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Biology Contribution

5-Androstene-3β,17β-diol Promotes Recovery of Immature Hematopoietic Cells Following Myelosuppressive Radiation and Synergizes With Thrombopoietin

Fatima S.F. Aerts-Kaya, MD, PhD,* Trudi P. Visser, BSc,* Shazia Arshad, BSc,* James Frincke, PhD,† Dwight R. Stickney, MD,† Chris L. Reading, PhD,† and Gerard Wagemaker, PhD*

**Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; and †Harbor Therapeutics, Inc, San Diego, California*

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Summary

5-Androstene-3β,17β-diol (5-AED) stimulates recovery of hematopoiesis after exposure to radiation. To elucidate its cellular targets, the effects of 5-AED alone and in combination with granulocyte-colony stimulating factor and thrombopoietin (TPO) on immature hematopoietic progenitor cells were evaluated following total body irradiation. 5-AED potently counteracts the hematopoietic effects of radiation-induced myelosuppression and promotes multilineage

Purpose: 5-Androstene-3β,17β-diol (5-AED) stimulates recovery of hematopoiesis after exposure to radiation. To elucidate its cellular targets, the effects of 5-AED alone and in combination with (pegylated) granulocyte colony-stimulating factor and thrombopoietin (TPO) on immature hematopoietic progenitor cells were evaluated following total body irradiation.

Methods and Materials: BALB/c mice were exposed to radiation delivered as a single or as a fractionated dose, and recovery of bone marrow progenitors and peripheral blood parameters was assessed.

Results: BALB/c mice treated with 5-AED displayed accelerated multilineage blood cell recovery and elevated bone marrow (BM) cellularity and numbers of progenitor cells. The spleen colony-forming unit (CFU-S) assay, representing the life-saving short-term repopulating cells in BM of irradiated donor mice revealed that combined treatment with 5-AED plus TPO resulted in a 20.1-fold increase in CFU-S relative to that of placebo controls, and a 3.7 and 3.1-fold increase in comparison to 5-AED and TPO, whereas no effect was seen of Peg-G-CSF with or without 5-AED. Contrary to TPO, 5-AED also stimulated reconstitution of the more immature marrow repopulating (MRA) cells.

Conclusions: 5-AED potently counteracts the hematopoietic effects of radiation-induced myelosuppression and promotes multilineage reconstitution by stimulating immature bone marrow cells in a pattern distinct from, but synergistic with TPO.

Reprint requests to: Prof. Dr. Gerard Wagemaker, Erasmus Medical Center, Institute of Hematology, H Ee 1314, 3015 GE Rotterdam, The Netherlands. Tel: 31-10-704 37 66; Fax: 31-10-704 47 45; E-mail: g.wagemaker@erasmusmc.nl

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Conflict of interest: At the time of manuscript preparation, J.F., D.R.S., and C.L.R. were employed by Harbor BioSciences Inc, and owned stock.

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reconstitution by stimulating immature bone marrow cells in a pattern distinct from but synergistic with TPO.

Introduction

Many hematopoietic growth factors have been tested for radiation mitigation potential after myelosuppressive therapy. The most potent among these are interleukin-11 (IL-11) (1), granulocyte-colony stimulating factor (G-CSF) (2, 3), granulocyte/macrophage-colony stimulating factor (GM-CSF) (2, 3), and, in particular, thrombopoietin (TPO), which was shown in preclinical studies to be the most effective radiation mitigation drug when used as a single agent (3, 4). Both GM-CSF and G-CSF are registered for treatment-related neutropenia and display modest effects on survival and neutrophil recovery when administered within 24 h after total body irradiation (TBI). However, these agents only alleviate recovery in a sublethal setting (3, 5) and are ineffective at a lethal dose (6, 7). TPO stimulates recovery of all bone marrow (BM) and spleen progenitor cells, affecting the complete hematopoietic compartment and promoting recovery of life-saving short-term multilineage spleen-repopulating cells (spleen colony-forming unit [CFU-S]) in mice, but at the transient expense of the more immature marrow repopulating cells (MRA) (4).

