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Radioprotective efficacy of delta-tocotrienol, a vitamin E isoform, is mediated through granulocyte colony-stimulating factor

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ABSTRACT

Aims: The objectives of this study were to determine the cytokine induction by delta tocotrienol (DT3, a promising radiation countermeasure) and to investigate the role of granulocyte colony-stimulating factor (G-CSF) in its radioprotective efficacy against ionizing radiation in mice.
Main methods: Multiplex Luminex was used to analyze cytokines induced by DT3 and other tocols (gamma-tocotrienol and tocopherol succinate) in CD2F1 mice. Mice were injected with an optimal dose of DT3 and a G-CSF antibody, and their 30-day survival against cobalt-60 gamma-irradiation was monitored. The neutralization of G-CSF by the administration of a G-CSF-specific antibody in DT3-injected mice was investigated by multiplex Luminex.
Key findings: Our data demonstrate that DT3 induced high levels of various cytokines comparable to other tocols being developed as radiation countermeasures. DT3 significantly protected mice against ionizing radiation, and the administration of a G-CSF neutralizing antibody to DT3-treated animals resulted in the complete abrogation of DT3's radioprotective efficacy and neutralization of G-CSF in peripheral blood.
Significance: Our study findings suggest that G-CSF induced by DT3 mediates its radioprotective efficacy against ionizing radiation in mice.

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Introduction

Although the search for suitable radiation countermeasures has been going on for the last 60 years, no safe and effective radiation countermeasure has been approved by the U.S. Food and Drug Administration (US FDA) for the acute radiation syndrome (Singh et al., 2012a; Dumont et al., 2010). It is now well recognized that free radicals formed by the radiolysis of cellular aqueous milieu, and their interaction with one another and with oxygen, are primary mediators of radiation injury (Hall and Giaccia, 2006). Most forms of ionizing radiation cause the production of reactive oxygen species through hydrolysis of water. These include superoxide, hydrogen peroxide, and hydroxyl radicals. Such reactive oxygen species induced by ionizing radiation can initiate oxidative cellular injury as well as activate intracellular signaling pathways and stimulate cytochrome *c* release from mitochondria that leads to apoptosis. This understanding has placed emphasis on the search for antioxidant agents that are suitable as radiation countermeasures (Singh et al., 2012a; Dumont et al., 2010; Weiss and Landauer, 2009). Exogenously supplemented antioxidants or agents that stimulate endogenous antioxidant systems within cells have shown promise in terms of suppressing the harmful effects of irradiation. If present in the cells at the time of radiation exposure, such antioxidants may protect cells from radiation damage by scavenging reactive oxygen species before they act on cellular components. A variety of reducing agents, such as vitamin E analogs, polyphenols, thiols, and superoxide dismutase mimetics have been described as potential radiation countermeasures in the recent past (Singh et al., 2012a; Dumont et al., 2010).

Vitamin E is well known for its established health benefits, including antioxidant, neuroprotective, and anti-inflammatory properties (Nesaretnam, 2008). It represents a family of compounds that is divided into two subgroups called tocopherols and tocotrienols, which act as important antioxidants that regulate peroxidation reactions and control free-radical production within the body (Palozza et al., 2006, 2008). This family of compounds has eight different isoforms that belong to two categories: four saturated analogues (α, β, γ, and δ) called tocopherols and four unsaturated analogues referred to as tocotrienols. These eight components are collectively known as tocols. Tocols and their derivatives have been evaluated for their radioprotective properties (Singh et al., 2013). The majority of these studies have been conducted with alpha tocopherol, the most commonly used vitamin E

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supplement and the most abundant vitamin E isoform in human and animal tissues (Weiss and Landauer, 2000, 2003, 2009). During the last decade, tocotrienol research has gained substantial momentum. For radioprotective efficacy, tocopherol succinate (TS), delta tocotrienol (DT3), and gamma-tocotrienol (GT3) are comparable and appear better than other tocols (Satyamitra et al., 2011; Singh et al., 2009, 2012b; Li et al., 2010). DT3 has been shown to have both radioprotective (administered before radiation exposure) and radiomitigative (administered after radiation exposure) efficacy (Satyamitra et al., 2011). Recently, delta-tocotrienol (DT3) was demonstrated to reduce activation of caspase-8, caspase-3, and caspase-7 while increasing autophagy-related beclin-1 expression in irradiated bone marrow cells (Satyamitra et al., 2012). DT3 also has been reported to increase cell survival and regeneration of hematopoietic microfoci and lineage$^-$/Sca-1$^+$/c-Kit$^+$ stem and progenitor cells in irradiated mouse bone marrow cells (Li et al., 2010). These changes were associated with activation of the mRNA translation regulator eIF4E and ribosomal protein S6. These findings suggest that DT3 protects mouse bone marrow and human CD34$^+$ cells from radiation-induced injury through Erk (extracellular signal-regulated kinase) activation associated with the mTOR (mammalian target of rapamycin) survival pathway.

Here we demonstrate that DT3 induces high levels of various cytokines comparable to cytokines induced by GT3 and TS. The administration of a G-CSF antibody completely neutralized DT3-induced G-CSF in peripheral blood, and leads to the abrogation of DT3's radioprotective efficacy.

Materials and methods

Mice

Six- to eight-week-old male, CD2F1-specific pathogen-free mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and housed in an air-conditioned facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Singh et al., 2012c). All mice were kept in rooms with a 12-h light/dark cycle. The mice holding room was maintained at 21 ± 2 °C having 10–15 hourly cycles of fresh air and a relative humidity of 50% ± 10%. Upon arrival, the mice were held in quarantine for 10 days. A microbiological examination of representative samples ensured the absence of *Pseudomonas aeruginosa*. Mice were provided certified rodent rations (Harlan Teklad Rodent Diet, Harlan Teklad, WI, USA) and acidified water (HCl, pH 2.5–2.8) ad libitum. Mice were 8–9 weeks old when experiments began. All animal procedures were performed according to a protocol approved by the Armed Forces Radiobiology Research Institute (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 Resources, National Research Council, U.S. National Academy of Sciences (National Research Council of the National Academy of Sciences, 2011).

