maintained at an AAALAC-accredited facility in plastic microisolator cages on hardwood chip bedding. Commercial rodent chow and acidified tap water were provided *ad libitum* at 12–20 weeks of age. Animal holding rooms were maintained at $21^\circ\text{C} \pm 1^\circ\text{C}$ with $50\% \pm 10\%$ relative humidity using at least 10 changes/h of 100%

conditioned fresh air. A 12-h, 0600 (light) to 1800 (dark) full-spectrum, lighting cycle was used. The AFRRI Institutional Animal Care and Use Committee approved all animal procedures. Euthanasia was performed in accordance with the recommendations and guidance of the American Veterinary Medical Association (33, 34).

Animals were randomly assigned to different groups ($N = 16$ –20 per group) and received the following treatment(s): sham; wounding alone; radiation alone; radiation with wounding (combined injury) along with one of the following: no treatment (control); vehicle; p-G-CSF alone; Alxn4100TPO alone; or combination of p-G-CSF and Alxn4100TPO.

Gamma Irradiation

Mice were restrained in well-ventilated Lucite® boxes (4 animals per box) and irradiated at 9.5 Gy ($LD_{50/30}$ for the radiation-alone group; $LD_{70/30}$ for the combined injury group) in a bilateral radiation field at a dose rate of 0.4 Gy/min in the AFRRI ^{60}Co gamma-irradiation facility (18). The alanine/electron spin resonance (ESR) dosimetry system (American Society for Testing and Material Standard E 1607) was used to measure dose rates to water in cores of acrylic mouse phantoms. The ESR signals were measured against a calibration curve based on standard calibration dosimeters provided by the National Institute of Standards and Technology (35). The accuracy of the calibration curves was verified by intercomparison with the National Physical Laboratory (London, UK). The radiation field was uniform within $\pm 2\%$.

Skin Injury

Skin-surface injuries were inflicted on the shaved dorsal surface of the mice. Animals receiving skin wounds were anesthetized by isoflurane inhalation. For animals receiving a skin wound, a 15% total-body-surface-area skin wound was inflicted within 1–2 h postirradiation, because the survival exacerbation enhanced by skin wound is diminished when the wound is inflicted later (after irradiation) (5, 6). A nonlethal total-body-surface-area wound was administered 19 ± 1.3 mm from the occipital bone and between the scapulae using a stainless-steel punch (9/16) on a Teflon-covered board cleaned with 70% alcohol before each use. The panniculus carnosus muscle and overlying skin (23.5 ± 1.1 mm in length and 14.9 ± 0.7 mm in width) were removed (7). For sham-treated animals, mice underwent the same procedure except without actual skin wounding. The stainless-steel punch and Teflon board were cleaned with 70% alcohol after each use.

Skin-wounded mice in the wound-alone and combined injury groups received a single dose of 150 mg/kg acetaminophen (for analgesia) in 0.5 ml 0.9% sodium chloride solution immediately after skin injury, while sham-treated and radiation-alone animals received a single dose of only 0.5 ml 0.9% sodium chloride solution immediately after a sham-skin procedure.

Administration of Pegylated G-CSF

Peg-G-CSF (Neulasta®; NDC: 555-13-019001) is a polyethylene glycol pharmaceutical formulated grade drug, also known as pegfilgrastim and was purchased from AmersourceBergan Corp. (Valley Forge, PA). A dose of 1,000 $\mu\text{g/kg}$ was injected s.c. (36, 37) in a volume of 0.2 ml at 4 h, day 8 and 15 after irradiation alone or combined injury, i.e., 25 $\mu\text{g}/25\text{-g}$ mouse. Administration of this drug was started 4 h postirradiation, based on the U.S. Department of Defense (DoD) requirement of offering drug treatment to military personnel as early as 4 h postirradiation. Neulasta® is supplied in 0.6-ml prefilled syringes for s.c. injection. Each syringe contains 6 mg Peg-G-CSF in a sterile, clear, colorless, preservative-free solution containing 0.35 mg acetate, 0.02 mg polysorbate 20, 0.02 mg sodium and 30 mg sorbitol in water for injection, USP. Vehicle-treated mice received 0.2 ml of vehicle containing 0.35 mg acetate, 0.02 mg polysorbate 20, 0.02 mg sodium and 30 mg sorbitol in 0.6 ml water.