5-AED, a naturally occurring adrenocortical steroid hormone, displays immune modulation (8) and myelopoiesis stimulation properties in irradiated mice (9, 10) and monkeys (11, 12), resulting in resistance to infections (13) and increased survival (9). The cellular mechanisms of 5-AED may be partly mediated by G-CSF (14) and IL-6 (15). We hypothesized that combination treatment of 5-AED with pegylated G-CSF (Peg-G-CSF) and TPO might result in an additive effect on hematopoietic cell recovery. We studied the effects of 5-AED with and without Peg-G-CSF and TPO on immature repopulating and progenitor cell subsets in myelosuppressed mice by enumerating early stem cells and their immediate progeny to gain insight into the cellular targets from which the radiation mitigation effects of 5-AED originate.

Methods and Materials

Animals

BALB/c mice, 8-12 weeks of age, were bred at the Experimental Animal Facility of the Erasmus Medical Center (Rotterdam, The Netherlands) and maintained under specific pathogen-free conditions. An independent ethical committee approved housing, experiments, and all other conditions in accordance with legal regulations in The Netherlands.

Experimental design

Mice were subjected to TBI at day 0, using two opposing ^{137}Cs-labled sources (Gammacell 40; Atomic Energy of Canada, Ottawa, Canada) at dose rates between 0.92 and 0.94 Gy/min. Mice received 5.5 or 6.0 Gy TBI delivered as a single dose or 9 Gy TBI delivered as 3 fractionated doses of 3 Gy each to measure peripheral blood (PB) reconstitution. At days 10, 14, 17, and 21, an experimental group of 3 mice was sacrificed (See Supplemental Table E1).

Test drugs

5-AED (25-200 mg/kg/day), prepared as a solution of 100 mg/ml in 7.4 mM sodium phosphate buffer, pH 6.0, containing 0.5% polysorbate 80, 0.02% benzalkonium chloride, and 4.0%-4.8% mannitol and its vehicle (12) was provided by Harbor BioSciences Inc, San Diego, CA and administered intramuscularly (IM). Recombinant murine TPO (Genentech Inc, South San Francisco, CA) was diluted in phosphate-buffered saline (PBS) supplemented with 0.01% Tween 20 and administered intraperitoneally (IP) at 9-28 μg/kg. These doses were effective in a similar model for myelosuppression (4). Pegylated recombinant human G-CSF (Amgen Europe, Breda, The Netherlands) was used at 400 μg/kg and administered IP. All drugs were administered 2 h after TBI.

Hematologic examinations

After being anesthetized with iosoflurane, mice were bled by retro-orbital puncture and killed by cervical dislocation. Complete blood cell counts were measured using a Vet ABC hematology analyzer (Scil Animal Care Co GmbH, Germany).

Measurements of surface antigens

BM, spleen, and PB cells of each mouse individually were collected for flow cytometric analysis (9). Monoclonal antibodies against murine CD4, CD8, CD11b, and B220 were purchased from BD Biosciences, San Jose, CA. Samples were measured using a FACSCalibur unit, and 10,000 events were analyzed using Cellquest (BD Biosciences, San Jose, CA).

In vitro clonogenic progenitor assays

On days 10, 14, 17, and 21 after TBI, mice were killed, and femurs and spleens were collected. Murine BM cells, 5×10^4, or 5% of the total cell content of the spleen were plated in serum-free methylcellulose culture medium, as described previously (4). Granulocyte/macrophage colony formation (CFU-GM) was stimulated with 10 ng/mL murine interleukin-3 (mIL-3), 100 ng/mL murine stem cell factor (mSCF), and 20 ng/mL GM-CSF; burst-forming erythroid (BFU-E) growth with 100 ng/mL mSCF and 4 U/mL human erythropoietin (hEPO; Behringwerke, Marburg, Germany); and colony-forming unit erythroid (CFU-E) with hEPO. Megakaryocyte progenitor cells (CFU-Meg) were stimulated in 0.275% agar cultures with 100 ng/mL SCF, 10 ng/mL mIL-3, and 10 ng/mL murine TPO (mTPO) (Genentech Inc San Francisco, CA).

Spleen colony-forming unit

To amplify 5-AED response on residual hematopoietic stem cells, donor mice were subjected to 3 × 2 Gy with 24-h intervals as previously described (4). Two hours after each fraction, mice were injected with vehicle or 40 mg/kg/day 5-AED IM for 3 days or 28 μg/kg mTPO, or 400 μg/kg Peg-G-CSF IP after the first fraction only. In addition, radiation controls did not receive injections. At 24 h after the last irradiation, mice were sacrificed, and one-tenth to one-half of the cell content of 1 femur was injected intravenously into lethally (8 Gy) irradiated BALB/c mice to perform the spleen colony assay as described by Till and McCulloch (16). At day 12, mice were sacrificed and macroscopically visible spleen colonies, designated CFU-S-12, were counted (see Supplementary Table E1, lower panel).