Drug preparation and administration

The optimal drug dose for this study, 200 mg/kg for all three agents, was selected based on published reports (Satyamitra et al., 2011; Li et al., 2010; Ghosh et al., 2009; Singh et al., 2010). DT3 and GT3 formulations in 5% Tween-80 in saline were purchased from Yasoo Health, Inc. (Johnson City, TN, USA). TS (Sigma-Aldrich, St. Louis, MO, USA) was administered as a suspension. For a 200-mg/kg dose (5 mg for a 25-g mouse), 100 mg of TS was dispersed in 1.9 ml of PEG-400 and 0.1 ml of Tween-80 for a total volume of 2.0 ml. Olive oil was used as vehicle control (equivalent to the quantity of tocols) in 5% Tween-80. The final tocol concentration (200 mg/kg) was adjusted to administer 0.1 ml, with control mice receiving 0.1 ml of vehicle. The subcutaneous (sc) injections of the drug and vehicle were done at the nape of the neck with a 23-G needle 24 h before irradiation.

Irradiation

Mice were placed in ventilated Plexiglas boxes compartmentalized to accommodate eight mice per box and exposed to bilateral irradiation in the AFRRI cobalt-60 facility at a dose rate of 0.6 Gy/min (Singh et al., 2011). Animals were exposed to a dose of 9.2 Gy ($LD_{90/30}$ dose for CD2F1 mice). After irradiation, mice were returned to their cages and monitored. Sham-irradiated mice were treated in the same manner as irradiated animals except that the facility's cobalt-60 rods were not raised from their pool of shielding water. Radiation dosimetry was based primarily on the alanine/EPR (electron paramagnetic resonance) system (Nagy, 2000; ISO-ASTM, 2004), currently accepted as one of the most accurate methods and used for intercomparison between national metrology institutions. The calibration curves (spectrometer e-Scan, Burker Biospin, Inc., Madison, WI, USA) used in dose measurements at the AFRRI are based on standard alanine calibration sets purchased from the United States National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA. The alanine dosimeters obtained from NIST had been calibrated in terms of absorbed dose to water using the US national standard radiation sources. At AFRRI, identical alanine dosimeters were irradiated in mice phantoms (Plexiglas 1″ diameter, 3″ length) for a predefined period of time. Measurement of their EPR signals using the calibration curve constructed with alanine dosimeters from NIST provided dose rates to water in the cores of mice. A small correction was subsequently applied for the difference in mass energy absorption coefficients between water and soft tissue.

G-CSF neutralization

Mice were administered DT3 (200 mg/kg) sc 24 h before blood harvest or irradiation. The DT3-treated mice then received either the G-CSF antibody (0.2 ml, 1000 μg/mouse) or the isotype control (0.2 ml, 1000 μg/mouse) intraperitoneally (ip), 8 h after DT3 administration as described earlier (Singh et al., 2010; Kulkarni et al., 2013). Before injection, G-CSF antibody and isotype were tested for 12 viral agents by BioReliance (Rockville, MD, USA) by MAP-IT (molecular antigen PCR-identification test for mice) assay (cat no. 104253) and found negative for all agents tested. Blood samples were collected 16 h after G-CSF antibody injection (24 h after DT3 administration) to analyze cytokine induction.

Blood collection and Luminex analysis of cytokines

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

Luminex 200 (Luminex Corp., Austin, TX, USA) was used to detect 40 cytokines, chemokines, and growth factors. Mouse serum samples were analyzed for interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17α, IL-17 F, IL-18, IL-21, IL-22, IL-23p19, IL-31, IL-33, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interferon-γ (IFN-γ), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1(MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-2, MIP-3α, RANTES (regulated on activation, normal T cell expressed and secreted), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (FGF-basic), leukemia inhibitory factor (LIF), monokine induced by γ-IFN (MIG), platelet-derived growth factor subunit B (PDGF-bb), vascular endothelial growth factor (VEGF), and cluster of differentiation

40 ligand (CD40L) as described earlier (Singh et al., 2010) using multiplex kits (Bio-Rad Inc., Hercules, CA, USA). Cytokine quantification was performed using Bio-Plex Manager software, version 6.1 (Bio-Rad Inc.).

Statistical analysis

For survival data, a log-rank test was used to compare survival curves. Fisher's exact test was used to compare survival rates at the end of 30 days, with a Bonferroni correction used to control for type I error if multiple comparisons were used. For cytokine and G-CSF 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. Statistical software SPSS version 19 was used for analyses.