Administration of Alxn4100TPO

The drug, Alxn4100TPO, is a thrombopoietin receptor agonist, acquired from Alexion® Pharmaceuticals, Inc. (Cheshire, CT). Mice received Alxn4100TPO (1 mg/kg, s.c.) or vehicle (sterile 0.9% sodium chloride solution for injection, USP; 0.2 ml/mouse s.c.) 4 h after sham treatment, wounding, radiation alone or combined injury, based on the DoD requirement of offering drug treatment to military personnel as early as 4 h postirradiation.

Administration of Antimicrobial Agents

Gentamicin sulfate cream, 0.1% (generic, NDC 0168-007-15; E. Fougera and Co., Melville, NY), was applied daily for 10 days to the skin injuries on days 1–10. Levofloxacin (generic, NDC 65862-537-50; Aurobindo Pharma Ltd., Mahaboob Nagar, India), 100 mg/kg in 0.2 ml/mouse, was administered p.o. daily for 14 days on days 3–16. Briefly, a 500-mg tablet was crushed by mortar and pestle. The levofloxacin powder was dissolved in a volume of sterile water approximately one-third of the total volume required to prepare the concentration needed for the average body mass of the mice to be treated. The suspension was centrifuged to remove the particulate filler and the supernatant solution was passed through a 0.45- μm membrane filter into a sterile amber bottle, which was sealed with a sterile rubber stopper.

Survival and Body Weight

Animals ($N = 16$ –22 per group) were monitored at least twice daily for their general health and survival for 30 days. Their body weights were measured on days 0, 1, 3, 7, 14, 21 and 28 (7, 24).

Water Intake

Water intake was measured daily for each cage containing 4 mice, with graduated bottles containing water until day 7 postirradiation. Daily volumes were divided by 4 and the data were presented with ml/day/mouse (7, 24).

Wound Closure

Assessments of wound closure were performed on days 1, 7, 14, 21 and 28. Wounds were measured to within 0.01 mm by a caliper with an electronic digital display. The average area of each wound was calculated as π (diameter $A/2$) $\times$ (diameter $B/2$), where π is 3.1416; A and B represent diameters at right angles to each other (7, 24).

Assessment of Blood Cell Profile in Peripheral Blood

Blood samples ($N = 6$ per group) were collected in EDTA tubes 30 days after irradiation alone or combined injury and assessed with the ADVIA® 2120 Hematology System (Siemens, Deerfield, IL). Differential analysis was performed using the peroxidase method and light scattering techniques recommended by the manufacturer.

Histology of Bone Marrow and Ileum

Sternum and ileal tissue specimens were collected from mice at day 30 ($N = 4$ mice per group). Specimens were rinsed in cold saline solution and immediately fixed in 10% phosphate-buffered formalin. The tissue was then embedded in paraffin, sectioned transversely and stained with hematoxylin and eosin (H&E). For sternum, numbers of fat cells and megakaryocytes on each $20\times$ field and 3–4 fields per sample were obtained (38). For ileum, villous heights, villous width, crypt heights and crypt counts were measured (7) using Nano-Zoomer2.0-RS (Hamamatsu Corp., Bridgewater NJ). The mucosal damage of ileum for each slide was graded on a six-tiered scale defined by Chiu *et al.* (39) as follows: grade 0, normal mucosa; grade 1, development of subepithelial spaces near the tips of the villi with capillary congestion; grade 2, extension of the subepithelial space with

A Peg-G-CSF + Alxn4100TPO

B Peg-G-CSF

C Alxn4100TPO

FIG. 1. Peg-G-CSF and Alxn4100TPO improved survival after 9.5 Gy whole-body irradiation alone or combined with skin wound. Mice were treated with vehicle (veh) or peg-G-CSF and Alxn4100TPO (P + A; panel A), peg-G-CSF alone (panel B) or Alxn4100TPO alone (panel C) after irradiation alone or combined injury. Their survival was monitored for 30 days. $N = 16\text{--}22$ per group. * $P < 0.05$ vs. sham-treated and wound-alone vehicle treatment groups; ^ $P < 0.05$ vs. irradiation-alone vehicle-treated group; # $P < 0.05$ vs. respective vehicle group.

moderate epithelial lifting from the lamina propria; grade 3, significant epithelial lifting along the length of the villi with a few denuded villous tips; grade 4, denuded villi with exposed lamina propria and dilated capillaries; and grade 5, disintegration of the lamina propria, hemorrhage, and ulceration.