Marrow repopulating ability

BM from 3 × 2-Gy-irradiated control or 5-AED-treated mice was collected 24 h after the last fraction of TBI and injected into lethally (8-Gy) irradiated recipients. After 13 days, BM of recipient mice was assayed for the presence of CFU. MRA was expressed as the number of CFU per recipient femur. Control mice were injected with the standard number of 10^5 BM cells (see Supplementary Table E1, lower panel).

Statistics

Standard deviations of the mean were calculated on the assumption of a normal distribution. The standard error of CFU-S-12 was calculated on the assumption that crude colony counts are Poisson distributed. Differences in repopulating abilities between each group were evaluated using 1-way ANOVA. Colony assays were performed in duplicate for individual mice. Results of colony assays are expressed as the means ± SD per femur or spleen for at least 3 mice per group.

Results

Efficacy of 5-AED after exposure to a single myelosuppressive dose of radiation

5-AED stimulates multilineage hematopoiesis

Exposure of BALB/c mice to 5.5 or 6 Gy TBI induced BM suppression and pancytopenia. Treatment with 40 mg/kg 5-AED IM resulted in accelerated multilineage recovery (Fig. 1). Levels of white blood cells ($P<.001$), platelets ($P<.01$), and red blood cells ($P<.05$) were significantly higher in the 5-AED-treated group at days 14, 17, and 21 postirradiation. Recovery of PB was preceded by accelerated recovery of BM cellularity (Fig. 2) with $7.8 \pm 2.8 \times 10^6$ cells in the 5-AED-treated group vs $3.3 \pm 2.0 \times 10^6$ cells in the placebo control group as early as 10 days after irradiation ($P<.001$) and accompanied by accelerated normalization of spleen cellularity at day 14 in the 5-AED group on average $97.5 \pm 76.4 \times 10^6$ cells/spleen in comparison to the placebo-treated group with $14.7 \pm 9.9 \times 10^6$ cells/spleen ($P<.01$). The robust multilineage reconstitution can be explained by the prominent effect of 5-AED on reconstitution of BM progenitor cells along the neutrophil, erythroid and megakaryocytic lineages (see Supplementary Fig. E1). Femoral