Results

Induction of various cytokines by DT3, GT3, and TS in mice

Our ongoing studies with different radiation countermeasures have shown a relationship between survival efficacy against ionizing radiation and an increase in cytokines circulating in the bloodstream. We, therefore, wanted to compare the levels of various cytokines following the administration of DT3, GT3, and TS. These levels were compared with levels of such cytokines in mice injected with vehicle (Fig. 1). Blood was collected 24 h after the administration of drug or vehicle, and serum samples were analyzed for 40 cytokines listed above. Mice injected with a 200-mg/kg dose of DT3 had significantly higher levels of 4 out of 40 cytokines compared to vehicle. These cytokines were G-CSF, KC, MCP-1 and IL-17 F. Mice injected with GT3 had significantly higher levels of 7 out of 40 cytokines evaluated as compared to the vehicle treated group. These cytokines were IL-9, G-CSF, KC, PDGF-bb, IL-17 F, CD40L, and MIP3-α. Mice injected with 200 mg/kg dose of TS had higher levels of 2 out of 40 cytokines tested: KC, and MCP-1. It is important to note that cytokines were evaluated at only one time point (24 h after drug injection). This may not be the optimal time point for cytokine induction for all drugs. We decided to measure cytokines 24 h after drug injection since these drugs are most effective when administered 24 h prior to radiation exposure (Satyamitra et al., 2011; Singh et al., 2009, 2010; Li et al., 2010; Ghosh et al., 2009). We were interested to know the levels of various cytokines in mice treated with these tocols at the time of radiation exposure. Our results suggest that the administration of DT3, GT3, or TS induces significantly higher levels of several cytokines compared to vehicle. These cytokines may play a critical role in radioprotective efficacy of these tocols.

Induction of various cytokines by DT3, GT3, and TS in irradiated mice

To determine the effect of DT3, GT3, and TS in irradiated mice, drug or vehicle was injected 24 h before irradiation with 9.2 Gy (dose rate 0.6 Gy/min). This time point was selected since these drugs are most effective when administered 24 h before irradiation as stated above. Blood was collected as described above at 6 h post-irradiation. Cytokine analysis data are presented in Fig. 2. Mice treated with DT3 had significantly higher levels of eleven cytokines compared to vehicle: IL-1β, IL-2, IL-5, IL-13, eotaxin, G-CSF, KC, MCP-1, M-CSF, MIG, and IL-22. The mice receiving GT3 showed higher levels of the following nine cytokines: IL-1β, IL-2, IL-5, IL-13 IL-17α, eotaxin, G-CSF, MCP-1, and MIG. Mice injected with TS had significantly higher levels of six cytokines compared to the vehicle-treated mice: IL-1β, IL-5, IL-13, eotaxin, GM-CSF, and TNF-α. Our results also suggest that radiation exposure induces various cytokines (cytokine levels observed in irradiated mice compared to unirradiated mice). Data presented in Fig. 3 suggest that radiation exposure induced the following cytokines 6 h after irradiation: G-CSF, KC, PDGF-bb, and MIP-3α. G-CSF is one of those cytokines and it has been studied extensively with various radiation countermeasures.

Role of G-CSF stimulated by DT3 in its radioprotective efficacy against ionizing radiation

We conducted an experiment to neutralize G-CSF in mice to determine whether G-CSF induction by DT3 is a key factor in the protection against radiation injury. Three groups of CD2F1 mice ($n = 16$) were administered DT3 (200 mg/kg) 24 h before exposure to cobalt-60 γ-radiation. One group of DT3-treated mice was administered G-CSF neutralizing antibody 16 h before irradiation. The second group received the isotype control 16 h prior to irradiation, and the third group did not receive the antibody or the isotype. A fourth group received only vehicle. All four groups were irradiated (9.2 Gy at 0.6 Gy/min) and monitored for survival for 30 days after irradiation. Data presented in Fig. 4 demonstrate that mice receiving only DT3 or DT3 plus the isotype control were protected significantly ($p < 0.01$) from ionizing radiation compared to vehicle control and DT3 plus the G-CSF neutralizing antibody. There was no significant difference between DT3-treated and DT3 plus isotype-treated mice.

Induction of cytokines by DT3 and its neutralization by G-CSF antibody in mice

Serum G-CSF levels in mice treated with DT3 were analyzed after administering the G-CSF neutralizing antibody to determine whether the increase in 30-day post-irradiation survival in the DT3-mice resulted from increased G-CSF levels. Three groups of mice ($n = 8$) were injected sc with DT3 (200 mg/kg). One group received the G-CSF antibody and another received its isotype ip 8 h after injection. The third group did not receive a second injection (G-CSF antibody or isotype), and a fourth group received only vehicle. Blood was harvested from mice 24 h after DT3 injection (or 16 h after G-CSF neutralizing antibody or isotype administration) based on previously published work (Singh et al., 2011; Kulkarni et al., 2013). To analyze whether the G-CSF antibody specifically neutralized circulating G-CSF in peripheral blood, serum samples were analyzed for IL-1β, IL-6, IL-10, IL-12(p70), G-CSF, GM-CSF, KC, and TNF-α by multiplex Luminex. Significant levels of all tested cytokines were observed in DT3-treated mice ($p < 0.01$, Fig. 5). Our data show that administering the G-CSF antibody specifically neutralized circulating G-CSF in peripheral blood and that neutralization was complete ($p < 0.01$). The administration of the isotype had no effect either on serum G-CSF levels or any other cytokine stimulated by DT3.

Neutralization of DT3-induced G-CSF by the administration of its specific antibody in irradiated mice

Various cytokines were analyzed in serum samples of four groups of mice ((a) vehicle, (b) DT3, (c) DT3 + isotype, and (d) DT3 + G-CSF antibody) receiving radiation exposure (9.2 Gy, 24 h after DT3 injection). Blood samples were collected at 6 h after irradiation (30 h after DT3 administration) for analysis of neutralization of G-CSF by use of the G-CSF antibody. Data presented in Fig. 6 further suggest that DT3 significantly stimulated production of all eight cytokine/growth factors evaluated in irradiated mice compared to the vehicle-treated irradiated group ($p < 0.001$). This study also confirmed that the G-CSF antibody completely neutralized G-CSF induced in response to DT3 administration ($p < 0.001$). Administering the isotype control antibody did not affect levels of any cytokine tested in DT3-treated and irradiated mice.