Statistical Analysis

Parametric data are expressed as the mean $\pm$ SEM. For each survival experiment, 16–20 mice per group were tested on an individual basis. Survival analyses were performed using a Kaplan-Meier curve and one-way analysis of variance (ANOVA). For experiments for hematological analysis ($N = 6$ per group) and histopathological analysis ($N = 4$ per group), one-way ANOVA, two-way ANOVA, studentized-range test and Student's t test were used for comparison of groups, with a 5% significance level.

RESULTS

Peg-G-CSF and Alxn4100TPO Treatments Increase Survival after Irradiation Alone or Combined Injury

Skin wounding alone (15% total-body-surface area) resulted in 94.4% survival over a 30-day observation period due to septic infection (Fig. 1A). However, combined injury

reduced survival to 35%, which was less than survival observed in mice subjected to radiation alone (60%), as shown in Fig. 1A. In the radiation-alone group, treatment with peg-G-CSF and Alxn4100TPO enhanced 30-day survival to 95% (Fig. 1A, $P < 0.05$). In combined injury mice, treatment with peg-G-CSF and Alxn4100TPO increased survival by 20% after combined injury (Fig. 1, $P < 0.05$). Treatment with peg-G-CSF alone increased survival after irradiation alone and combined injury by 33% and 15%, respectively, above the respective vehicle-treated groups (Fig. 1B). Treatment with Alxn4100TPO alone increased survival after irradiation alone and combined injury by 4% and 23%, respectively, above the respective vehicle-treated groups (Fig. 1C). Mice in the sham-treated and wounded-alone groups, treated with either drug individually or in combination all survived (data not shown).

Peg-G-CSF and Alxn4100TPO Treatments Mitigate Weight Loss after Irradiation Alone or Combined Injury

It is evident that irradiation alone reduced body weights starting on day 2 postirradiation (1). Skin wounding alone did not induce body-weight loss but did enhance the

A Peg-G-CSF + Alxn4100TPO

B Peg-G-CSF

C Alxn4100TPO

FIG. 2. Peg-G-CSF and Alxn4100TPO significantly mitigated body-weight loss after 9.5 Gy whole-body irradiation alone or combined with skin wound. Mice were treated with vehicle or peg-G-CSF and Alxn4100TPO (P + A; panel A), peg-G-CSF alone (panel B) or Alxn4100TPO alone (panel C) after sham, wounding, irradiation or combined injury. Body weights were measured on days 1, 3, 7, 14, 21 and 28. N = 18–22 per group. Data are presented as mean $\pm$ SEM. * P < 0.05 vs. sham and wound, vehicle-treated groups; ΔP < 0.05 vs. irradiation, vehicle-treated group; # P < 0.05 vs. respective vehicle group.

radiation-induced body-weight loss beginning on day 1 after combined injury (Fig. 2A). Treatment with peg-G-CSF and Alxn4100TPO mitigated body-weight loss in both the radiation-alone and combined injury groups (Fig. 2A) on days 21 and 28, respectively, but had no effect on mice subjected to only skin wounding, compared to the respective vehicle-treated groups (data not shown). Treatment with peg-G-CSF alone failed to mitigate the body-weight loss after irradiation alone or combined injury, compared to the respective vehicle-treated groups (Fig. 2B). Treatment with Alxn4100TPO alone mitigated weight loss on day 7 after combined injury (Fig. 2C). Sham-treated and wounded-alone mice administered with either drug or both drugs combined did not lose body weight (data not shown).

Peg-G-CSF and Alxn4100TPO Increases Water Intake after Irradiation Alone or Combined Injury

It is evident that, compared to the sham-treated group, wounding alone significantly increased water intake since day 1 after wounding (1). Water intake was significantly

decreased on days 1, 2, 3, 5 and 7 after irradiation alone and on days 1, 2, 6 and 7 after combined injury (Fig. 3A). Treatment with peg-G-CSF and Alxn4100TPO, combined, increased water intake on days 3 and 7 after irradiation alone and combined injury (Fig. 3A). Wounded mice drank more water on days 1, 2, 3, 5 and 6, and water intake returned to the basal volume on day 7 (Fig. 3A). Treatment with peg-G-CSF alone failed to mitigate changes in water intake volumes after irradiation alone or combined injury, compared to that of the respective vehicle-treated groups (Fig. 3B). Treatment with Alxn4100TPO alone mitigated water intake volumes on days 4, 5 and 7 only after combined injury (Fig. 2C). Sham-treated and wounded-alone mice that were administered either drug or both drugs combined did not change their water intake volumes (data not shown).