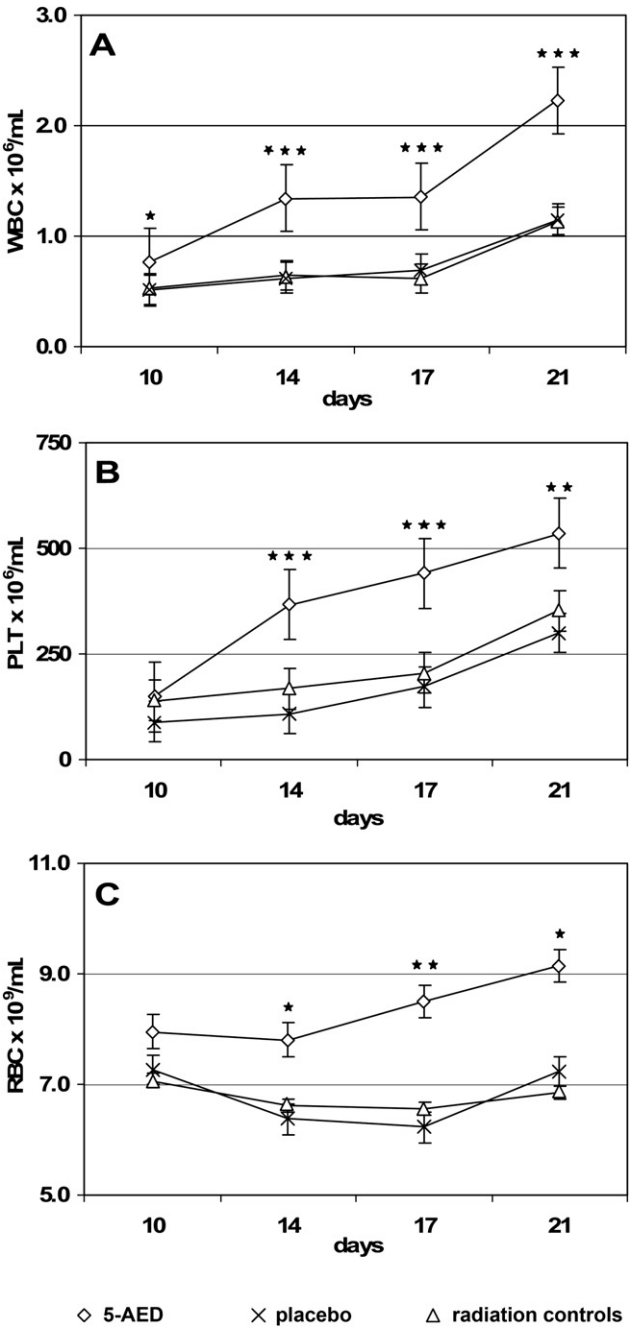

Fig. 1. Regeneration patterns of peripheral blood cells after a single myelosuppressive dose of TBI Regeneration pattern of white blood cells (A), platelets (B), and red blood cells (C) in mice subjected to 5.5-6.0 Gy TBI and treated with a single dose of 40 mg/kg 5-AED (diamonds), placebo (crosses), and radiation controls (triangles), data are the average ± SD of 5 different experiments, n = 3 mice per data point per experiment. Significantly different from time matched placebo controls: *$P<.05$; **$P<.01$; ***$P<.001$.

CFU-GM ($P<.001$), BFU-E ($P<.01$), CFU-E ($P<.001$), and CFU-Meg ($P<.05$) reached consistently higher numbers from day 10 after irradiation in the 5-AED-treated group. Fluorescence-activated cell sorting analysis of PB, BM, and spleen cells revealed a consistent 2- to 3-fold increase in absolute numbers of CD11b-positive cells in 5-AED-treated mice in comparison to

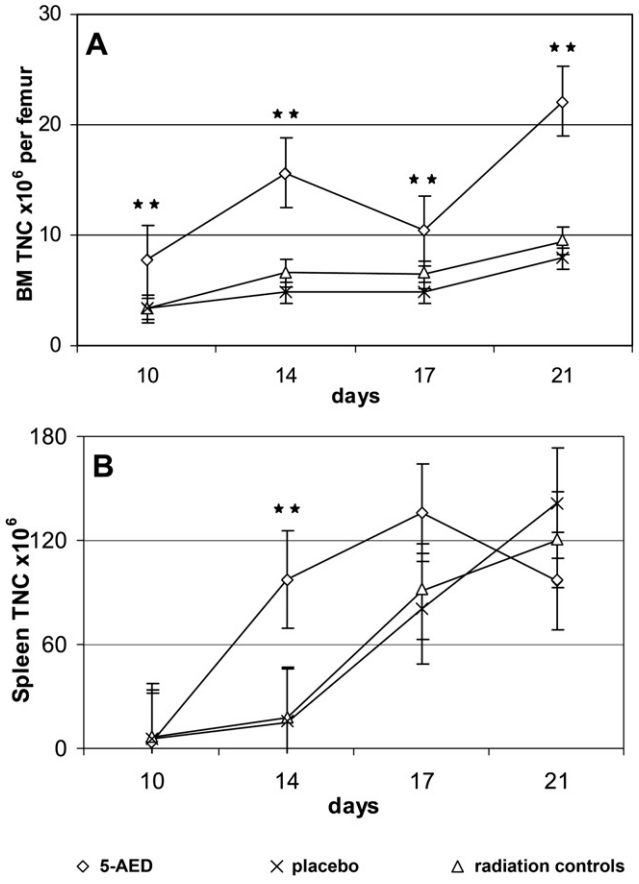

Fig. 2. Regeneration of bone marrow (BM) and spleen cells after a single myelosuppressive dose of TBI Regeneration of total nucleated cells (TNC) in BM per femur (A) and spleen (B) in mice subjected to 5.5-6.0 Gy TBI and treated with a single dose of 40 mg/kg 5-AED (diamonds), placebo (crosses), and radiation controls (triangles). Data are presented as the average ± SD of 5 different experiments, n = 3 mice per data point per experiment. Significantly different from time matched placebo controls $^{**}P<.01$.

radiation controls and placebo animals but no clear effect on lymphocytes.