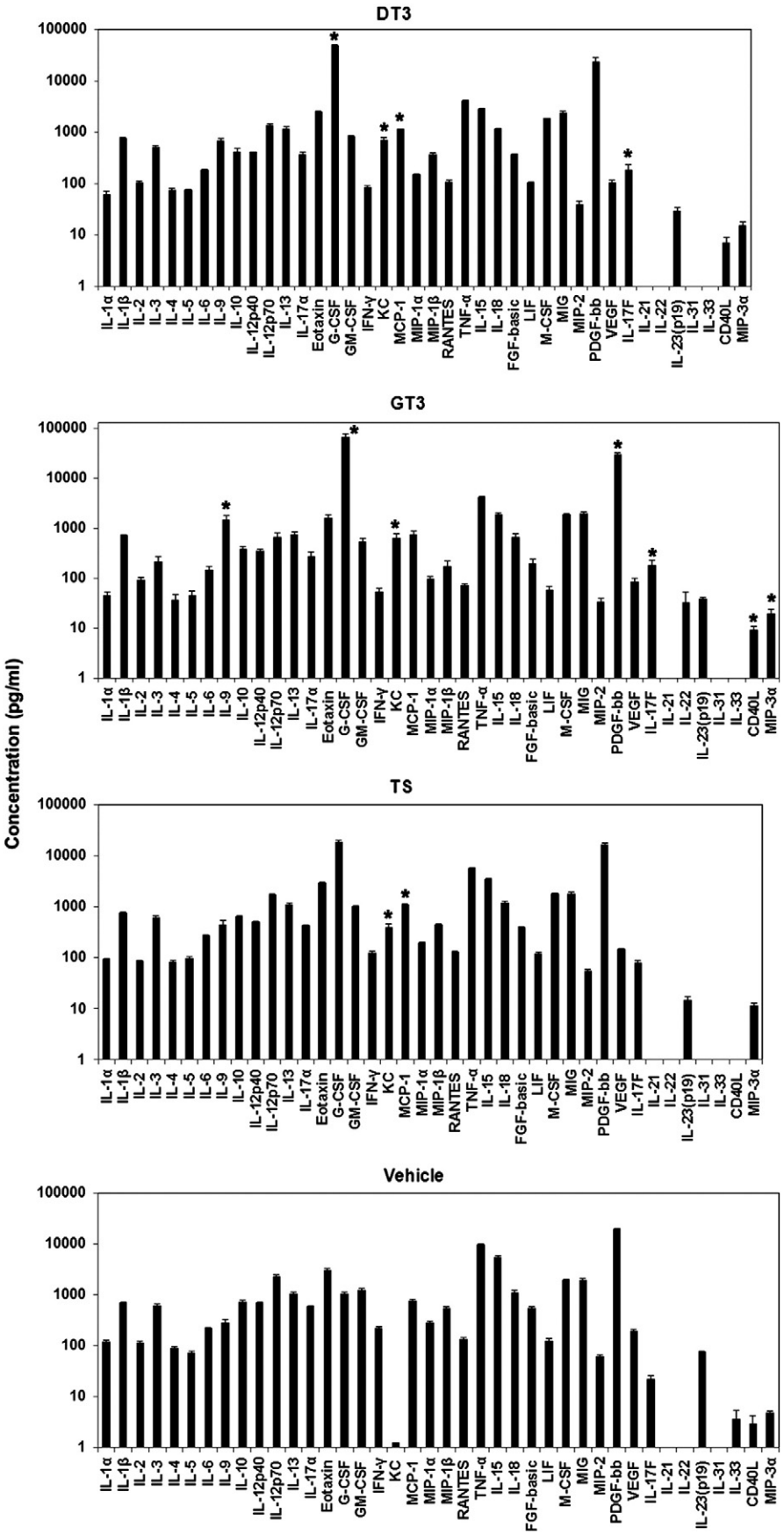

Fig. 1. Induction of cytokines by various tocols after sc administration to mice. Four groups of mice were injected either with 200 mg/kg of DT3, GT3, TS or vehicle. Blood samples were collected 24 h after drug or vehicle injection. Serum samples were analyzed by multiplex Luminex assay for cytokines. *Significantly higher levels compared to the vehicle control group ($p < 0.05$).