Peg-G-CSF and Alxn4100TPO do not Accelerate Wound Healing after Wounding Alone or Combined Injury

It is evident that wound healing in unirradiated mice took 14 days, but radiation treatment delayed wound healing by

A Peg-G-CSF + Alxn4100TPO

B Peg-G-CSF

C Alxn4100TPO

FIG. 3. Peg-G-CSF and Alxn4100TPO significantly improved water consumption after 9.5 Gy whole-body irradiation alone or combined with skin wound. Mice were treated with vehicle (veh) or peg-G-CSF and Alxn4100TPO (P + A; panel A), peg-G-CSF alone (panel B) or Alxn4100TPO alone (panel C) after irradiation or combined injury. Water intakes were measured daily for the first 7 days after sham, wounding irradiation or combined injury. N = 16–22 per group. Data are presented as mean $\pm$ SEM. * P < 0.05 vs. sham vehicle-treated group; ΔP < 0.05 vs. sham and irradiated vehicle-treated groups; # P < 0.05 vs. respective vehicle group.

another 14 days (Fig. 4A). Treatment with peg-G-CSF and Alxn4100TPO did not accelerate the wound-healing rate in the wounded-alone or combined injury mice (Fig. 4B). Treatment with peg-G-CSF alone slowed down the wound-healing time in wounded-alone mice by 7 days, although the combined injury mice were fully healed on day 28, as was the case in the vehicle-treated combined injury mice (Fig. 4C). Treatment with Alxn4100TPO alone did not affect the wound-healing time in wounded-alone mice, but the combined injury mice took longer than 28 days to fully heal from the wound (Fig. 2D).

Peg-G-CSF and Alxn4100TPO Mitigate Bone Marrow Histopathology

As shown in Fig. 5, on day 30 after irradiation alone and combined injury, wounding alone did not change bone marrow cellularity. The numbers of fat cells and megakar-

yocytes present in the bone marrow were not different from the sham-treated group. Radiation alone, however, significantly reduced the bone marrow cellularity in vehicle-treated mice, indicated by increased fat cell counts and reduced megakaryocyte counts. As with radiation alone, combined injury significantly increased the numbers of fat cells and further decreased the numbers of megakaryocytes in vehicle-treated mice. However, treatment with peg-G-CSF and Alxn4100TPO, combined, significantly reduced fat cell counts and increased megakaryocyte counts in both the radiation-alone and combined injury groups.

Peg-G-CSF and Alxn4100TPO Mitigate WBC and RBC Loss in Peripheral Blood after Combined Injury

Radiation alone and combined injury are known to deplete WBCs and RBCs [(1); Figs. 6 and 7]. Skin wounding alone did not affect either WBC (Fig. 6) or

FIG. 4. Peg-G-CSF and Alxn4100TPO did not improve the wound healing rate after 9.5 Gy whole-body irradiation combined with skin wound. Mice were treated with vehicle (panel A), peg-G-CSF and Alxn4100TPO (P + A; panel B), peg-G-CSF alone (panel C) or Alxn4100TPO alone (panel D) after irradiation or combined injury. Their wound areas were measured on days 1, 3, 7, 14, 21 and 28 after wounding or combined injury. N = 16–22 per group. Data are presented as mean $\pm$ SEM. * P < 0.05 vs. the wound group; ^ P < 0.05 vs. respective drug-treated wound groups.

RBC (Fig. 7) profiles. In radiation-alone and combined injury mice, the combined treatment of peg-G-CSF and Alxn4100TPO, significantly mitigated WBC depletion (Fig. 6), mainly numbers of neutrophils, lymphocytes, monocytes and eosinophils, in both radiation-alone and combined injury groups. This treatment did not alter basophil counts (Fig. 6). Treatment with peg-G-CSF and Alxn4100TPO also significantly mitigated reduction of RBC numbers, hemoglobin and hematocrit (Fig. 7).

Treatment with peg-G-CSF alone mitigated radiation-induced WBC depletion, mainly numbers of neutrophils, lymphocytes, monocytes and eosinophils, after irradiation alone, and lymphocytes and monocytes after combined injury (Fig. 6). In addition, peg-G-CSF alone mitigated radiation-induced reductions in RBC counts, hemoglobin concentrations and hematocrit values in the irradiated-alone group, while mitigating only the hematocrit values in the combined injury group (Fig. 7).