Effect of combined treatment of 5-AED and Peg-G-CSF or TPO

Exposure of BALB/c mice to 5.5 or 6.0 Gy TBI and treatment with 40 mg/kg 5-AED, 10 μg Peg-G-CSF, 0.225 μg TPO, or combinations thereof resulted in significantly enhanced numbers of white blood cells, platelets, BM, and spleen cellularity ($P<.01$) at day 14 after irradiation in comparison to placebo mice (see Supplementary Table E2). Combined treatment of 5-AED with Peg-G-CSF or TPO dampened the lineage-specific response of either drug in favor of a more general response (see Supplementary Table E3).

Efficacy of 5-AED after fractionated irradiation

Effects of 5-AED or Peg-G-CSF on hematopoietic recovery

To amplify the 5-AED response, mice were subjected to 3 × 2 Gy TBI (4). This regimen induces a more profound pancytopenia than a single mid-lethal dose of TBI. Mice treated with 5-AED or Peg-G-CSF showed higher counts of white blood cells ($P<.05$) and platelets ($P<.0001$) at day 17 after TBI in comparison to placebo controls (Fig. 3). BM and spleen cellularity was significantly increased (Fig. 4) at day 17, with $6.9 \pm 1.1 \times 10^6$ BM cells ($P<.01$) in the 5-AED-treated group and $12.9 \pm 2.0 \times 10^6$ BM cells ($P<.001$) in the Peg-G-CSF-treated group in comparison with $2.9 \pm .9 \times 10^6$ for placebo controls, paralleling $138.0 \pm 15.1 \times 10^6$ cells/spleen ($P<.001$) in the 5-AED group and $92.4 \pm 6.2 \times 10^6$ cells/spleen ($P<.01$) in the Peg-G-CSF-treated group relative to $35.9 \pm 22.8 \times 10^6$ spleen cells in placebo mice.

Effect of 5-AED on MRA

Eight-Gy-irradiated mice transplanted with 5-AED-treated BM cells showed 100% survival vs 80% in the radiation control group. The number of secondary GM-CFU present in the BM of lethally irradiated recipients was increased for the 5-AED group in comparison to radiation controls, that is, 10^4 (10 mice) vs 1.4×10^3 colonies (8 mice), respectively. Spleen GM-CFU were increased ($P<.001$) for the 5-AED-treated group in comparison to radiation controls (Fig. 5).

Effect of 5-AED on short-term repopulating stem cells (CFU-S-12)

Fig. 6 demonstrates that in comparison to mice transplanted with untreated irradiated BM cells, administration of 5-AED to irradiated bone marrow donors (see Materials and Methods) resulted in a 5.4-fold increase of CFU-S-12 in recipient mice ($P<10^{-5}$). Treatment with TPO resulted in a 6.5-fold increase in CFU-S-12 ($P<10^{-5}$). 5-AED synergized with TPO, resulting in a 20.1-fold increase in CFU-S-12 ($P<10^{-10}$) but not with Peg-G-CSF. Differences among mice treated with TPO plus 5-AED and TPO or 5-AED alone were highly significant, at $P<10^{-7}$ and $<10^{-10}$, respectively.

Discussion

Immune response modulation and protection from lethal microbial challenge, attributes previously ascribed to dehydroepiandrosterone (DHEA) (17), have now been shown to be mediated by 5-AED, a metabolite of DHEA (13, 17, 18). Protective effects of 5-AED are more pronounced under conditions of increased susceptibility to infections, increased age (18), or after myelosuppression (8, 9). 5-AED ameliorates neutropenia and thrombocytopenia in irradiated mice (9, 14) and nonhuman primates (11, 12) resulting in increased survival and a radiation dose reduction factor of 1.3 (9).

The present study demonstrates that 40 mg/kg 5-AED after a single dose of 6 Gy TBI or 25 mg/kg/d 5-AED after 3 × 3 Gy fractionated TBI adequately counteracts radiation-induced pancytopenia and promotes multilineage hematopoietic reconstitution and an increase of BM cellularity and elevated numbers of hematopoietic progenitor cells at 14 days postirradiation (6 Gy TBI) or at 17 days postirradiation (3 × 3 Gy TBI).