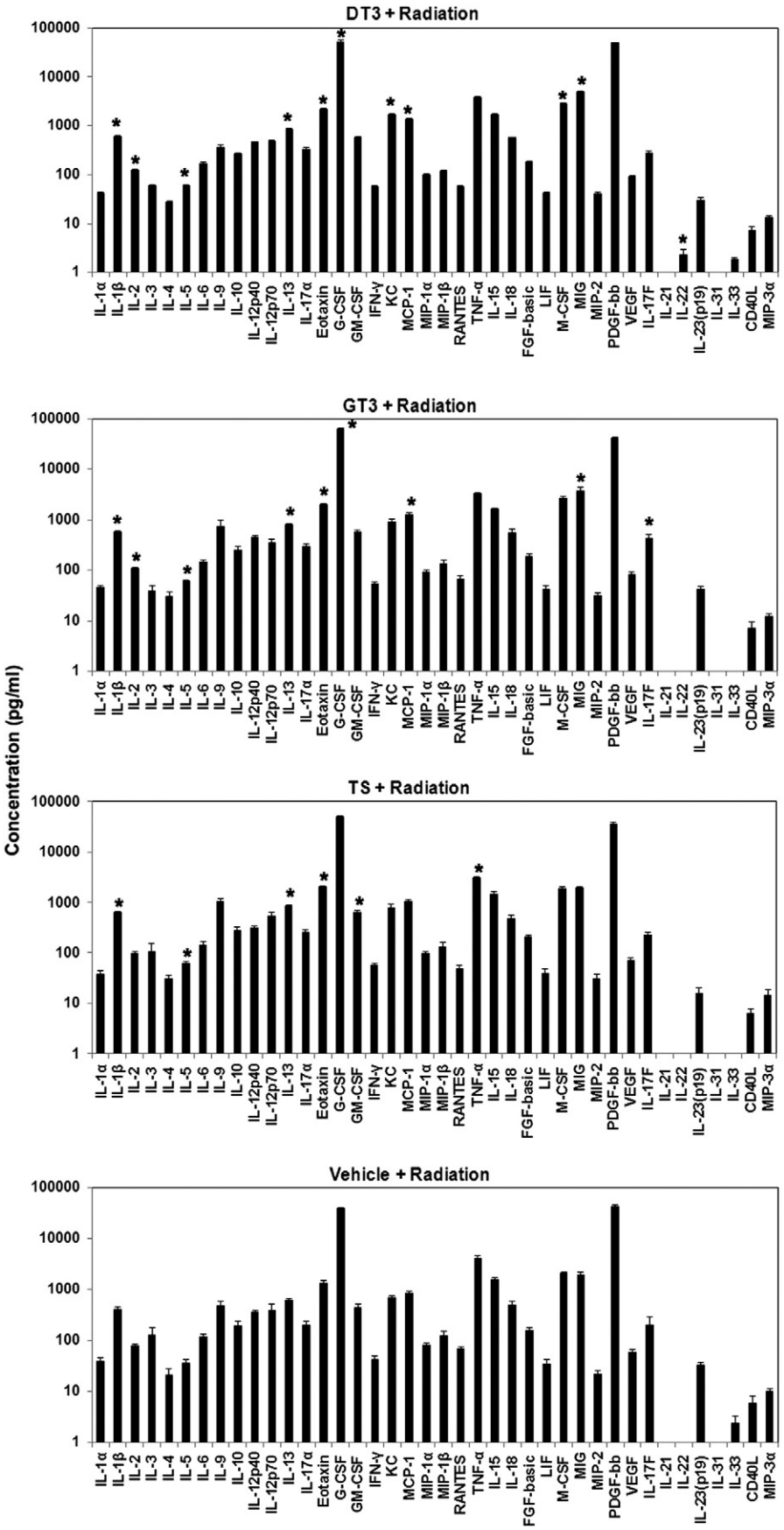

Fig. 2. Induction of cytokines by various tocols in ^{60}Co γ-irradiated mice. Four groups of mice were administered sc either 200 mg/kg of DT3, GT3, TS, or vehicle. Mice were irradiated at 9.2 Gy (0.6 Gy/min) 24 h after drug or vehicle injection. Blood samples were collected 6 h after irradiation and serum was analyzed for cytokines by multiplex Luminex assay. *Significantly higher levels compared to the vehicle control group ($p < 0.05$).

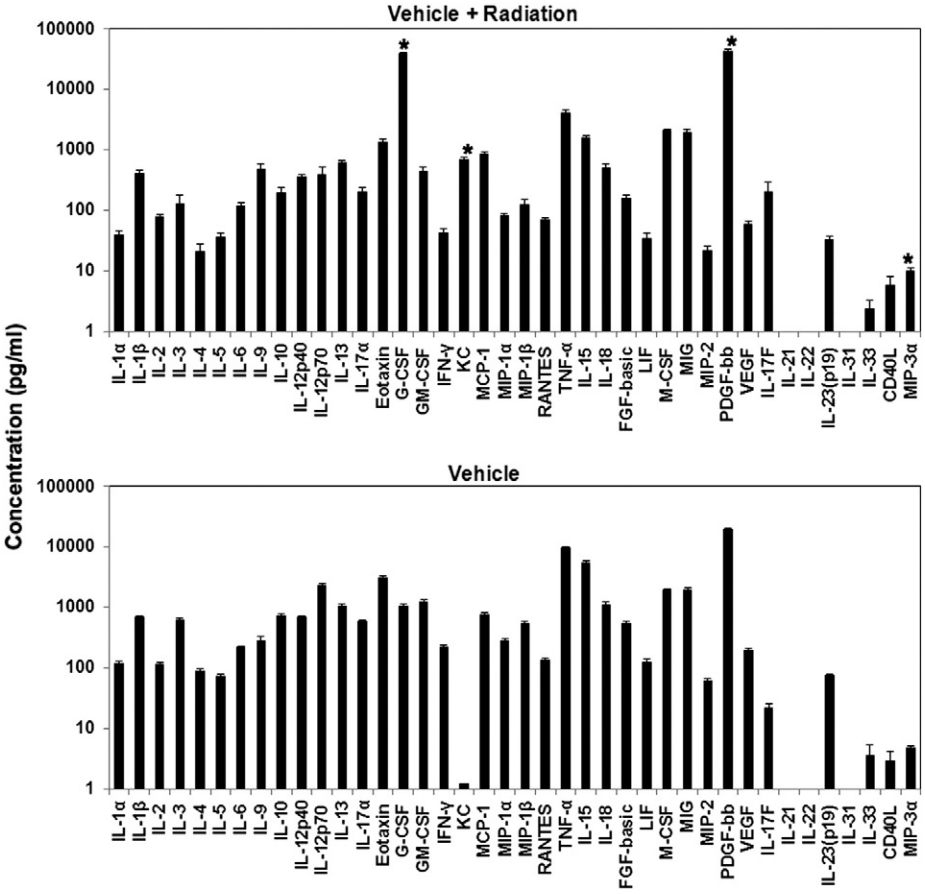

Fig. 3. Induction of cytokines in mice exposed to ^{60}Co γ-irradiated mice. One group of mice treated with vehicle was irradiated (9.2 Gy, dose rate 0.6 Gy/min), and other group was sham irradiated. Blood samples were collected 6 h after irradiation and serum was analyzed for cytokines by multiplex Luminex assay. *Significant difference compared to sham radiation control group ($p < 0.05$).