Treatment with Alxn4100TPO alone did not affect WBC depletion after irradiation alone or combined injury (Fig. 6). However, in these mice, Alxn4100TPO mitigated reduc-

tions of RBC counts, hemoglobin concentrations, and hematocrit values after irradiation alone but not combined injury (Fig. 7).

Peg-G-CSF and Alxn4100TPO Mitigate Radiation-Induced Platelet Loss after Irradiation Alone or Combined Injury

Skin wounding did not alter the numbers of platelets (Fig. 8A). Treatment with peg-G-CSF and Alxn4100TPO together significantly mitigated reduction of platelet numbers in surviving mice from irradiation-alone and combined injury groups (Fig. 8A). Treatment with peg-G-CSF alone significantly mitigated platelet reduction only after irradiation alone (Fig. 8A). Treatment with Alxn4100TPO alone significantly mitigated platelet reductions after irradiation alone and combined injury (Fig. 8A).

The number of platelets in peripheral circulation correlates with the number of megakaryocytes. As shown in Fig. 8B, the positive correlation is represented with $y = 0.011x$ and a correlation coefficient of 0.76.

FIG. 5. Peg-G-CSF and Alxn4100TPO significantly improved bone marrow cellularity after 9.5 Gy whole-body irradiation alone or after irradiation combined with skin wound. Data are presented as mean $\pm$ SEM. Panels A–D: Histology with H&E staining of bone marrow samples 30 days after irradiation or combined injury. N = 4 per group. Panel E–F: Fat cells and megakaryocytes per 20 $\times$ field were counted. For panel E: * P < 0.05 vs. sham and wound groups; ^ P < 0.05 vs. irradiation vehicle-treated group. For panel F: * P < 0.05 vs. sham and wound groups; ^ P < 0.05 vs. irradiation or combined injury groups treated with vehicle.

FIG. 6. Peg-G-CSF and Alxn4100TPO significantly mitigated WBC depletion after whole-body irradiation alone or combined with skin wound. Mice were treated with either vehicle, peg-G-CSF and Alxn4100TPO, peg-G-CSF alone or Alxn4100TPO alone after 9.5 Gy irradiation alone or combined with skin wound (combined injury). Panels A–F: WBC profiling of blood samples collected 30 days after irradiation or combined injury. N = 6 per group. Data are presented as mean $\pm$ SEM. * P < 0.05 vs. sham group; ^ P < 0.05 vs. irradiation vehicle-treated group. # P < 0.05 vs. combined injury vehicle-treated group. P+A: peg-G-CSF+Alxn4100TPO; P: pegylated G-CSF; A: Alxn4100TPO

Peg-G-CSF and Alxn4100TPO Mitigate Ileum Histopathology

The gastrointestinal (GI) tract is known to be very sensitive to ionizing radiation (I). Combined injury at LD_{70/30} causes hematopoietic injury and GI damage (I); ileum was therefore examined as well. Radiation alone induced short yet edematous villi and crypt count reduction but did not alter crypt depth even 30 days after 9.5 Gy exposure (Fig. 9E–H).

Combined injury resulted in further changes in GI morphology (Fig. 9D) and significantly increased the mucosal injury score (Fig. 9I). Wounding alone did not appear to alter the morphology, but the apparent villous suppression was likely caused by feces in the lumen (Fig. 9B) compared to the sham-treated group (Fig. 9A).

Treatment with peg-G-CSF and Alxn4100TPO significantly improved GI morphology, with demonstrated increases in villous heights, decreases in villous widths,

FIG. 7. Peg-G-CSF and Alxn4100TPO fully recovered RBC counts, hemoglobin levels and hematocrit readings after 9.5 Gy whole-body irradiation alone or combined with skin wound. Mice were treated with vehicle or peg-G-CSF and Alxn4100TPO, peg-G-CSF alone or Alxn4100TPO alone after irradiation or combined injury. Panels A–C: RBC profiling of blood samples collected 30 days after irradiation or combined injury. $N = 6$ per group. Data are presented as mean $\pm$ SEM. * $P < 0.05$ vs. sham group; $^{\wedge}P < 0.05$ vs. irradiation vehicle-treated group. $^{\#}P < 0.05$ vs. combined injury vehicle-treated group.

increases in crypt counts and increases in crypt depths (Fig. 9E–H). This combined treatment significantly lowered mucosal injury scores (Fig. 9I).