Effects of G-CSF in humans are lineage-restricted and result in expansion of surviving neutrophil precursors rather than in increased survival of immature cells (19, 20), whereas in mice, treatment may result in enhanced multilineage recovery. Maximum stimulation of hematopoietic progenitor cells by Peg-G-CSF may have concealed possible synergism of 5-AED and Peg-G-CSF. Saturation of G-CSF-receptors by Peg-G-CSF may

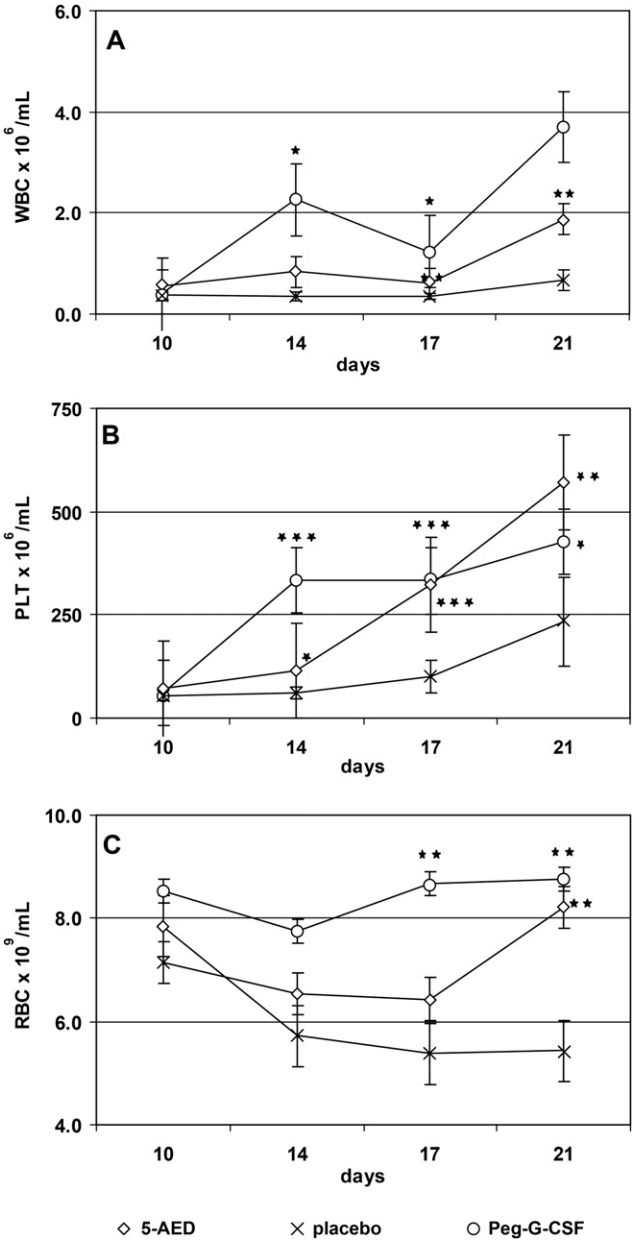

Fig. 3. Regeneration pattern of peripheral blood cells after fractionated TBI Regeneration pattern of white blood cells (A), platelets (B), and red blood cells (C) in mice irradiated with 3 × 3 Gy TBI and treated with 25 mg/kg/d 5-AED (diamonds) 2 h after each fraction, or placebo (triangles) IM or 400 μg/kg Peg-G-CSF (crosses) IP after the first irradiation only. Data are the average ± SD of 2 separate experiments, n = 3 mice per data point per experiment. Significantly different from time matched placebo controls *$P<.05$; **$P<.01$; ***$P<.001$.