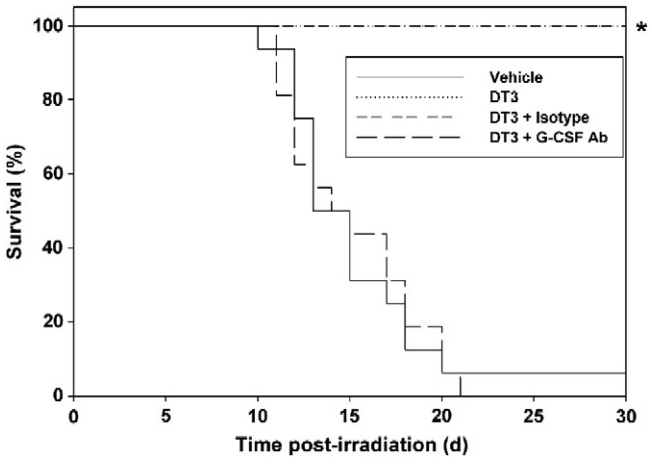

Fig. 4. Abrogation of the protective effect of DT3 by the administration of a G-CSF-specific antibody in irradiated mice. Three groups of mice were given sc injections of DT3 (200 mg/kg) 24 h before irradiation. Then 8 h after DT3 injection (16 h before irradiation), two of the DT3-treated groups were administered (ip) either the G-CSF antibody or an isotype control. A fourth group was the vehicle control. Mice were irradiated with 9.2 Gy (0.6 Gy/min) γ-radiation and observed for 30 days. DT3 and DT3 plus isotype control lines are superimposed in the figure. Mice treated with either DT3 or DT3 plus isotype control were (n = 16) protected significantly with respect to vehicle control. A significant difference in survival was observed between the DT3 plus G-CSF antibody-treated mice compared to either DT3- or DT3 plus isotype control-treated mice ($p < 0.05$). *Significant differences among indicated groups ($p < 0.05$).

Discussion

We have tested several promising radiation countermeasures (5-androstenediol, CBLB502, CBLB612, and CBLB613, tocopherol succinate, and gamma-tocotrienol) and reported stimulation of G-CSF and other cytokines by these drugs in mice and also in nonhuman primates (CBLB502 (Singh et al., 2012a) and gamma-tocotrienol (unpublished observation)). DT3 induces high levels of cytokines comparable to other tocols, which are being developed as radiation countermeasures (GT3 and TS) (Singh et al., 2010, 2011; Kulkarni et al., 2012). DT3 stimulated significantly high levels of G-CSF in unirradiated as well as irradiated mice compared to respective vehicle controls. Using rodent, canine, and nonhuman primate experimental models, G-CSF and IL-6 have been suggested as candidate biomarkers of CBLB502's radioprotective/mitigative efficacy. G-CSF has been demonstrated to enhance the survival of irradiated mice and to minimize the effect of radiation on gastrointestinal injury in a dose-dependent manner when administered after radiation exposure (radiomitigator) (Kim et al., 2012; Patchen et al., 1990; Tanikawa et al., 1989, 1990; Fushiki et al., 1990; Hosoi et al., 1992). G-CSF has also been shown to mitigate radiation injury in nonhuman primates (Farese et al., 2012, 2013). There is general agreement in literature that G-CSF is an acceptable treatment for human subjects exposed to ≥3 Gy of total body irradiation or ≥2 Gy of total-body irradiation in combined injury (radiation plus wound/trauma/burn) (Waselenko et al., 2004; Dainiak et al., 2011a, 2011b; Dainiak, 2010). G-CSF has been assigned a pre-emergency-use authorization (EUA) package held by the Centers for Disease