DISCUSSION

Here we report that skin wounding significantly increased radiation-induced mortality and body-weight loss, elevated water consumption and delayed wound healing ability in B6D2F1/J mice. These results are consistent with previously reported observations in rats (40, 41), guinea pigs (42), dogs (43), swine (44) and mice (1, 5, 6, 45–48). Consequences of either radiation alone or combined injury include acute myelosuppression, thrombocytopenia, immune system inhibition, fluid imbalance, macro/microcirculation failure, massive cellular damage and disruption of vital organ functions, which lead to multiple organ dysfunction syndrome and multiple organ failure, the most frequent causes of death after irradiation (42–44).

FIG. 8. Peg-G-CSF and Alxn4100TPO significantly mitigate platelet counts after 9.5 Gy whole-body irradiation alone or combined with skin wound. Mice were treated with vehicle or peg-G-CSF and Alxn4100TPO, peg-G-CSF alone or Alxn4100TPO alone after irradiation or combined injury. Panel A: Platelet counts on day 30 in surviving sham, wounded, irradiation and combined injury groups. $N = 6$ per group. Data are presented as mean $\pm$ SEM. Panel B: Correlation between platelet counts in peripheral blood and megakaryocyte counts in bone marrow. $N = 31$. * $P < 0.05$ vs. sham group; $^{\wedge}P < 0.05$ vs. irradiation vehicle group; $^{\#}P < 0.05$ vs. combined injury vehicle group; $^{\&}P < 0.05$ vs. wounded only group.

Drugs such as 5-androstenediol (5-AED) (49, 50), G-CSF (8, 18), peg-G-CSF (9, 18) and captopril (51), were found to be effective in mitigating radiation-induced lethality. When these drugs were applied for treating combined injury, however, 5-AED and peg-G-CSF were noted to be ineffective in improving survival (18, 51), and captopril even decreased the survival after combined injury (52). On the other hand, Alxn4100TPO, effective in improving survival after combined injury, was found to be ineffective after irradiation alone (Fig. 1C) (24). For these reasons, we investigated the combined treatment of peg-G-CSF and Alxn4100TPO resulting in 95% survival after irradiation alone and 20% survival after combined injury. This combined therapy significantly mitigated body-weight losses after irradiation alone and combined injury, but

FIG. 9. Peg-G-CSF and Alxn4100TPO significantly improved ileal morphology after 9.5 Gy whole-body irradiation alone or combined with skin wound. Panels A–D: Histology slides with H&E staining of ileums collected 30 days after irradiation and combined injury. N = 4 per group. Panels E–I: Villous heights, villous width, crypt depth, crypt counts and mucosal injury scores were measured. Data are presented as mean ± SEM. **P* < 0.05 vs. sham group; ^*P* < 0.05 vs. irradiation (irrad.) vehicle group; #*P* < 0.05 vs. respective vehicle group.

failed to accelerate the delayed wound healing, suggesting that nonwound-healing mechanisms play essential roles in these two drugs, with limited efficacy. However, our results of enhanced survival induced by peg-G-CSF treatment are in agreement with data obtained from reported studies in which mice are subjected to radiation combined with burn trauma (18). Treatment was initiated 4 h after irradiation alone or combined injury, in accordance with the DoD requirement of offering therapeutics to military personnel as early as 4 h after nuclear accident occurrences.

We reported that radiation alone and combined injury induced increases in G-CSF concentrations in serum (on the order of 100–1,000 pg/ml in irradiated-only mice and 2,000–10,000 pg/ml in combined injury mice) (7). These increases were important for recovery from exposure to radiation (50, 53, 54). In comparison, a dose of 25 µg/mouse would yield a maximum serum concentration on the order of 1,000 pg/ml (55). Peg-G-CSF has a longer biological half-life than G-CSF (12). Therefore, daily injections were not necessary, which improved 30-day survival by 100% (18). However, the original G-CSF peptide was effective in improving survival of mice subjected to combined injury by an incremental difference of 20% above the control (18). In contrast to the mice subjected to radiation alone, peg-G-CSF failed to improve survival after combined injury with burn. This could be due to the complexity of mechanisms of combined injuries, involving the enhancement of serum cytokines/chemokines and systemic bacterial infection (7, 47, 48), which requires more than peg-G-CSF to manage the imbalance of homeostasis. The G-CSF survival improvement after irradiation alone in our study is consistent with that observed in nonhuman primates (8, 20). The peg-G-CSF survival improvement after irradiation alone is in agreement with that observed in nonhuman primates (9). Whether radiation alone or combined injury would alter the pharmacokinetic profile of peg-G-CSF is unknown. C_{max} and T_{max} were not measured. They are certainly confounding factors that need to be addressed.