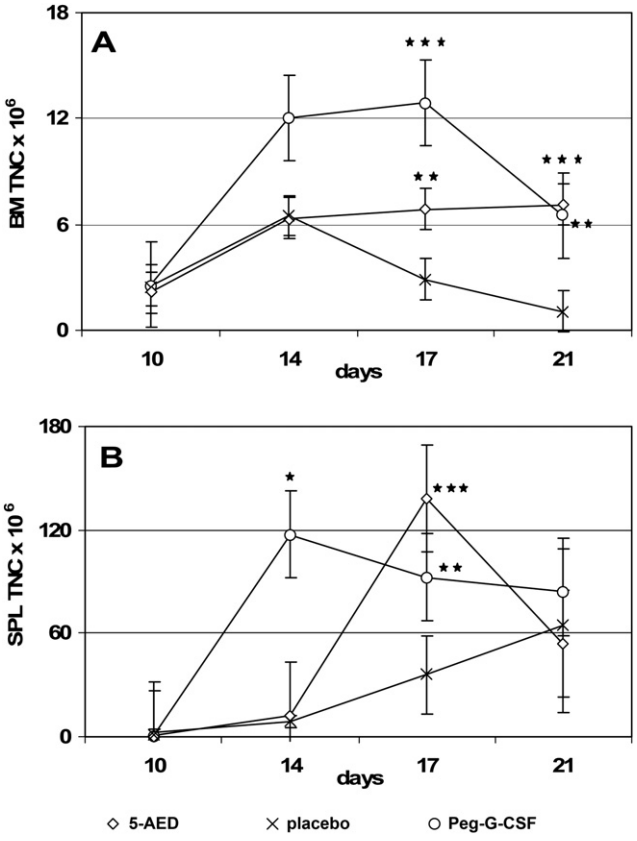

Fig. 4. Regeneration of bone marrow and spleen cells after fractionated TBI Regeneration of BM (A) and spleen (B) total nucleated cells (TNC) in mice subjected to 3 × 3 Gy TBI and treated with 25 mg/kg/d 5-AED (diamonds), Peg-G-CSF (crosses), and placebo controls (triangles). Data are the average ± SE, n = 3 per group. Significantly different from time matched vehicle treated controls *$P<.05$; **$P<.01$; ***$P<.001$.

have prevented 5-AED- induced endogenous G-CSF from exerting additive effects. Combined treatment with 5-AED and Peg-G-CSF resulted in a shift from a lineage-specific to a more generally enhanced recovery. A possible working mechanism of 5-AED might involve modulation of G-CSF levels. Indeed, accelerated hematopoietic recovery and increased survival after treatment of irradiated mice with 5-AED was accompanied by increased levels of circulating G-CSF (14). NFκB is essential in the regulation of many hematopoietic growth factors and data demonstrating that 5-AED promotes survival of irradiated hematopoietic progenitor cells by NFκB induced G-CSF secretion *in vitro* (15) would be consistent with this mechanism.

A single injection of TPO after TBI was shown to give multilineage recovery of PB cells and BM progenitors (4). Here, we used a suboptimal dose of TPO to uncover additive or synergistic effects of combined 5-AED and TPO treatment. As plasma erythropoietin levels are reciprocally related to levels of peripheral red blood cells, the highest level of red blood cells in the TPO-plus-5-AED-treated group would consequently result in the lowest levels of erythropoietin. This is consistent with the observation that TPO plus 5-AED combination treatment resulted in an increased number of BFU-E in comparison to treatment with 5-AED alone ($P<.05$) but with a decreased number of CFU-E, which for their maturation strongly depend on the presence of erythropoietin. TPO affects progenitor cell viability by preventing apoptosis and can affect erythropoiesis by binding to the TPO receptor c-mpl or through activation of the erythropoietin receptor. The additive effect observed on BFU-E of 5-AED and TPO may be the result of TPO-related stimulation of increased numbers of viable erythroid progenitor cells. Combined treatment with 5-AED and TPO resulted in an increase of 20.1-fold of CFU-S-12 in comparison to radiation controls ($P<10^{-10}$), and a 3.1 and