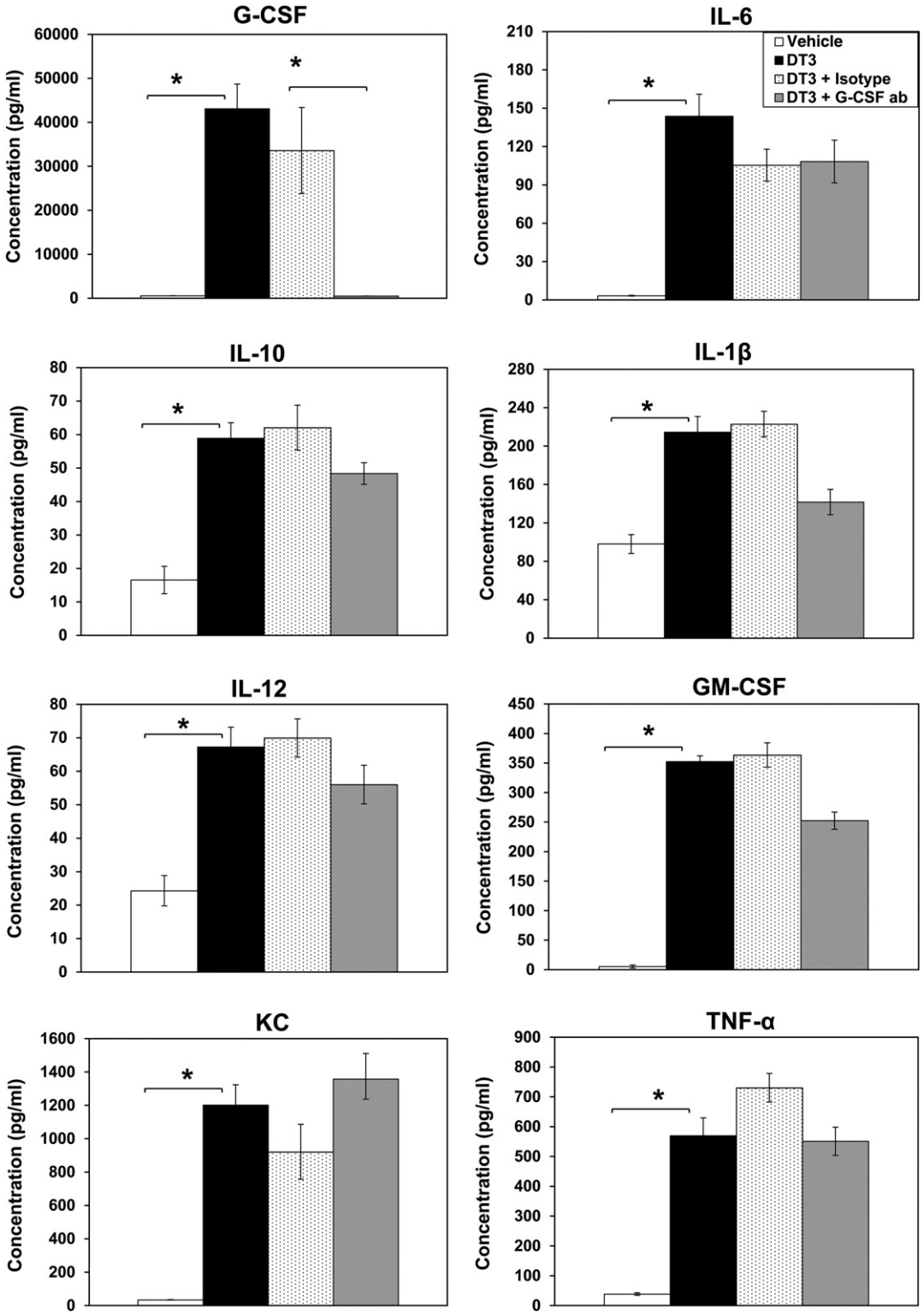

Fig. 5. Neutralization of DT3-induced G-CSF by the administration of exogenous G-CSF antibody in mice. Four groups of mice ($n = 6$) were given sc injections of DT3 (200 mg/kg). Then 8 h after DT3 administration, two of the groups were administered either G-CSF antibody or its isotype. Blood samples were collected 24 h after DT3 injection. Serum samples were analyzed by multiplex Luminex for eight cytokines. Error bars indicate the standard error of the mean (SEM). *Significant differences among indicated groups ($p < 0.01$).

Control and Prevention, which allows the FDA to authorize emergency use of an experimental drug outside the traditional investigational new drug (IND) and study protocol requirements. EUA allows the FDA to authorize use of an experimental drug in an emergency situation that does not allow time for submission of an IND in accordance with 21CFR, Sec. 312.23 or Sec. 312.34. G-CSF is available as a treatment agent in current radiation countermeasure strategic national stockpiles that have been developed in the United States and by the World Health Organization (Dainiak, 2010).

All tested agents (DT3, GT3, and TS) are radioprotectors, and their radioprotective efficacy are comparable (Satyamitra et al., 2011; Singh et al., 2009, 2010; Li et al., 2010; Ghosh et al., 2009). These agents are optimally effective when administered in a single dose at 24 h prior to radiation exposure. All three agents protect almost 100% of mice against