Radiation alone and combined injury significantly reduced WBC counts (1, 2). At day 30 after irradiation alone or combined injury, surviving mice still displayed low WBC counts, mainly neutrophils, lymphocytes, monocyte, eosinophils and basophils (Fig. 6). However, the radiation and combined injury induced decreases were mitigated significantly in peg-G-CSF and Alx4100TPO-treated mice. The data are consistent with observations in irradiated nonhuman primates treated with peg-megakaryocyte growth and development factor combined with G-CSF (32). Peg-G-CSF is known to initiate proliferation and differentiation of myeloid progenitors into mature granulocytes and to induce hematopoietic stem cell mobilization from the bone marrow into the bloodstream, making it effective for recovery from infection (13, 14) and wound healing (15). Peg-G-CSF, when combined with stem cell factors and erythropoietin, was used to treat a technician, who was exposed to gamma

radiation (16). From our study, we postulate further, that peg-G-CSF mobilizes hematopoietic progenitor cells in addition to myeloid cells to peripheral blood to mitigate the blood cell depletion (Fig. 6), which is reinforced with the recovered cellularity of bone marrow (Fig. 5).

Reports from our laboratory and others indicate that an IL-3/G-CSF receptor agonist in nonhuman primates (56), G-CSF administration in canines (20) and peg-G-CSF treatment alone in mice (18) increase platelet counts after irradiation alone. Even Alx4100TPO treatment alone increases platelet counts after combined injury in addition to irradiation alone (24). In the current study, we observed that the combined treatment increased platelet counts after irradiation alone and combined injury. Taken together, these data suggest that platelet recovery probably contributes at least partially to the survival in mice subjected to combined injury. The reason that peg-G-CSF administration yielded differential effects after irradiation alone from those of combined injury is unclear. It is evident that G-CSF administration to healthy humans can induce an inflammatory process with endothelial cell activation that increases platelet counts (57). We postulate that the endothelial cell activation response to radiation alone is different from that of combined injury, thereby leading to peg-G-CSF's incapability to induce thrombopoiesis after combined injury.

Radiation alone and combined injury significantly reduced RBC counts, hemoglobin levels and hematocrit values (1, 2). At day 30 after irradiation alone and combined injury, surviving mice still displayed significantly low RBC counts, hemoglobin levels and hematocrit values (Fig. 6). However, the decreased induced by radiation alone and combined injury were fully recovered in peg-G-CSF and Alx4100TPO-treated mice, suggesting that increases in erythropoietin production may have occurred after this combined therapy. This warrants further exploration.

Radiation alone and combined injury significantly reduced platelet counts (1, 2). At day 30 after irradiation alone or combined injury, surviving mice still displayed significantly low platelet counts (Fig. 8A), which correlated with the low megakaryocyte counts in bone marrow (Fig. 5). Peg-G-CSF administration alone increased platelet counts after irradiation alone, but decreased these counts after combined injury (18), whereas Alx4100TPO alone increased platelet counts in mice subjected to either radiation alone or combined injury (24). Peg-G-CSF and Alx4100TPO treatment improved platelet counts (Fig. 8) in peripheral blood and megakaryocyte counts in bone marrow (Fig. 5) of surviving mice in both the radiation-alone and combined injury groups, a result similar to platelet recovery resulting from IL-12 treatment (58). Our data also show a positive correlation between platelet counts in peripheral blood and megakaryocyte counts in bone marrow (Fig. 8B). The bone marrow recovery by peg-G-CSF and Alx4100TPO treatment is similar to the observations with bone marrow-derived mesenchymal stem

TABLE 1
Effects of Peg-G-CSF, Alxn4100TPO and
Combination of Both on Survival, Wound Healing,
WBCs, RBCs and Platelets in Mice after Irradiation
Alone, Wound Alone or Combined Injury