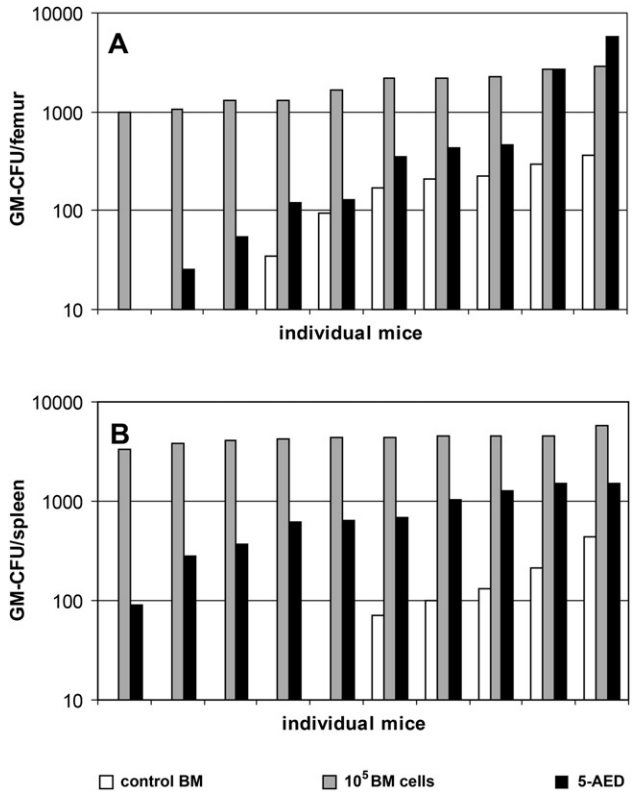

Fig. 5. Secondary marrow repopulating ability after fractionated TBI Marrow repopulating ability (MRA): GM-CFU per femur (A) or spleen (B), ranked in ascending order on day 13 of recipients of the cellular content of 1 femur at 24 h after the last radiation fraction of mice treated with 40 mg/kg 5-AED IM 2 h after each fraction of 2 Gy TBI (black bars, survival 10/10) in comparison with recipients receiving control BM derived from mice that did not receive treatment (white bars, survival 8/10) and with recipients transplanted with 10^5 BM cells from normal, unirradiated and untreated donors (striped bars, survival 10/10). A total of 10 mice per group were injected with BM cells. Bars represent the data from individual mice.

3.7-fold increase in comparison to TPO ($P<10^{-7}$) and 5-AED ($P<10^{-10}$), respectively. Such a dramatic response was not observed after treatment with Peg-G-CSF alone or in the presence of 5-AED. This confirms that the observed effects of Peg-G-CSF cannot be attributed to increased survival of short-term repopulating stem cells, but rather by amplified expansion of surviving progenitors (19, 20), whereas 5-AED selectively enhances the properties of TPO to protect transplantable short-term repopulating immature hematopoietic stem cells.

The pattern through which 5-AED promotes radiation mitigation is distinct from that of TPO, as TPO promoted CFU-S but at the transient expense of MRA (4), whereas 5-AED promotes CFU-S to a similar extent as TPO but simultaneously promotes recovery of MRA. Cytokine array tests showed an increase of circulating G-CSF and IL-6 but not TPO after 5-AED treatment *in vivo* (14) and *in vitro* (15). These data support the hypothesis that the observed synergistic effect of 5-AED in presence of TPO is the result of activation of an alternative pathway, whereas the lack of additive effect in presence of G-CSF strongly suggests the use of a common shared pathway. Thus, in addition to TPO other hematopoietic growth factors may also act in concert with 5-AED,

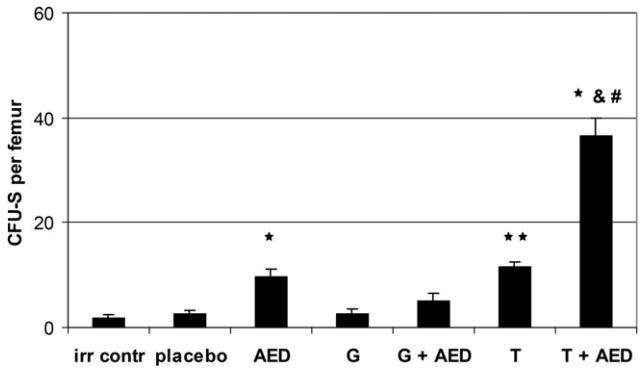

Fig. 6. Colony forming unit-spleen after fractionated TBI Colony forming unit-spleen (CFU-S): 8 Gy irradiated recipient mice were injected with BM cells from donor mice subjected to 3 × 2 Gy, followed by treatment with placebo, 40 mg/kg/d 5-AED (AED) IM or 9 μg/kg TPO (T) IP at 2 h after each fraction, 400 μg/kg Peg-G-CSF (G) IP at 2 h after the first fraction only, or combinations. Recipients were killed at 12 days after transplantation and spleen colonies were enumerated. Data are depicted as average plus SD of 1-3 separate experiments, n = 10 mice per group per experiment. Significantly different from time matched placebo controls: $*P<10^{-5}$; $**P<10^{-10}$; from TPO alone: $\#P<10^{-7}$ and from 5-AED alone: & $P<10^{-10}$.

promoting the radiation mitigation effect on HSCs. Detailed mechanistic studies are warranted to optimize the radiation mitigation effects of 5-AED alone or in concert with hematopoietic growth factors.

Conclusions

In conclusion, 5-AED stimulates multilineage hematopoiesis and increases BM and spleen cellularity following TBI. This effect is mediated by increased survival and/or reconstitution of immature repopulating cells in a pattern distinct from that of TPO. 5-AED strongly synergizes with TPO at the level of immature progenitor cells, thus revealing a novel mechanism of BM protection. In view of these results, the steroid 5-AED should be classified as a powerful radiation mitigation agent, the effects of which occur primarily at the level of immature stem cell subsets.

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