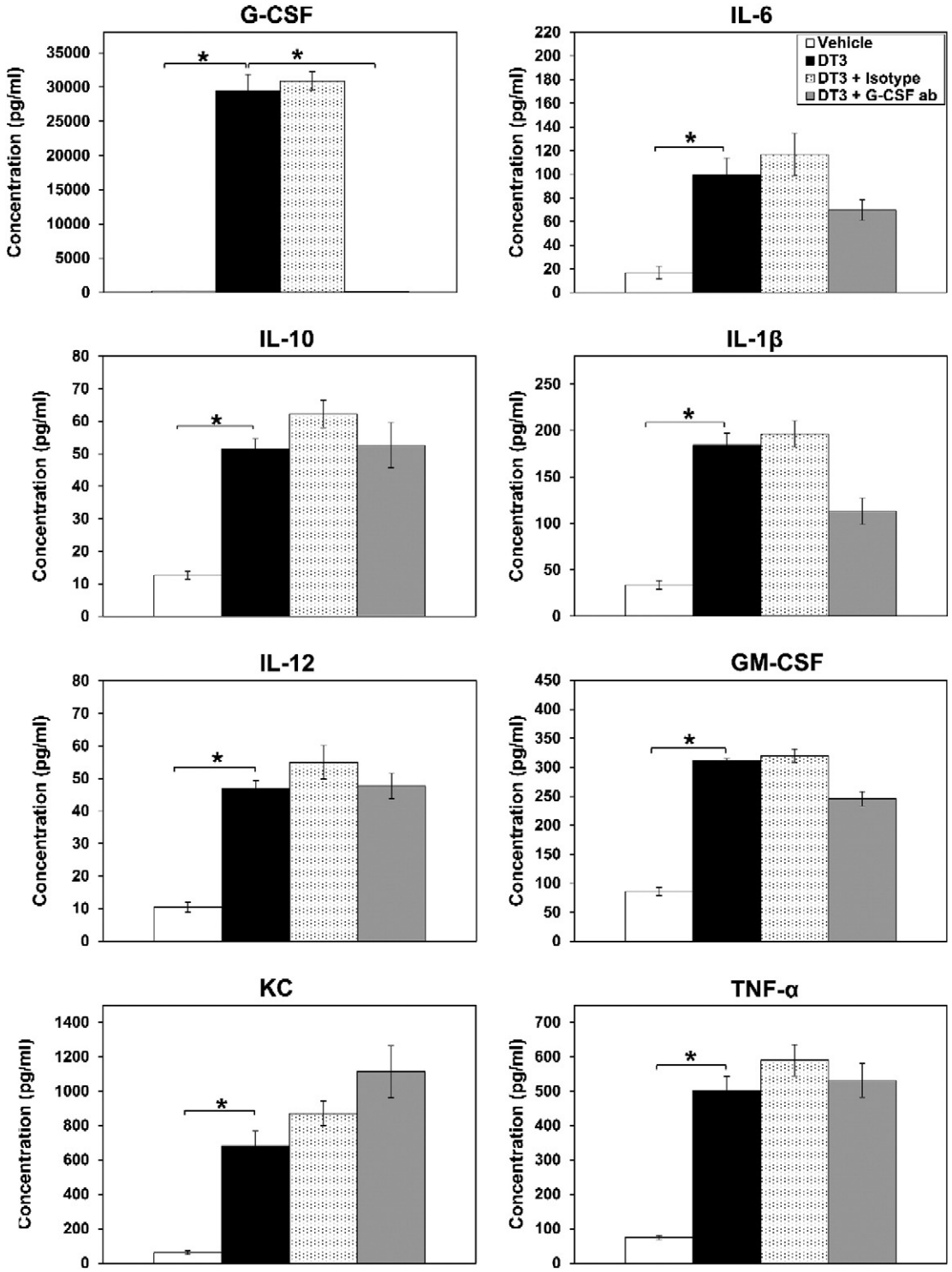

Fig. 6. Neutralization of DT3-induced G-CSF by the administration of G-CSF-specific antibody in irradiated mice. Animals were treated as described in Fig. 1 and irradiated with a dose of 9.2 Gy γ-radiation 24 h after DT3 injection. Blood samples were collected 6 h after radiation exposure. Serum samples were analyzed by multiplex Luminex for eight cytokines. Error bars indicate the standard error of the mean (SEM). *Significant differences among indicated groups ($p < 0.01$).

$LD_{90/30}$ dose of radiation. The highest dose of radiation against which significant protection is achieved by these agents is 11.5 Gy total body irradiation. All three agents induce high, comparable levels of G-CSF as presented in Fig. 1. Recently, we have demonstrated that levels of G-CSF induced by TS are directly related to the degree of mouse protection against total body irradiation (Singh et al., 2013). With increasing doses of TS, higher levels of G-CSF are induced. These higher levels of G-CSF are associated with increased radioprotective efficacy of TS. Exogenous G-CSF is effective only as a radiomitigator and needs to be administered in multiple doses for a few days post radiation exposure. Although G-CSF's optimal dose is less compared to tocols, tocols have the advantage of being cheap and stable at room temperature, making them more practical.

Although the exact mechanism of radioprotection or G-CSF induction by DT3 (or other tocols) is not completely understood, there are recent publications demonstrating its potential mode of

action. Apart from its antioxidant activity, DT3 elicits survival by modulating signaling pathways. The mechanism of DT3-mediated effects may be attributed to stimulation of the Erk activation-associated mTOR survival pathway (Li et al., 2010). DT3 activates Erk 1/2 phosphorylation and inhibits formation of DNA-damage marker γ-H2AX foci in mouse bone marrow (in vivo) and human $CD34^+$ cells (in vitro). DT3 also up-regulates mTOR and phosphorylation of its downstream effector, 4EBP-1. These changes are associated with activation of mRNA translation regulator eIF4E and ribosomal protein S6, which are responsible for cell survival and growth. DT3 has also been shown to suppress apoptotic death pathways and modulate autophagic markers (Satyamitra et al., 2012). Further study is needed to understand exact mechanism of G-CSF induction by DT3.

We also have reported that radiation exposure induces elevation of circulating G-CSF and that administering a neutralizing antibody to G-CSF exacerbates the deleterious effects of radiation exposure, suggesting that G-CSF induced in response to irradiation plays an important protective role in recovery (Singh et al., 2012d). Recently, we have demonstrated that the use of the G-CSF antibody abrogates the radioprotective efficacy of few radiation countermeasures (Singh et al., 2010; Kulkarni et al., 2013; Krivokrysenko et al., 2012; Grace et al., 2012). Our current study demonstrates that DT3 is a potent stimulator of several cytokines including G-CSF, and that the radioprotective efficacy of DT3 is mediated through G-CSF. To the best of our knowledge, this is the first report demonstrating induction of various cytokines by this promising radiation countermeasure and abrogation of its radioprotective efficacy by G-CSF antibody. The administration of a neutralizing G-CSF antibody completely neutralized G-CSF in peripheral circulation of DT3-treated irradiated or unirradiated mice. The neutralization of DT3-induced G-CSF by G-CSF antibody was specific and associated with the complete abrogation of the radioprotective efficacy of DT3 against gamma-radiation exposure. Because another isomer of DT3 (GT3, unpublished observation) and CBLB502 (Krivokrysenko et al., 2012) are being evaluated in a preclinical nonhuman primate model for radioprotection and/or radiomitigation with an objective to develop them for future human use, it will be interesting to investigate the effects of the G-CSF antibody in other species to better understand the mechanism of radioprotection afforded by these radiation countermeasures. This is particularly important as the above radiation countermeasures are being developed under the US FDA Animal Efficacy Rule which requires demonstration of efficacy in two animal models and thorough understanding of the countermeasures' mechanisms of action.

Conclusions

DT3 induces high levels of several cytokines comparable to other tocols in the mouse model. The administration of an antibody to G-CSF completely neutralizes DT3-induced G-CSF and abrogates its radioprotective efficacy against ionizing radiation in mice suggesting that radioprotective efficacy of DT3 is mediated through G-CSF.

Conflict of interest statement

The authors declare no conflict of interest.

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