	Peg-G-CSF	Alxn4100TPO	Peg-G-CSF + Alxn4100TPO
Increase in survival (%)			
Radiation alone	33	4	35
Combined injury	15	23	20
Wound healing (days)			
Wound alone	21	14	14
Combined injury	28	≥28	28
WBCs			
Radiation alone	↑↑	-	↑↑
Combined injury	-	-	↑↑
RBCs			
Radiation alone	↑↑	↑↑	↑↑
Combined injury	↑	-	↑↑
Platelets			
Radiation alone	↑↑	↑↑	↑↑
Combined injury	-	↑↑	↑↑

Notes. Survival, wound healing time and numbers of WBCs, RBCs and platelets were reduced after radiation alone and combined injury and remained low at day 30 after treatment. Comparisons are made from data pooled from three separate experiments. ↑ = Increase; ↑↑ = further increase; ↓ = decrease; - = no change.

cells injected into mice subjected to combined injury (26, 27).

It is very important to keep in mind that peg-G-CSF alone mitigated reductions of WBC, RBC, hemoglobin levels, hematocrit values and platelets only in the radiation-alone group, and not in the combined injury group (18). On the other hand, Alxn4100TPO alone mitigated RBC reduction in irradiated-alone mice, while mitigating platelet reductions in both irradiated-alone and combined injury groups. Therefore, this combined therapy provided the optimal recovery of WBC, RBC and platelets for both irradiated-alone and combined injury groups, thereby perhaps leading to increases in survival after irradiation alone or combined with injury. Regardless of the differences between these two animal groups in their responses to individual treatment with either peg-G-CSF or Alxn4100TPO, the combination of these two drugs synergistically mitigated depletion of WBCs, RBCs and platelets in both radiation-alone and combined injury mice. These findings suggest that combined therapy with peg-G-CSF and Alxn4100TPO is effective for the mitigation of acute hematopoietic syndrome induced either by radiation alone or in combination with injury.

Combined peg-G-CSF or Alxn4100TPO treatment did not accelerate the wound healing rate after combined injury (Fig. 4). This deficiency could be one of the key factors that limits the efficacy of this approach for survival improvement after combined injury. Drugs such as ghrelin (28) or ciprofloxacin (29–31) significantly accelerate wound healing and reduce the time required for wound healing, greatly improving survival after irradiation and injury combined.

Based on the FDA two-animal rule, FDA has approved G-CSF (Neupogen®) and pegylated G-CSF (Neulasta®) for acute hematopoietic syndrome (8, 9). Therefore, we postulate that peg-G-CSF combined with either ghrelin or ciprofloxacin could be an even better strategy to concurrently mitigate both radiation and combined injuries, given the high risk of tissue trauma from radiological accidents or nuclear weapon detonation. It would be worthwhile to investigate this further in nonhuman primates in addition to mice.

At a dose of 9.5 Gy, radiation induced GI injury in addition to the hematopoietic injury (1, 2). On day 30 after irradiation alone and combined injury, surviving mice still displayed injured ileal morphology, indicated by shorter villous heights, wider villous widths and lower crypt counts (Fig. 9). Peg-G-CSF and Alxn4100TPO treatment significantly improved the ileal morphology, suggesting that the combined treatment is effective in mitigating the gastrointestinal component of the ARS (GI-ARS), in addition to the hematopoietic component of the ARS (H-ARS).

Table 1 shows comparisons on percentage survival increases, wound healing and numbers of WBCs, RBCs, and platelets in 30-day surviving mice from irradiated-only and combined injury groups treated with peg-G-CSF alone, Alxn4100TPO alone or combination of both. The combined therapy increased survival, WBCs, RBCs and platelets in both groups, although it did not accelerate wound healing. These data suggest that this combined treatment is likely an effective strategy for saving victims. In particular, G-CSF or peg-G-CSF combined with a drug capable of wound repair would likely optimize the survival improvement after both irradiation alone or combined injury.

In summary, skin wounding increased radiation-induced mortality and body weight loss. Combined treatment of peg-G-CSF and Alxn4100TPO in mice after irradiation alone or combined injury enhanced 30-day survival and significantly mitigated body weight loss, hematopoietic and GI-ARS in both groups. These results demonstrate the efficacy of peg-G-CSF and Alxn4100TPO as a combined treatment for exposure to radiation alone or combined with injury. A combined therapy such as this could optimize efficiency by saving time from triage after a radiological accident and providing timely treatment for victims of both radiation alone or combined injury.

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